THE COMPARISON OF THE EFFECTS OF DIETARY ARACHIDONIC ACID AND EXOGENOUS PROSTAGLANDIN E_2 ON BONE METABOLISM AND MINERALIZATION IN THE NEW BORN PIGLET

BY

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A thesis submitted to the Department of Foods and Nutrition in partial fulfillment of the requirements for the Degree of

MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

THE COMPARISON OF THE EFFECTS OF DIETARY ARACHIDONIC ACID AND EXOGENOUS PROSTAGLANDIN E_2 ON BONE METABOLISM AND MINERALIZATION IN THE NEW BORN PIGLET

Both prostaglandin E_2 (PGE_2) administration and dietary arachidonic acid (AA), the precursor to endogenous PGE_2 synthesis, have been independently shown to elevate bone mass. The research objective is to compare the effects of dietary AA versus PGE_2 on the fatty acid composition of plasma and liver tissue, bone cell metabolism, ex vivo PGE_2 release and bone mineral content (BMC) and density (BMD) of the new born piglet. Twenty-eight five-day old piglets from seven litters were randomized to one of four treatments (n=7 per treatment) for fifteen days: Fatty acid supplemented formula (FA; 0.8 % of total fatty acids as AA and 0.1 % of total fatty acids as DHA) plus PGE_2 injections (0.1 mg/kg/day), FA plus saline injections, standard formula (STD; n-6:n-3 of 8:1) plus PGE_2 injections or STD plus saline injections. After fifteen days, plasma and liver tissue were analyzed for fatty acids via gas chromatography. Biochemical bone markers, plasma osteocalcin, urinary calcium and cross-linked N-telopeptide of type I collagen (NTX) were measured and ex vivo PGE_2 release in bone organ culture was determined. Whole body, femur and total lumbar 1-4 BMC and BMD were measured using dual energy x-ray absorptiometry. All data are mean $\pm$ SD with differences among groups detected using two-way ANOVA within a randomized complete block design. Compared to those fed STD, piglets fed FA diet had higher AA content in plasma ($P<0.05$) yet lower levels of LA in plasma and liver ($P<0.05$), lower liver EPA ($P<0.05$), as well as reduced NTX ($P<0.05$) and ex vivo PGE_2 production ($P<0.05$). Piglets given 0.1 mg/kg/day PGE_2 had higher plasma total n-3 fatty acids ($P<0.05$) and DHA ($P<0.05$), increased levels of EPA in plasma and liver ($P<0.05$), elevated plasma osteocalcin

($P<0.05$) and reduced urinary calcium ($P<0.05$) compared to those given saline. Whole body, femur and lumbar spine BMC and BMD were similar between FA fed and PGE_2-treated piglets. Piglets fed FA combined with PGE_2 injections had greater lumbar BMC ($P=0.05$) and BMD ($P=0.05$) compared to those fed the same formula but given saline. In contrast, lumbar BMC ($P=0.05$) and BMD ($P=0.05$) were lower in piglets fed STD combined with PGE_2 treatment compared to STD diet, saline-treated piglets. Dietary AA and PGE_2 treatment did not affect bone equally. Dietary AA suppressed bone resorption, whereas PGE_2 elevated formation. Dietary AA and DHA plus PGE_2 synergistically elevated BMC and BMD of the lumbar spine. Supplementation of formula with AA and DHA did not elevate ex vivo PGE_2 release suggesting that the response of bone to dietary AA may have not been due to endogenous PGE_2 synthesis. Further research is required to investigate the effects of dietary AA and DHA on the synthesis of eicosanoids besides PGE_2 and their potential impact in regulating bone metabolism.

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A heart-felt thank you is extended to my mom, Maria Lucia for providing me with the strength in overcoming every challenge faced, my dad, Joe Lucia for teaching me the importance of hard work and my sister ("My SU") for truly being their when I needed you the most!

DEDICATION

This thesis is dedicated to my grandparents- Antonio and Vienna Lucia and Antonio and Francesca Torchia.

Questo libro e stato dedicato a le miei cari Nonni.

Nonno Antonio e Nonna Vienna Lucia

Nonno Antonio e Nonna Francesca Torchia

Grazie tanto per tutto il tuo amore. Vi voglio bene.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

LA	linoleic acid
α LNA	alpha linolenic acid
AA	arachidonic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
DGLA	dihomo-gamma-linolenic acid
GLA	gamma-linolenic acid
DPA	docosapentaenoic acid
PUFA	polyunsaturated fatty acids
BM	breast milk
STD	standard formula
FA	fatty acid supplemented formula
RBC	red blood cells
LBW	low birth weight infant
VLBW	very low birth weight infant
PGE_2	prostaglandin E_2
PGG_2	cyclic 9-11 endoperoxide 15-hydroperoxide
PGH_2	prostaglandin H_2
$1,25\ (OH)_2D_3$	1,25 dihydroxycholecalciferol
PTH	parathyroid hormone
NTX	cross-linked N-telopeptide of type I collagen
DEXA	dual energy x-ray absorptiometry
BMC	bone mineral content
BMD	bone mineral density
Cr	creatinine

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

- SECTION I -

LITERATURE REVIEW

I. LITERATURE REVIEW

Introduction

Whether or not the long-chain polyunsaturated fatty acids (PUFA), arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6 n-3) should be supplemented into milk formula has become a major issue in infant nutrition. Omega-6 and omega-3 long-chain PUFA are essential in the human diet, however it is uncertain as to the amount and type of omega-6 and omega-3 fatty acids that are needed in the diets of young infants. In North America, currently available infant formulas contain similar to breast milk or even higher levels of the eighteen carbon precursor essential fatty acids, linoleic acid (LA, 18:2 n-6) and alpha linolenic acid (α LNA, 18:3n-3). In contrast, infant formulas differ from breast milk in that they do not provide the longer, more unsaturated omega-6 and omega-3 fatty acids, AA and DHA, respectively.

Infants who are fed formula without AA and DHA depend on active endogenous long-chain PUFA synthesis from LA and α LNA provided in the formula (Innis, 1991). Both premature (Carnielli et al., 1996) and term infants (Demmelmair et al., 1995; Salem et al., 1996) are capable of converting precursor essential fatty acids, LA and α LNA to their respective end products in the first few weeks after birth. Despite this evidence, infants fed standard, unsupplemented formula (containing LA and α LNA, but no AA and DHA) have lower AA and DHA levels in plasma and red blood cell phospholipids compared to infants fed breast milk or supplemented formula (Kohn et al., 1994; Koletzko et al., 1995; Koletzko et al., 1996; Boehm et al., 1996; Bondia-Martinez et al.,

1998; Vanderhoof et al., 1999). Although infants are capable of endogenously synthesizing AA and DHA, the amounts synthesized from dietary precursors, LA and α LNA may be insufficient to meet tissue accretion needs and to completely fulfill their high demands during early growth and development (Gibson and Makrides, 1998; Bondia-Martinez et al., 1998). The presence of AA and DHA in breast milk, the high concentrations of AA and DHA in structural membranes of growing tissues, such as the brain and retina and lower circulating levels of AA and DHA in plasma and red blood cells of infants fed formula rather than breast milk has raised questions about the potential role of these fatty acids in facilitating optimal growth and development.

AA and DHA are important for rapid growth and development of the infant during the late fetal and early postnatal period (Innis, 1991). The role of long-chain PUFA in infant nutrition has been extensively investigated for their possible implications in early cognitive and visual development (Hoffamn et al., 1993; Carlson et al., 1993; Werkman and Carlson, 1996; Birch et al., 1998). However, very little is known on the effects of feeding formula supplemented with both AA and DHA on the growth and development of tissues, including bone during early infancy.

AA is the precursor to prostaglandin E_2 (PGE_2), an eicosanoid that serves as a potent regulator of both bone formation and resorption. Both dietary AA and long-term administration of PGE_2 have been shown to stimulate bone formation and mineral density in rodent and piglet models (Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Jee et al., 1991; Ke et al., 1992; Akamine et al., 1992; Ito et al., 1993; Weiler, 2000). Although several theories have been postulated, the exact mechanism by which dietary AA and PGE_2 affect bone metabolism and subsequent mineralization is not known since no study

has compared and investigated the combined effects of diet and PGE_2 on bone during periods of rapid growth and development.

The main objective of this thesis was to compare the effects of dietary AA versus PGE_2 on 1) the fatty acid composition of plasma and liver tissue, 2) bone cell metabolism, 3) ex vivo PGE_2 synthesis, and 4) bone mineral content (BMC) and density (BMD) of the new born piglet. The following literature review will describe the present state of knowledge for these topic areas. The hypothesis is that dietary AA will support greater PGE_2 synthesis and elevate BMC and BMD similar to that observed with low dose PGE_2 injection.

The fatty acid composition of breast milk and infant formula

After birth, breast milk or formula is the recommended sole food for infants during the first four to six months of life. The total fat content in breast milk ranges from 31 to 53 g/L or 45 to 58 % of total energy intake (LSRO Report, 1998). Current infant formulas contain 36 g/L, providing 48% of total energy from fat (Aggett et al., 1991; LSRO Report, 1998). Breast milk and formula contain a variety of different fatty acids, and the fatty acid composition of commercially available formulas differs from that of breast milk.

In addition to LA and α LNA, breast milk naturally contains longer-chain PUFA, AA and DHA with levels varying according to the quality and amounts of fat consumed by the mother during pregnancy and lactation (Innis, 1992; Hamosh and Salem, 1998; LSRO Report, 1998; Agostoni et al., 1999). The ratio of AA to DHA in breast milk is most commonly 1:1 to 2:1 (Uauy et al., 2000). Breast milk contains (in % of total fatty

acids) approximately 8-30 % LA, 0.5-2.0 % α LNA, 0.5-0.8 % AA and 0.1-1.0 % DHA (Innis, 1991; Innis, 1992; Gibson and Makrides, 1998).

The ratio of omega-6 to omega-3 fatty acids in breast milk is approximately 1.0:0.5 (Aggett et al., 1991; Decsi and Koletzko, 1994), however, ratios may range from 5:1 to 10:1, up to 18:1 if corn, sunflower or safflower oils are consumed (Uauy et al., 2000). Women that consume typical North American diets have relatively low levels of DHA in their breast milk (0.2-0.4 % of total fatty acids) compared to women who eat large amounts of fish (1.4 % of total fatty acids) (Innis, 1992; LSRO Report, 1998; Gibson and Makrides, 1998; Agostoni et al., 1999).

Commercially available vegetable oil based formulas differ from breast milk in that they do not provide longer-chain PUFA, AA and DHA. Table 1 describes the minimum and maximum fatty acid specifications for term infant formulas as recommended by the Food and Drug Administration (FDA), European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) and the Department of Health of Canada.

Standard infant formula is a cow's milk based formula, containing skim milk powder, lactose and a mixture of vegetable oils. The fatty acid composition of infant formula depends on the quality and proportion of oils used in the fat blend. Infant formulas contain a mixture of coconut, corn, soybean and safflower oils (Innis, 1991) providing a fatty acid composition nearly identical to breast milk for saturated, monounsaturated, and for the essential fatty acids, LA and α LNA. Corn, safflower and soybean oils contain abundant amounts of LA, approximately 45 to 70 % of total fatty acids. Soybean oil typically contains 6-9 % of total fatty acids as α LNA, whereas

safflower and corn oils provide substantially lower amounts of this essential fatty acid (Innis, 1991; LSRO Report, 1998). The omega-6 and omega-3 fatty acid composition of commonly used vegetable oils in infant formula is presented in Table 2.

Importance of dietary fat

Breast-fed and formula fed infants require dietary fat as a major source of energy for rapid growth, facilitation of the absorption and transport of fat-soluble vitamins, essential fatty acids and eicosanoid synthesis. Long-chain PUFA, AA and DHA are major components of membrane phospholipids, such as in the brain and the retina (Innis, 1991), and therefore are important in maintaining the structure and integrity of cell membranes. More specifically, they are involved in cell membrane lipid synthesis, membrane order, fluidity and permeability, activity of membrane-bound receptors and enzymes and signal transduction mechanisms (Innis, 1991; Decsi and Koletzko, 1994).

In addition to their structural role in cell membranes, AA, dihomo-gamma linolenic (DGLA, 20:3 n-6) and eicosapentaenoic acids (EPA, 20:5 n-3) serve as precursors to eicosanoids, biologically active, hormone like compounds including prostaglandins, thromboxanes, prostacyclins and leukotrienes. Eicosanoids further regulate numerous physiological processes including reproduction, blood pressure, heart rate, blood clotting, vasoconstriction, immune and inflammatory responses (reviewed in Mann and Skeaff, 1999; Berdanier, 2000).

Table 1: Minimum and maximum fatty Acid specifications for term infant formulas

Nutrient (units[1])		FDA	ESPGAN	CANADA
Total Fat (g, % Kcal)	Minimum	3.3, 30%	4.4, 40%	3.3, 30%
	Maximum	6.0, 54%	6.0, 54%	6.0, 54%
LA (mg, % Kcal) [ratios]	Minimum	300, 2.7%	500, 4.5% [LA: α LNA, 5:1]	500, 4.5% [LA: α LNA, 4:1]
	Maximum	*	1200, 10.8% [LA: α LNA, 15:1]	[LA: α LNA, 16:1]
α LNA (% Kcal)	Minimum	*	*	0.7 %
	Maximum	*	*	*
AA (% of total fat)	Minimum	*	*	*
	Maximum	*	*	*
DHA (% of total fat)	Minimum	*	*	*
	Maximum	*	*	*
EPA (% of total fat)	Minimum	*	*	*
	Maximum	*	*	*

(Adapted from LSRO Report, 1998; Health Canada, 1995)
* No values specified.

Table 2: Fatty Acid composition (% of total fatty acids) of oils used in infant formulas

	Coconut	Corn	Soybean	Safflower
N-6 Series				
18:2 n-6	2.0	58.0	55.1	78.4
20:2 n-6				
20:4 n-6				
22:5 n-6				
N-3 Series				
18:3 n-3		1.1	7.0	0.2
20:5 n-3				
22:5 n-3				
22:6 n-3				

(Adapted from Innis, 1991)

Structure and nomenclature of fatty acids

Fatty acids consist of a hydrocarbon chain (even numbered chain of carbon atoms with hydrogens attached), a methyl group at one end and a carboxylic acid group at the other. The fatty acid chain may be saturated, containing no double bonds or unsaturated, containing one or more double bonds. Omega-6 and omega-3 fatty acids are defined according to the number of carbon atoms in the chain, the number of double bonds and by the location of the first double bond from the terminal methyl end of the fatty acid molecule. Fatty acids with their first double bond between carbon number six and seven are designated omega-6 (n-6) fatty acids, whereas fatty acids with their first double bond between carbon three and four are named omega-3 (n-3) fatty acids (reviewed in Innis, 1991; Xiang and Zetterstrom, 1999).

LA and α LNA cannot be synthesized in the body, as humans do not have the enzymes to insert double bonds at the n-6 or n-3 position of the fatty acid carbon chain. As a result, LA and α LNA are considered to be essential and they must be supplied in the diet (Aggett et al., 1991). In contrast, longer-chain PUFA, such as AA and DHA can be derived from the metabolism of LA (n-6 precursor) and α LNA (n-3 precursor).

Fatty acid synthesis

Long-chain PUFA of the n-6 and n-3 series are synthesized in the liver, brain and retina from parent essential fatty acids, LA and α LNA via a series of alternating desaturation (addition of double bonds) and elongation (addition of two carbon units) steps (Figure 1). Initially, it was suggested that long-chain PUFA synthesis occurred

solely in the endoplasmic reticulum. Although twenty-carbon chain fatty acids, AA and EPA are formed in the endoplasmic reticulum, the synthesis of DHA involves delta-6 desaturation and chain shortening which occurs in the peroxisomes of the liver (Sprecher, 1992; Sprecher et al., 1995; reviewed in Gibson and Makrides, 1998; Hamosh and Salem, 1998; Uauy et al., 2000; Innis, 2000; Crawford, 2000).

a) Omega-6 pathway

LA is desaturated by delta-6 desaturase to form gamma-linolenic acid (GLA,18:3n-6). An elongase enzyme adds two more carbon atoms to GLA to form DGLA, precursor of series 1 prostaglandins (PGE_1, PGF_{1a}, PGD_1). Delta-5 desaturase converts DGLA to the 20-carbon PUFA, AA, precursor to series 2 prostaglandins (PGE_2, PGf_{2a} and PGD_2). Docosapentaenoic acid (DPA, 22:5 n-6) is the final long-chain PUFA synthesized from the omega-6 pathway.

b) Omega-3 pathway

α LNA is desaturated twice and elongated once to form EPA, precursor of series 3 prostaglandins (PGE_3, PGH_3 and PGI_3). EPA is further elongated to 24:5 n-3 followed by delta-6 desaturation to form 24:6 n-3. Two carbons are cleaved from the fatty acid to form DHA.

Delta-6 desaturation is the initial and rate-limiting step in both the n-6 and n-3 metabolic pathways, as the enzyme controls the entry of LA and α LNA into the desaturation-elongation sequence (Innis, 1991). N-6 and n-3 fatty acids compete for the same desaturase and elongase enzymes, however the enzymes favour the n-3 pathway

over n-6. The preferential substrate binding affinity for delta-6 desaturase is in the order of n-3>n-6>n-9.

Considering n-6 and n-3 fatty acids compete with each other for the same enzymes, a balanced addition of AA and DHA into milk formula in amounts above a minimal threshold may be more important than the total or absolute amount of each fatty acid. Supplementing infant formula with excess DHA may competitively inhibit n-6 fatty acid metabolism, reducing their incorporation into tissue phospholipids (Boyle et al., 1998; Ward et al., 1998; Dela Presa-Owens et al., 1998), which could potentially alter the synthesis of eicosanoids (Hwang et al., 1988). Ward et al., 1998 studied the competitive interaction of dietary n-6 and n-3 PUFA in new born rats fed formula with 10 % LA and 1% α LNA and one of three levels of both AA and DHA (0, 0.4 and 2.4% fatty acids) from day five to eighteen of life. On day eighteen, rats fed formula supplemented with additional AA (2.4%) had higher levels of AA, but lower levels of DHA in red blood cells and brain tissue. In contrast, rats fed formula with additional DHA had higher levels of DHA, but lower levels of AA in brain and red blood cells. The researchers demonstrated that feeding formula with AA or DHA increased deposition of the long-chain PUFA in brain and red blood cells while decreasing levels of the long-chain PUFA of the other series. Similarly, Dela Presa-Owens et al., 1998 demonstrated that feeding piglets from birth to eighteen days of life a formula with (in % of total fatty acids) 20% LA, 2% α LNA and 0.8% AA, with no DHA resulted in higher AA content in plasma, liver, heart and kidney tissue, however lower plasma DHA and reduced levels of EPA in liver, heart, and kidney. Interestingly, supplementation of formula with 0.8% AA resulted in a greater reduction in EPA than in DHA in tissue phospholipids of piglets.

The researchers suggested that the decrease in EPA rather than DHA after dietary AA supplementation might have occurred as result of preferential formation of DHA from α LNA or conservation (recycling) of tissue DHA during inhibition of DHA synthesis. Piglets fed formula containing 0.3% DHA, with no AA had higher levels of DHA and EPA in plasma, liver, heart and kidney yet lower levels of AA in plasma and brain. The decrease in blood and tissue levels of AA with the feeding of n-3 fatty acids without AA suggests that formulas that are unbalanced in n-3 and n-6 PUFA are not compatible with normal biochemical development of tissue structural lipids (Innis, 1991).

A young infants' ability to synthesize AA and DHA from eighteen carbon essential fatty acids depends on their energy intake, enzymatic activity and if fed formula, content of LA and α LNA (Innis, 1991). Recent studies have demonstrated using oral doses of stable isotope labelled eighteen carbon precursors that both premature (Carnielli et al., 1996) and term infants (Demmelmair et al., 1995; Salem et al., 1996) are capable of converting LA and α LNA to longer chain fatty acids, AA and DHA, respectively in the first few weeks after birth. Demmelmair et al., 1995 studied the conversion of LA to AA after birth in four, eighteen-day old term infants that were fed for four days a formula containing corn oil (devoid of AA), a natural source of LA with a high carbon 13 (^{13} C) content. The researchers estimated that 6% of total plasma AA was renewed each day by endogenous synthesis. By day four, 23 % of plasma AA was endogenously synthesized from LA. Corn oil does not contain AA therefore the researchers concluded that these term infants were capable of endogenously synthesizing AA.

Carnielli et al., 1996 measured in vivo conversion of ^{13} C labelled LA and α LNA to AA and DHA in five one-month old formula fed premature infants. Both tracers were

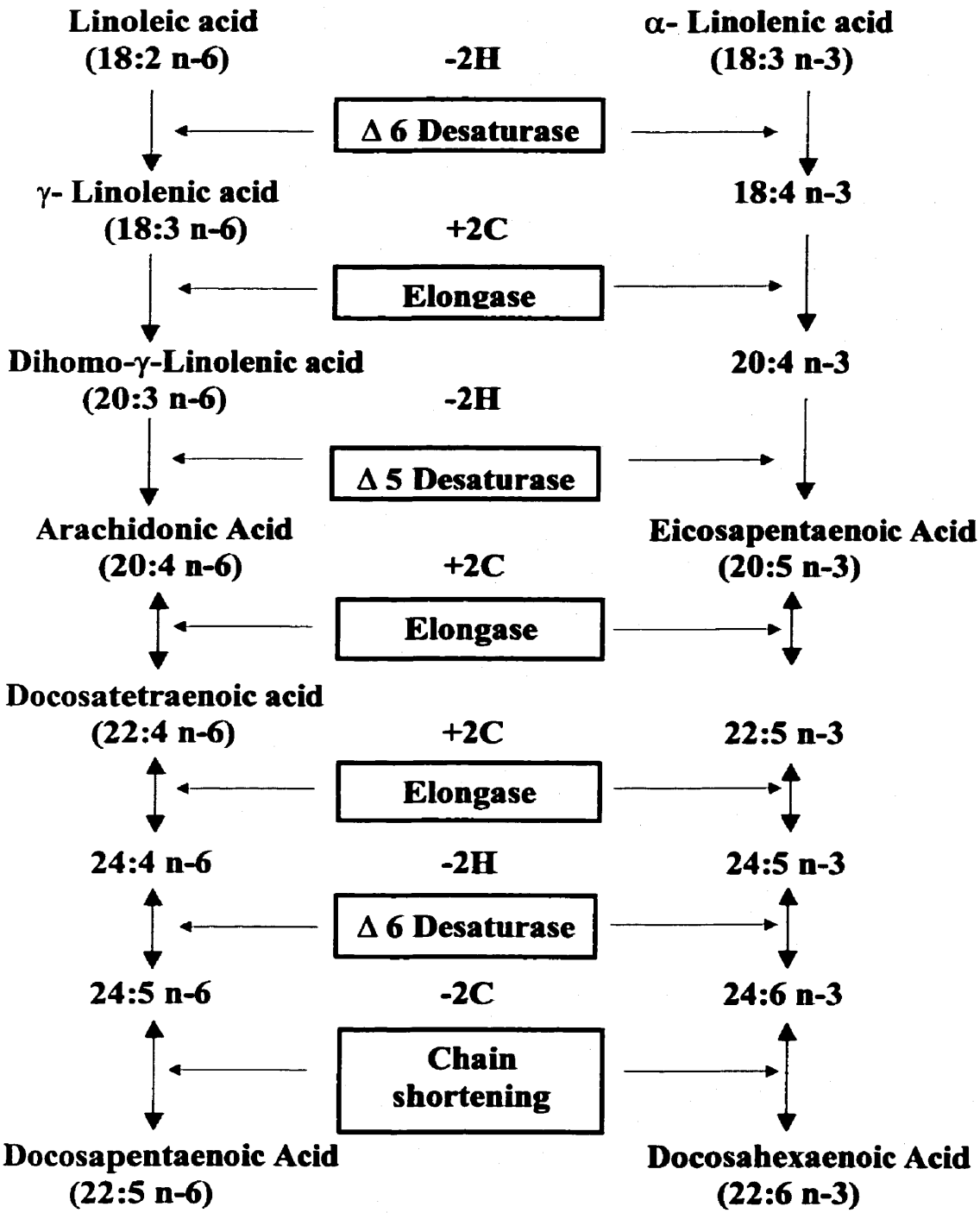
mixed in formula, which contained no AA and DHA, and was administered continuously for forty-eight hours. The researchers calculated that 6.05 ± 2.26% and 14.07 ± 4.20 % of the total dose of carbon ^{13} C LA and α LNA were converted into plasma phospholipid AA and DHA, respectively. Based on these studies, it remains unclear whether young infants synthesize AA and DHA from LA and α LNA provided in the formula in amounts sufficient to support adequate deposition of AA and DHA in growing tissues.

Effect of long-chain PUFA supplementation on blood levels of AA and DHA

The fatty acid composition of blood is most commonly used to measure the fatty acid status of infants. The quality and amount of fatty acids supplied in the diet influence the n-6 and n-3 fatty acid composition of blood. The rationale for measuring circulating PUFA levels is based on the assumption that levels reflect recent dietary intake, fatty acid deficiencies, imbalances and the transport of fatty acids through the blood stream to tissues.

Different approaches have been tried to improve the lipid supply of infant formula fed to both premature and term infants. Since LA and α LNA are the precursors to AA and DHA, respectively, it was initially assumed that formula fed infants would receive sufficient amounts of these long-chain PUFA, as long as both LA and α LNA were provided in the formula. Multiple studies have shown that AA and DHA levels in plasma and red blood cell phospholipids rapidly decline after birth, within two weeks of feeding in both term (Kohn et al., 1994; Koletzko et al., 1996; Bondia-Martinez et al., 1998) and premature infants (Koletzko et al., 1995; Boehm et al., 1996) fed standard milk formulas

Figure 1: Metabolic pathways of the n-6 and n-3 fatty Acids



(Adapted from Hamosh and Salem, 1998; Uauy, Mena and Rojas, 2000)

containing LA (11-13 % total fatty acids) and α LNA (0.4- 1.0 % of total fatty acids), but no AA and DHA (Table 3).

There are a number of studies that have shown that infants fed formulas supplemented with AA and DHA in the ratios of 1.4:1.0 to 2.0: 1.0 (with AA ranging from 0.2-0.8% of total fatty acids and DHA, 0.1-0.4% of total fatty acids) have plasma and red blood cell levels of AA and DHA similar to breast fed infants and significantly higher than infants fed formulas without these fatty acids (Table 3: Kohn et al., 1994; Koletzko et al., 1995; Koletzko et al., 1996; Boehm et al., 1996; Bondia-Martinez et al., 1998; Vanderhoof et al., 1999). Infants fed formula containing only the precursor fatty acids, LA and α LNA (in amounts similar to that provided in breast milk) were not able to match the AA and DHA status of breast fed infants. These studies suggest that infants fed formula might require a dietary supply of preformed AA and DHA during the first few months of life if the fatty acid status of breast fed infants is to be achieved.

Supplementation of formula with AA (Boyle et al., 1998) and DHA (Carlson et al., 1987) is more effective in maintaining blood AA and DHA levels similar to those of breast-fed infants than increasing the amounts of LA and α LNA. In 1998 Boyle and colleagues examined the interaction of n-3 and n-6 fatty acids in new born pups whose dams received a constant level of dietary n-3 PUFA (2% of total fat) and increasing levels of either LA (1, 2 or 5% of total fatty acids) or AA (1,2 or 5% of total fatty acids) for fourteen days. After fourteen days of feeding, pups that received increasing levels of AA had higher levels of AA in liver and plasma phospholipids than pups that received LA as the predominant source of n-6 fatty acids.

Several investigators have described the effects of supplementing fish oil, a rich source of DHA and EPA into infant formula in attempt to improve the DHA status of term (Makrides et al., 1995; Auestad et al., 1997; Birch et al., 1998; Lapillonne et al., 2000) and premature infants (Carlson et al., 1991; Carlson, 1996; Ryan et al., 1999). The fish oil supplemented formulas contained DHA in the range of 0.1 to 0.36% of total fatty acids and 0.1 to 0.58% of total fatty acids as EPA, with no AA. These studies demonstrated that supplementation of infant formula with fish oil increases the levels of n-3 PUFA, DHA and EPA, while reducing AA content in plasma and red blood cell phospholipids (Table 3).

Dietary n-3 fatty acids including α LNA, DHA and EPA can down regulate chain desaturation and elongation of LA to AA, compete with n-6 fatty acids for incorporation into membrane phospholipids and elevate synthesis of EPA derived eicosanoids with reduced production of eicosanoids from AA (Innis, 1991; Alam et al., 1993; Whelan et al., 1993; Whelan, 1996). Figure 2 describes the impact of dietary n-3 fatty acids on tissue AA content and eicosanoid synthesis.

The long-chain PUFA of fish oils and seafood, EPA and DHA are more effective than α LNA in depressing tissue AA levels and competitively reducing synthesis of AA derived eicosanoids (Hwang et al., 1988). Hwang et al., 1988 studied the ability of dietary α LNA compared with longer chain n-3 fatty acids found in fish oil to inhibit the formation of eicosanoids derived from AA in rat tissues. Weanling rats were fed α LNA or fish oil concentrate in the presence of a fixed amount of LA as safflower oil. Dietary α LNA at a ratio of 0.28 n-3 to n-6 fatty acids suppressed levels of AA in lung and plasma phospholipids and the capacity of tissues to synthesize eicosanoids. At similar

ratios of n-3 to n-6 dietary fatty acids, longer-chain n-3 fatty acids in fish oil were more effective than α LNA in reducing AA levels and eicosanoid synthesis.

The relationship between dietary PUFA intake and blood AA and DHA concentrations is well known and does not necessarily provide evidence that AA and DHA are essential or of benefit to infants. In order to determine whether or not these fatty acids are essential, one must look at the relationship between dietary intake and functional outcomes, such as growth. AA may have a growth promoting effect during early life. The positive impact of AA on infant growth may be related to its structural function in membrane phospholipids or to its role as precursor to eicosanoid synthesis, however the mechanism by which this occurs is still unknown. Research has shown that fetal and infant growth is associated with blood levels of AA. There is a significant positive correlation between birth weight and the AA content in plasma and red blood cells (Koletzko and Braun, 1991; Carlson et al., 1993; Jensen et al., 1997). Reduced blood levels of AA have been associated with impaired growth of infants fed formula containing excess n-3 fatty acids without AA (Carlson et al., 1993; Jensen et al., 1997; Ryan et al., 1999).

Carlson and colleagues reported that supplementation of formula with fish oil containing (in % of total fatty acids) 0.2% DHA and 0.3% EPA decreased plasma AA (1991) and first year growth of premature infants (1993). Compared to those fed the unsupplemented diet, infants that received fish oil supplemented formula had lower weight, length and head circumference that persisted throughout the first year of life. The researchers indicated that the impaired growth of these infants was associated with poor AA status, which resulted from long-term feeding of n-3 PUFA. The potential for

adverse effects on n-6 fatty acid metabolism and growth due to feeding fish oils high in DHA or EPA has led to the suggestion that formula with DHA should also contain AA.

Long-chain fatty acids and bone

The type of dietary fat supplied in the diet has been shown to reflect the fatty acid composition of bone in rats (Alam et al., 1993; Claassen et al 1995), chicks (Watkins et al., 1996; Watkins et al., 1997; Watkins et al., 2000) and piglets (Weiler, 2000). Claassen et al. (1995) investigated the effect of feeding rats different dietary ratios of GLA:EPA + DHA (3:1, 1:1 and 1:3) or LA:α LNA (3:1) on calcium absorption, calcium balance, calcium excretion, femur growth rate and calcium content in bone. After six weeks of feeding, rats fed the 3:1 diet had higher urinary calcium levels, intestinal calcium absorption and calcium content in bone compared to rats receiving the other dietary groups. The researchers suggested that the higher calcium levels in bone of rats fed the 3:1 diet might have been the result of the increased intestinal calcium absorption seen in this group. Rats fed the 1:3 diet had reduced urinary calcium excretion after six weeks of supplementation. Dietary EPA was negatively correlated with urinary calcium excretion suggesting that increased dietary EPA supplied in the 1:3 diet was associated with reduced output of urinary calcium. Whether this reflects elevated use for bone mineralization or enhanced reabsorption in the kidney requires clarification.

Kruger and colleagues (1997) studied similar dietary ratios of GLA:EPA + DHA (9:1, 3:1, 1:3, 1:9) on calcium homeostasis of eleven week old, ovariectomized Sprague Dawley rats. After six weeks of feeding, red blood cell levels of GLA and DGLA increased in rats fed the 9:1 and 3:1 diet. EPA and DGLA red blood cell levels increased

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Carlson et al., 1991	Premature infant n=67	STD : LA-33.2%, α LNA-4.8% AA-none, DHA-none EPA-none FA : LA-32.6%, α LNA-4.9% AA-none, DHA-0.2% EPA-0.3%	Birth to 79 weeks post conception	Infants fed FA had higher plasma and RBC DHA and EPA between 38 and 79 weeks post-conception. Several months after feeding trial was completed (93 weeks post conception), plasma and RBC DHA and EPA remained significantly higher in infants fed FA compared to STD. Infants fed FA had lower AA in plasma throughout study and still at 93 weeks post conception.
Carlson et al., 1992	VLBW infant n=65	Premature STD : LA-19.1%, α LNA-3.0%, AA-none, DHA-none, EPA-none Premature FA: LA-18.7%, α LNA-3.1%, AA-none, DHA-0.2%, EPA-0.3% Term STD: LA-33.2%, α LNA-4.8%, AA-none, DHA-none, EPA-none Term FA: LA-32.6%, α LNA-4.9%, AA-none, DHA-0.2%, EPA-0.3%	Birth to 79 weeks post conception	Beginning at 40 weeks post conception and throughout the first year of life, infants fed FA had lower weight, length and head circumference compared to infants fed STD.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Clandinin et al., 1992	Premature infant n=34	BM ; not provided STD : LA-14.9%, α LNA-1.6% AA-none, DHA-none, EPA-none FA: LA-14.1% LA, α LNA-1.6% AA-0.21%, DHA-0.35%, EPA-0.2%	Birth to 4 weeks of age.	No difference in plasma AA and DHA levels between BM and FA fed infants. Infants fed FA had higher plasma levels of AA and DHA than infants fed STD.
Kohn et al., 1994	Term infant n=not given	BM: LA-10.76%, α LNA-0.81% AA-0.36%, DHA-0.22% EPA-0.04% STD: LA-12.28%, α LNA-0.74% AA-0.02%, DHA-<0.01% EPA-none FA: LA-12.09%, α LNA-0.77% AA-0.25%, DHA-0.18% EPA-0.02%	Birth to 3 months of age	No difference in plasma and RBC AA and DHA levels between BM and and FA fed infants Compared to BM and FA fed infants, infants fed STD had: -Lower AA in plasma and RBC phospholipids on days 7, 30 and 90. -Lower DHA in plasma and RBC phospholipids on days 30 and 90. -Reduction of AA and DHA in plasma and RBC over three months.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Koletzko et al., 1995	LBW infant n=27	BM: LA-11.0%, α LNA-0.9% AA-0.5%, DHA-0.3% EPA-0.2% STD: LA-10.8%, α LNA-0.4% AA-0.05%, DHA-none EPA-none FA: LA-13.8%, α LNA-0.8% AA-0.5%, DHA-0.3% EPA-0.3%	3 weeks	No difference in plasma AA and DHA status between BM and FA fed infants. At 3 weeks, STD fed infants showed a significant reduction of AA in plasma phospholipid (reduced to 74% of levels measured in BM fed infants), DHA (reduced to 64%) and total long-chain PUFA (reduced to 74%).
Koletzko et al.,1996	Term infant n=20	BM: not provided STD: LA-13.2%, α LNA-1.0% AA-none, DHA-none EPA-not given	Birth to 2 months of age	After birth, plasma AA levels in infants fed BM were stable over two months. Plasma AA decreased in infants fed STD between two weeks and two months of age. Plasma AA was lower in infants fed STD at the ages of two weeks, one month and two months compared to BM fed infants.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Koletzko et al., 1996	Term infant n=22	STD: LA-13.2%, α LNA-1.0% AA-none, DHA-none EPA-not given FA: LA and α LNA-similar to STD, AA-0.4%, DHA-0.3% EPA-not given	Birth to 4 months of age	From one to four months of age, plasma AA was lower in infants fed STD compared to FA.
Carlson et al., 1996	Term infant n=58	BM: LA-15.8%, α LNA-0.8% AA-0.6%, DHA-0.1% EPA-0.1% STD: LA-21.9%, α LNA-2.2% AA-none, DHA-none EPA-none FA: LA-21.8%, α LNA-2.0% AA-0.43%, DHA-0.1%, EPA-none	Birth to 4 months of age	Infants fed FA had higher levels of AA and DHA in plasma at 2,4 and 6 months of age compared to infants fed STD. At 4, 6 and 12 months of age, infants fed FA had higher AA and DHA content in RBC compared to infants fed STD. Infants fed BM and FA had better visual acuity at 2 months of age compared to those fed STD.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Carlson, 1996	Premature infant n=67	Premature STD : LA-19.1%, α LNA-3.0%, AA-none DHA-none, EPA-none Premature FA: LA-18.7%, α LNA-3.1%, AA-none DHA-0.2%, EPA-0.3% Term STD: LA-33.2%, α LNA-4.8%, AA-none DHA-none, EPA-none Term FA: LA-32.6%, α LNA-4.9%, AA-none DHA-0.2%, EPA, 0.3%	Birth to 9 months of age	Infants fed FA had lower levels of plasma AA throughout study compared to infants fed STD. At 12 months of age, plasma AA remained lower in infants that had been fed FA until 9 months of age.
Boehm et al., 1996	LBW infant n=41	BM: LA-10.56%, α LNA-0.88% AA-0.36%, DHA-0.22% EPA-not given STD: LA-11.30%, α LNA-0.56% AA-none, DHA-none EPA-not given FA: LA-12.75%, α LNA-0.82% AA-0.25%, DHA-0.15%, EPA-not given	2 to 10 weeks of age	No difference in plasma and RBC AA and DHA between BM and FA fed infants. Compared to infants fed BM and FA, infants fed STD had: -Lower AA in plasma and RBC phospholipids within 2 weeks of feeding. -Lower DHA in plasma after 6 weeks of feeding. -Reduction in plasma AA between 2-10 weeks of age.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Jensen et al., 1997	Term infant n=80	Formula 1 : LA-17.6%, α LNA-0.4% LA:α LNA-44:1 Formula 2: LA-17.3%, α LNA-0.95% LA:α LNA-18.2:1 Formula 3 : LA-16.5%, α LNA-1.7% LA:α LNA-9.7:1 Formula 4 : LA-15.6%, α LNA-3.2% LA:α LNA-4.8 :1	Birth to 4 months of age	Infants fed formula 4 had the highest plasma levels of α LNA, DHA and EPA, but lower levels of AA at 21, 60 and 120 days of age. Infants fed formula 1 had the greatest plasma AA content. At 120 days, body weight of all infants correlated positively with plasma AA levels at 120 days. Average body weight of infants fed formula 4 was 760 g less than the mean weight of infants fed formula 1.
Bondia-Martinez et al., 1998	Term infant n=49	BM: LA-6.57-27.58% α LNA-0.58-1.2% AA-0.22-0.79% DHA-0.15-1.03% EPA-0.06-0.42% STD: LA-12%, α LNA-1.2% AA-none, DHA-none EPA-none FA: LA-11.5%, α LNA-1.1% AA-0.3%, DHA-0.15% EPA-0.05%	Birth to 3 months of age	No difference in plasma and RBC AA and DHA levels between BM and FA fed infants. Compared to infants fed BM and FA, infants fed STD had: -Lower AA and DHA levels in plasma and RBC at 1 and 3 months of age. -Reduction of plasma AA (by 47 %) between 7 days and 1 month of age. -Reduction of plasma DHA (by 54 %) between 7 days and 1 month of age. -Reduction of RBC AA (by 26 %) between 7 days and 3 months of age. -Reduction of RBC DHA (by 44%) between 7 days and 3 months of age.

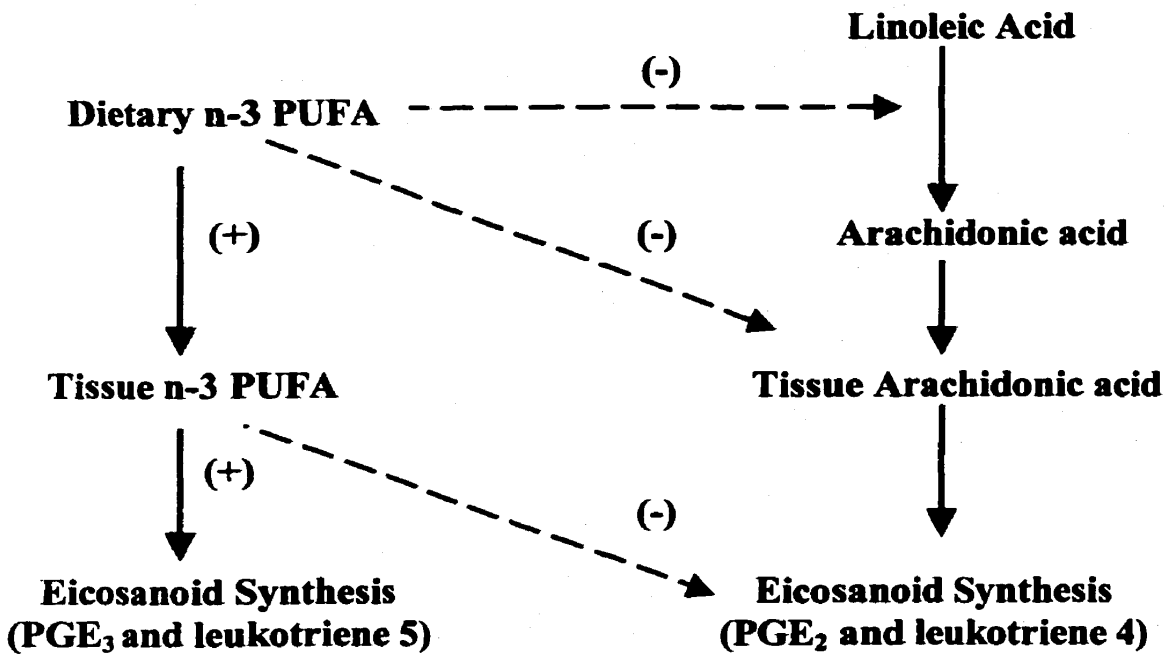
Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Birch et al., 1998	Term infant n=108	BM: LA-12.7%, α LNA-0.80% AA-0.56%, DHA-0.29%, EPA-0.10% STD : LA-14.6%, α LNA-1.49 AA-none, DHA-none, EPA-none DHA formula : LA-15.1%, α LNA-1.54%, AA-0.02%, DHA-0.35%, EPA, none AA + DHA formula : LA-14.9%, α LNA-1.53%, AA-0.72%, DHA-0.36%, EPA-none	Birth to 4 months of age	No difference in RBC fatty acids and visual acuity between breast-fed and supplemented formula fed infants. At 4 months of age, infants fed STD had lower DHA in RBC compared to infants fed BM and supplemented formulas. At 52 weeks of age, RBC DHA levels remained lower in infants that had been fed STD for their first 4 months of life. Infants fed DHA formula had lower RBC AA levels compared to infants fed BM and AA + DHA formula. Infants fed supplemented formulas had better visual acuity than infants fed STD at 6, 17 and 52 weeks of age.
Vanderhoof et al., 1999	Premature infant	BM: not provided STD: LA-12.8%, α LNA-1.4% AA-none, DHA-none EPA-not given FA: LA-12.1%, α LNA-1.5% AA-0.5%, DHA-0.35% EPA-not given	enrolment to 48 weeks post-conception age	No difference in plasma AA and DHA between BM and FA fed infants. Compared to infants fed BM and FA infants fed STD had: -Lower AA and DHA in plasma at 40 and 48 weeks post-conception age.

Table 3: The effect of diet on blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA)

Reference	Subjects	Diets (% of total fatty acids)	Length of Feeding	Major Findings
Ryan et al., 1999	LBW infant n=63	Premature STD : LA-16.8%, α LNA-2.5%, AA-not given, DHA-none, EPA-none Premature FA: LA-15.8%, α LNA-2.3%, AA-not given, DHA-0.2%, EPA-0.04% Term STD: LA-22.4%, α LNA-2.4%, AA-not given, DHA-none, EPA-none Term FA: LA-20.9%, α LNA-2.0%, AA-not given, DHA-0.2%, EPA-0.07%	7-10 days prior to hospital discharge to 6 months of age	Infants fed FA had higher plasma DHA at 2 and 4 months of age compared to those fed STD. At 4 months of age, plasma AA and other n-6 fatty acids were lower in infants fed FA compared to STD. Between enrolment and 6 months of age, male infants fed FA had smaller gain in weight, length and head circumference compared to infants fed STD. Male infants fed FA had lower fat free mass at 4 and 6 months of age. No differences between female infants in measures of growth or body composition.
Lapillonne et al., 2000	Term infant n=37	BM : LA-6.9-16.4%, α LNA-0.7-1.3%, AA-0.2-1.2%, DHA-0.1-0.6%, EPA-0-0.6% STD: LA-17.35%, α LNA-1.59% AA-none, DHA-none, EPA-none FA: LA-17.62%, α LNA-1.07% AA-0.03%, DHA-0.31%, EPA-0.08%	3 days to 4 months of age	Infants fed FA had higher DHA and EPA in RBC at 4 months of age compared to infants fed STD and BM. RBC AA levels declined in infants fed FA between 3 days and 4 months of age. Infants fed FA had lower RBC AA levels at 4 months of age compared to infants fed STD and BM.

Figure 2: Flow chart describing the negative effect of dietary n-3 PUFA on tissue arachidonic acid (AA) content and eicosanoid synthesis



(Adapted from Whelan, 1996)

in rats fed 1:3 and 1:9 diets, however AA decreased. Red blood cell DGLA was positively correlated to femur calcium content.

Weiler (2000) studied the effects of AA and DHA on growth and bone mineralization of newborn piglets. Ten day old piglets were fed either standard formula containing (in % of total fatty acids) 32 % LA and 6.5 % α LNA or the same formula supplemented with 0.5% AA and 0.1 % DHA for fourteen days. After fourteen days of feeding, piglets fed formula supplemented with 0.5 % AA and 0.1 % DHA had lower levels of LA, yet higher levels of AA in liver and cortical bone. End study body weight and BMD of the whole body, lumbar spine and femur were greater in piglets fed supplemented formula compared to those fed the unsupplemented diet. In addition, liver AA correlated positively to piglet growth, AA content in bone, BMC of the whole body, lumbar spine and femur, and BMD of lumbar and femur regions. The researcher suggested that feeding formula supplemented with 0.5 % AA and 0.1 % DHA resulted in greater whole body weight, BMC and BMD in piglets after fourteen days compared with feeding a standard formula containing only LA and α LNA as the sole sources of n-6 and n-3 PUFA.

Watkins et al. (1996, 1997 and 2000) demonstrated that the dietary ratio of n-6:n-3 PUFA influences the bone tissue content of AA and EPA, which in turn can modulate bone eicosanoid synthesis. Watkins et al. (1996) examined the effects of dietary n-6 and n-3 PUFA on bone ash content, morphometry, fatty acid composition, ex vivo PGE_2 biosynthesis and serum alkaline phosphatase activity in chicks. Twenty-day old newborn chicks were fed a semi-purified diet containing soybean oil (rich in n-6 fatty

acids) or menhaden + safflower oils (rich in n-3 fatty acids) for nineteen days. Chicks fed the soybean oil diet had lower EPA, yet higher AA and ex vivo PGE_2 release in liver and bone compared to chicks fed menhaden + safflower oils. However, bone formation rate and plasma alkaline phosphatase activity (marker of bone formation) was higher in chicks fed menhaden + safflower oil compared to those fed the soybean oil diet. The researchers suggested that feeding a diet high in n-6 fatty acids elevated PGE_2 synthesis in bone to a level that depressed bone formation and stimulated bone resorption. Watkins et al. (1997) compared liver and bone fatty acid composition of one day old chicks fed one of four semi-purified diets: soybean oil diet, butter + corn oil diet, margarine + corn oil diet, or menhaden + corn oil diet for fifty-six days. At 21 and 42 days of age, chicks fed the soybean oil diet had higher concentrations of LA, AA, 22:4 n-6 and total n-6 PUFA in bone polar lipids compared with those fed the menhaden + corn oil diet. At 21 and 42 days of age, chicks fed the menhaden + corn oil diet had the highest concentration of n-3 fatty acids (20:5 n-3, 22:5 n-3 and 22:6 n-3), total n-3 PUFA and ratio of n-3 to n-6 PUFA in polar and neutral lipids of cortical bone and liver, but the lowest amount of AA in these tissues. Tibia periosteal bone formation rate was highest in chicks fed the butter + corn oil diet at 21 days of age compared to those fed soybean oil and menhaden + corn oil diets. The same researchers carried out a second study (1997) where one-day old chicks were fed either soybean oil diet or butter oil diet for sixteen days. Chicks fed the butter oil diet had lower AA concentration, reduced ex vivo PGE_2 synthesis in tibia and greater trabecular bone formation rate compared to those fed the soybean oil diet. These researchers suggested that dietary fat altered trabecular bone formation rate by controlling the synthesis of PGE_2 in bone. Moderate levels of PGE_2

synthesis in rats fed the butter oil diet stimulated bone formation, whereas the soybean oil diet led to excess PGE_2 production from the higher AA content in bone polar lipids, which depressed bone formation.

Watkins et al. (2000) studied the effects of dietary PUFA on ex vivo PGE_2 release and bone formation rate in rats. Twenty-one day old rats were fed diets with varying ratios of n-6:n-3 fatty acids (23.8, 9.8, 2.6 or 1.2) for forty-two days. Rats that were fed diets with a lower dietary ratio of n-6 to n-3 fatty acids had reduced AA concentration in bone and lower ex vivo PGE_2 release in liver homogenates and bone organ cultures (femur and tibia) compared with rats fed diets with a higher dietary ratio. Bone alkaline phosphatase and bone formation rate was highest in rats fed diets high in n-3 fatty acids or low ratio of n-6 to n-3 fatty acids compared with those fed the higher dietary ratio. The researchers showed a positive correlation between dietary n-6:n-3 ratio and AA content of bone. Bone PGE_2 was positively correlated with AA:EPA ratio, whereas bone formation rate was negatively correlated with AA:EPA ratio and PGE_2 in bone.

Structure and function of bone

Bone is a specialized connective tissue of mineralized collagen matrix characterized by a cylinder-like tube in the center known as the shaft or diaphysis. The center of the diaphysis is filled with hematopoietic and marrow-producing tissues and is called the medullary or marrow cavity. At both ends of the diaphysis is the epiphysis and metaphysis, which are joined together by the epiphyseal cartilage known as the growth plate.

Cortical or compact bone is a thick, dense layer of calcified tissue located on the diaphysis and outer surfaces and it becomes progressively thinner near the metaphysis and epiphysis of long tubular bones. The inner section of bone is composed of a network of thin spicules of calcified bone known as trabeculae or cancellous bone (O'Flaherty, 1991), primarily located in vertebrae, flat bones and in the epiphysis of adult long bones. Under normal conditions, trabecular bone appears as a honeycomb structure with thick walls, however, trabecular bone is more prone to bone loss than cortical bone. This is because bone turnover depends on a large surface area and trabecular bone has a higher surface to volume ratio in comparison to cortical bone (Manolagas and Jilka, 1995). Approximately 80% of the human skeleton is composed of cortical bone with the remaining 20% being trabecular (Gong et al., 1964; Johnson, 1964).

Skeletal bone serves three primary functions for the body, which include:

1. Mineral reservoir: Bone tissue acts as a storage organ for ions such as calcium, phosphate and magnesium and functions to maintain a balance of these minerals in the blood.
2. Protection and support: Bone provides mechanical support for soft tissues, as well as protects the bone marrow and vital organs.
3. Hematopoiesis: Bones are the major sites of hematopoiesis, the formation of blood.

In fulfilling these major functions for the body, bone must be continuously broken down and reformed by two highly regulated processes known as bone modeling and remodeling.

Bone formation and resorption

Throughout an individual's life span, old bone is continuously resorbed and new bone is constantly being laid down. Bone modeling is most active during infancy and adolescence and is characterized by first the formation of new bone followed by resorption. Remodeling of bone takes place throughout adult life whereby bone is first resorbed and then formed. Bone modeling increases the strength and overall skeletal mass of bone, whereas remodeling maintains calcium homeostasis within a normal range, alters bone shape and maintains adult bone mass (Anderson, 1996). When resorption and formation are balanced, no net change in bone density occurs. However, a negative balance, such as when break down exceeds formation is the basis of many skeletal diseases, such as osteoporosis, hyperparathyroidism and hypercalcemia of malignancy (Rodan, 1992). Bone formation and resorption are hormonally regulated and both processes are determined by the activity of osteoclasts, bone resorbing and osteoblasts, bone forming cells.

a) Osteoclast-bone resorbing cells

Osteoclasts are large, multinucleated cells located on bone surfaces. They are of hematopoietic origin derived from precursor cells of the macrophage-monocyte lineage. The primary function of osteoclasts is to resorb and remove bone, thereby allowing new bone to be formed (Reviewed in Rodan, 1992; Bronner, 1992; Price and Thompson, 1995). Bone resorption depends on the rate that osteoclasts are produced, the speed of which they function and their life span.

Osteoclast cells contain a nucleus, cytoplasm, golgi complex, mitochondria and transport vesicles filled with lysosomal enzymes. Bone resorption occurs within the osteoclast membrane called the ruffled border, which is surrounded by an organelle free region known as the clear zone. The ruffled border is highly convoluted with deeply enfolded finger like projections neighboring the mineralized surface of bone. The clear zone functions to maintain a favorable environment for bone resorption to occur. Osteoclast bone resorption depends on lysosomal enzyme secretion and an acidic environment. Protons via ATPase are secreted across the ruffled border membrane to dissolve the mineral and lysosomal proteases degrade the collagen matrix.

b) Osteoblast-bone forming cells

Osteoblasts are cuboidal, mononuclear spindle shaped cells of mesenchymal origin derived from stromal cells found in the bone marrow. These cells are responsible for the synthesis and deposition of the bone matrix, which eventually becomes mineralized. There are three forms of the osteoblast cell lineage, which include the progenitor osteoblasts or preosteoblasts, mature osteoblasts and osteocytes (Reviewed in Rodan, 1992; Bronner, 1992; Price and Thompson, 1995).

Preosteoblasts are precursor cells bound to become osteoblasts. They lie near bone forming surfaces where active mature osteoblasts are synthesizing bone. Osteoblasts consist of a round nucleus at the base of the cell, a strongly basophilic cytoplasm, golgi apparatus and a rough endoplasmic reticulum. They are located along the bone surface in clusters where they synthesize and secrete bone matrix proteins, such as type I collagen and non-collagenous proteins including osteopontin, osteonectin and

osteocalcin (Rodan, 1992). Osteoblasts deposit about 0.5 μm of matrix per day and their bone forming period lasts for about 100 days (Ericksen et al., 1990).

After bone formation has stopped at a particular site, osteoblast cells either become flat lining cells or osteocytes (Manolagas and Jilka, 1995). Bone lining cells protect bone surfaces from resorption. Osteocytes were originally osteoblast cells that became trapped in their own synthesis of bone matrix, which later became calcified (Raisz, 1993). They are found deeply embedded within bone in small lacunae where they communicate with each other via a system of canaliculi. Osteocytes are believed to be the sensor cells of bone that detect mechanical signals. They transmit signals to osteoblasts or osteoclasts, which in turn either lay down new bone or resorb the matrix.

Frost (1964) explained in a step-by step process the combined roles of osteoclast and osteoblast cells. Under normal conditions, osteoclasts adhere to bone and subsequently remove bone by acidification and proteolytic digestion. These cells degrade the bone surface for approximately three weeks eventually forming an erosion cavity. Following osteoclast resorption, osteoblasts invade the area and begin to deposit collagen (osteoid) at a rapid rate. At this time, the bone matrix is synthesized without corresponding mineralization and consequently a thick osteoid seam is produced. It may take osteoblasts three to four months to replenish the bone lost by osteoclast resorption. Eventually, the rate of mineralization increases to match collagen synthesis thereby maintaining a constant osteoid seam thickness. As mineralization proceeds, the rate of collagen synthesis decreases and the osteoid seam disappears.

Methods used to assess bone metabolism

a) Osteocalcin

Osteocalcin, also known as bone gamma-carboxyglutamate protein is a single chain polypeptide of 49 amino acids with a molecular weight of 5800 daltons. The protein consists of three residues of the vitamin K dependent, calcium binding amino acid, gamma carboxyglutamic acid at positions 17, 21 and 24 (Lian and Gundberg, 1988; Azria, 1989). These residues enable osteocalcin to bind to hydroxyapatite ($Ca_{10}H(PO_4)_6(OH)_2$), tiny crystals of carbonate-containing calcium phosphate (Azria, 1989; Risteli and Risteli, 1993).

The use of plasma osteocalcin as a biochemical marker of osteoblast activity and bone formation is becoming more widely accepted. Osteocalcin is found solely in bone tissue and dentine (Price and Thompson, 1995) and is the most abundant non-collagenous protein synthesized and secreted by osteoblast cells (Lian and Gundberg, 1988). The protein is incorporated into the bone matrix (Parviainen et al., 1991; Risteli and Risteli, 1993) in the late stage of differentiation and bone mineralization (Risteli and Risteli, 1993). Approximately 70% of the osteocalcin synthesized is secreted into blood where it can be measured in plasma by radioimmunoassy, and the rest is incorporated into the bone matrix (Lian and Gundberg, 1988; Risteli and Risteli, 1993; Price and Thompson, 1995). The circulating plasma levels of osteocalcin reflect a portion of newly synthesized protein that does not bind to hydroxyapatite, but rather was released directly into the blood stream (Lian and Gundberg, 1988; Rodan, 1992).

b) Cross-linked N-telopeptide of type I collagen

Bone resorption is measured by breakdown products of collagen. The excretion of cross-linked N-telopeptide of type I collagen (NTX) provides a direct and reproducible measure of osteoclast activity or bone resorption (Hanson et al., 1992; Price and Thompson, 1995; Arnaud, 1996; Tsukahara et al., 1998; Tsukahara et al., 1999). Tsukahara et al. (1999) and Naylor et al. (1999) showed that bone turnover assessed by measurement of urinary excretion of collagen cross links, pyridinoline and deoxypyridinoline and N-telopeptide of type I collagen (Naylor et al., 1999) are high at birth and preterm infants have higher bone turnover than term infants.

Type I collagen, a helical protein is the principal organic component of bone tissue comprising 90% of the organic matrix of bone tissue. Pyridinoline cross-links are formed at the N and C terminals of the collagen molecule. During osteoclast bone resorption, collagen is broken down and the cross-linking molecules are released and excreted in the urine where they can be measured by enzyme-linked immunosorbent assay (Price and Thompson, 1995; Arnaud, 1996; Garnero et al., 1996; Gfatter et al., 1997).

Dual energy x-ray absorptiometry

Dual-energy x-ray absorptiometry (DEXA) is a widely used technique in measuring bone mineral content of the whole body as well as at central skeletal sites, the lumbar spine and the femur. The machine uses two x-ray beams with different levels of energy. The absorption of each beam by bone is used to calculate bone mineral content

and density. DEXA offers excellent precision, low-radiation exposure and rapid scan acquisition (Koo and Hockman, 2000). Weiler, 2000 calculated the average coefficient of variation for triplicate scans of whole body, lumbar 2-4 and femur bone mineral content to be 3.8 %, 3.2 % and 1.2 %, respectively in piglets less than twenty-five days of age.

Calcium metabolism

The maintenance of plasma calcium concentration depends on dietary intake as well as intestinal absorption, renal calcium excretion and bone turnover. These three processes are closely regulated by calcium regulating peptide hormones including vitamin D, parathyroid hormone and calcitonin. 1,25 dihydroxycholecalciferol (1,25 $(OH)_2 D_3$), most active vitamin D metabolite functions in increasing intestinal calcium and phosphate absorption and renal reabsorption of calcium (Raisz, 1993). Receptors for 1,25 $(OH)_2D_3$ receptors are located on osteoblast cells.

Parathyroid hormone (PTH) is a single-chain polypeptide of 84 amino acids with a molecular weight of 9500. It is synthesized and secreted by chief cells of the parathyroid gland in response to low plasma calcium concentration. PTH regulates calcium and phosphorus concentration in extracellular fluids by binding to cAMP coupled plasma membrane surface receptors in the kidney and osteoblast cells (reviewed in Raisz, 1993; Boguslawski et al., 2000). PTH increases calcium and reduces phosphate concentration in the blood by:

1. stimulating osteoclasts to resorb bone mineral and release calcium and phosphate into the blood.

2. enhancing intestinal calcium absorption by stimulating synthesis of 1,25 dihydroxycholecalciferol in the kidney.
3. reducing urinary calcium excretion by stimulating renal reabsorption of calcium.
4. decreasing renal reabsorption of phosphate, thereby increasing its excretion.

The C cells of the thyroid gland synthesize calcitonin, thirty-two amino acid long peptide, when the concentration of calcium in the blood rises. Calcitonin binds to receptors located on osteoclasts and lowers blood calcium by inhibiting osteoclast bone resorption and hormones involved in bone resorption, such as PTH and vitamin D (reviewed in Raisz, 1993; Bowler et al., 1998).

The role of prostaglandin E_2 in bone metabolism

Prostaglandins are localized tissue hormones synthesized and released from bone cells. They perform autocrine (effecting the same cell that synthesized it), as well as paracrine (effecting adjacent cells) actions in bone, and other tissues, and thus they do not have to travel in the blood to reach their target. Prostaglandins are potent regulators of bone cell activity that may have both stimulatory and inhibitory effects on bone formation and resorption. Prostaglandin E_2 (PGE_2, $C_{20}H_{32}O_5$), a cyclooxygenase product of AA is currently one of the most widely investigated prostaglandins with respect to bone.

a) Prostaglandin E_2 synthesis

PGE_2 is synthesized by bone cells osteoblasts and osteocytes and by cells adjacent to the bone in the marrow and periosteal tissues via the cyclooxygenase pathway from

AA. The concentration of PGE_2 synthesized locally in bone is critical. At moderate levels, PGE_2 stimulates bone formation, but at higher levels it is inhibitory (Raisz and Fall, 1990; Watkins et al., 1997). The local production and secretion of PGE_2 by bone cells occurs in response to various stimuli, such as mechanical stress, cytokines, sex steroids and circulating hormones. Figure 3 describes the pathway for the synthesis of cyclooxygenase-derived eicosanoids.

Phospholipase A_2 is activated and AA is hydrolyzed from the sn-2 position and released from the phospholipid membrane. Prostaglandin endoperoxide H synthase enzymes are heme-containing glycosylated proteins that catalyze a cyclooxygenase reaction in which AA and O_2 are converted to cyclic 9-11 endoperoxide 15-hydroperoxide (PGG_2) and a peroxidase reaction in which PGG_2 undergoes a two-electron reduction to prostaglandin H_2 (PGH_2). PGH_2 is then converted to prostaglandins of the D, E, and F series, such as PGE_2, $PGF_2\alpha$, TXA_2 and PGI_2 (Smith et al., 1996). Phospholipase A_2 and prostaglandin endoperoxide H synthase enzymes represent the two rate limiting enzymes of the prostaglandin biosynthetic pathways.

b) Regulation of prostaglandin E_2 synthesis

PGE_2 synthesis is regulated by the:

1. concentration of AA in membrane phospholipids, which is modulated by dietary fatty acid composition
2. differential release of AA from membrane phospholipids and/or
3. altered activity of the prostaglandin endoperoxide H synthase enzymes

Whelan et al (1993) investigated whether dietary AA elevates phospholipid content of various tissues and whether the enrichment increases eicosanoid production. Syrian hamsters were divided into four groups and fed diets supplemented with ethyl esters of oleic acid, LA, AA or EPA for three weeks. The phospholipid content of AA was significantly higher in liver, lung, heart, spleen, kidney, testes, macrophages and platelets of hamsters fed the AA diet compared to rats receiving the other dietary groups. Rats fed the AA diet had the highest levels of eicosanoids, particularly PGE_2 and thromboxane B_2 in macrophages and platelets compared with cells from animals fed the other diets. The researchers suggested that feeding AA increases tissue AA levels and enhances AA derived eicosanoid synthesis.

The synthesis and release of PGE_2 from bone cells is regulated by a number of hormones, cytokines and growth factors that control AA release from the phospholipid membrane or alter prostaglandin endoperoxide H synthase enzyme activity. The inhibitors of PGE_2 synthesis inhibit the release of AA from the phospholipid bilayer and decrease prostaglandin endoperoxide H synthase enzyme synthesis, whereas the stimulators trigger AA release and increase prostaglandin endoperoxide H synthase enzyme activity (Kawaguchi et al., 1995).

Inhibitors of PGE_2 Synthesis	Stimulators of PGE_2 Synthesis
Interleukin 4	Arachidonic Acid
Glucocorticoids	Interleukin 1
Estrogen	Tumor necrosis factor
Androgen	Bradykinin
	Transforming growth factors α and β
	Basic Fibroblast growth factor
	Epidermal growth factor
	Parathyroid hormone
	Parathyroid-related peptide hormone
	1,25 dihydroxy vitamin D
	Thyroxin

c) Mechanism of action of prostaglandin E_2

Once PGE_2 is synthesized within a cell, it is not stored for very long but functions locally near the site of synthesis. At this time, it is uncertain whether prostaglandins can act in cells of tissues distant from the cells from which they were produced. PGE_2 exerts a wide variety of actions, which are mediated by specific G-protein coupled surface receptors. The multiple functions of PGE_2 indicate that there must be more than one receptor subtype present in cells. PGE_2 can bind to four distinct receptors known as EP_1, EP_2, EP_3 and EP_4, which are widely distributed in the body. The affinity constants or K_d of PGE_2 for these receptors range from 1-10 nM depending on the receptor subtype and tissue analyzed (Coleman, 1994). In determining which of the receptor subtypes mediate the various actions of PGE_2 in bone cells, specific agonists and antagonists have been developed (Table 4). Of the four PGE_2 receptors, osteoblast (Kasugai et al., 1995; Suda et al., 1996; Weinreb et al., 1999) and osteoclast cells (Mano et al., 2000) predominantly express EP_1, EP_2 and EP_4.

PGE_2 receptors are coupled to different signal transduction pathways, none of which are fully elucidated in bone. PGE_2 is coupled to both cyclic AMP (cAMP) and protein kinase C (PKC) signal transduction pathways. Some EP receptors act by increasing cAMP (EP_2 and EP_4), decreasing cAMP (EP_3) and some act through the phosphatidylinositol pathway (EP_1 and EP_3) to increase intracellular calcium concentration (Yamaguchi et al., 1988; Reich and Frangos, 1993; Bergmann and Schoutens, 1995; Bowler et al., 1998; Mano et al., 2000).

Several researchers have demonstrated that long-term daily administration of PGE_2 increased bone mass and stimulated bone formation in both human (Jorgensen et al., 1988) and rodent models (Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Jee et al., 1991; Ke et al., 1992; Akamine et al., 1992; Ito et al., 1993; Chen et al., 1998). These studies support that PGE_2 is an anabolic agent of bone in vivo, however the exact mechanism of its action is unknown. PGE_2 may alter bone metabolism either by its direct effect on formation and resorption or indirectly mediated by growth factors and cytokines (Chen et al., 1998).

Jee and colleagues (1985) showed that rats given daily subcutaneous injections of 1,3 and 6 mg PGE_2/kg/day for three weeks had increased growth cartilage thickness and proximal tibia metaphyseal bone mass compared to controls. The same researchers in 1987 demonstrated that rats treated for three weeks with 3 and 6 mg PGE_2/kg/day had an increase in trabecular bone and hard tissue mass along with elevated osteoblast precursor cells and osteoblast numbers, reduced osteoclast numbers and surface to volume ratio and increased woven bone formation in the proximal tibia metaphysis. The researchers suggested that PGE_2 increases bone mass by reducing resorption by decreasing osteoclast

numbers, increasing osteoblast numbers favoring bone formation and stimulating the replication and differentiation of osteoblast precursors to form more osteoblasts and subsequent formation of new woven bone.

Akamine et al. (1992) studied the effects of varying doses of PGE_2 on thirteen-month-old rats that were subjected to right hind limb immobilization. The rats were injected subcutaneously with 0, 1, 3 or 6 mg PGE_2/kg/day for two and six weeks. Daily injection of 1 mg PGE_2/kg/day activated woven bone formation after two weeks and decreased the bone-remodeling period after six weeks of treatment. Rats given 3 and 6 mg PGE_2/kg/day over two and six weeks had increased cancellous bone mass, activated woven bone formation, increased trabecular width, mineral appositional and bone formation rates, and suppressed bone remodeling period. Compared to untreated rats, plasma osteocalcin was higher in rats given 3 and 6 mg PGE_2/kg/day for two and six weeks suggesting elevated bone turnover, bone formation or both in PGE_2 treated animals. The researchers demonstrated that PGE_2 increases cancellous bone in a dose-response manner by stimulating bone formation associated remodeling, whereby formation exceeds resorption.

Jee et al. (1991) used single photon absorptiometry to measure bone mineral content, density and bone width of seven-month-old rats that were given 0, 1,3 and 6 mg PGE_2/kg/day for 60, 120 and 180 days. Bone width of rats was significantly increased after 180 days of treatment with 3 mg PGE_2/kg/day and after 60, 120 and 180 days with 6 mg/kg/day PGE_2. Bone mineral density was increased in rats given 3 mg PGE_2/kg/day after 60 and 120 days and after 60, 120 and 180 days with 6 mg/kg/day PGE_2. There was no difference observed in bone width and bone mineral density in tibia diaphyses of rats

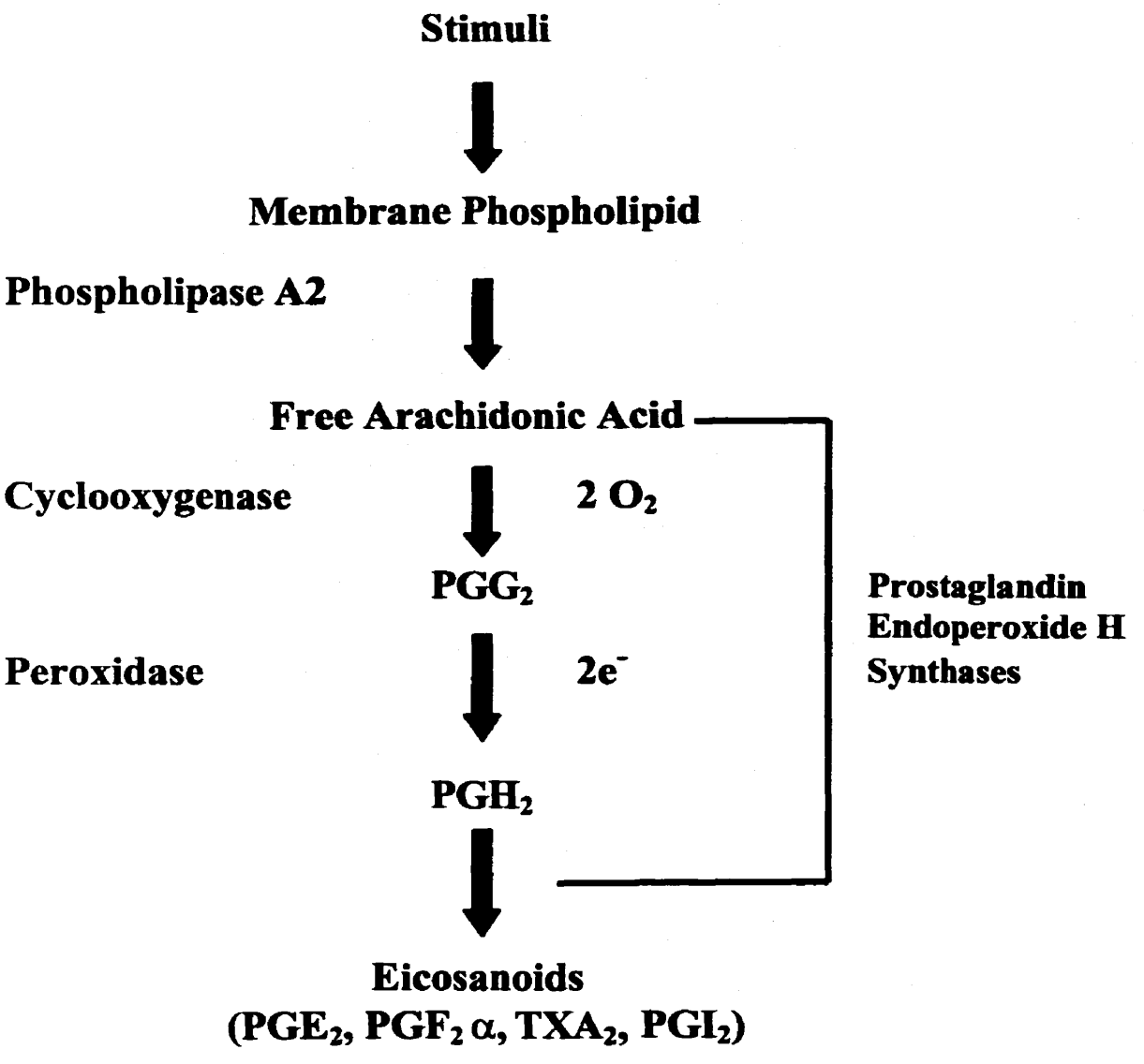
treated with 1 mg PGE_2/kg/day compared to controls. However, rats given this dose for 60 days had increased bone area, periosteal and corticoendosteal bone formation and activated corticoendosteal lamellar and woven trabecular bone formation compared to controls. The researchers demonstrated that 3 and 6 mg PGE_2/kg/day increased bone width and mineral density of rats after 60 days of treatment, which was maintained for an additional 120 days by continuous daily administration. Similarly, Ke and colleagues (1992) showed that the anabolic effects of PGE_2 on bone mineral density of seven-month-old rats could be maintained with daily administration of PGE_2 for 180 days. Compared with age-matched controls, rats given daily injections of 3 mg/kg/day PGE_2 had increased proximal tibia bone mineral density at 120 and 180 days and at 60, 120 and 180 days for rats given 6 mg/kg/day dose level.

Piglet model

Newborn piglets are useful animals in which to evaluate early n-6 and n-3 fatty acid requirements, as lipid metabolism and the fatty acid composition of milk from piglets are similar to that received by human infants. Other similarities existing between the newborn piglet and infant include intestinal maturation, fat digestion, absorption and transport, and similarity in essential nutrient requirements (Innis, 1991; Innis, 1993). In addition, piglets can easily adapt to milk formula as they are taught to eat within two days after removal from the sow.

Weight gain is faster in the newborn piglet in comparison to the human infant. The piglet may double or triple in body weight within two weeks (Weiler et al., 1995), whereas, a healthy infant's birth weight doubles by about four months of age and triples

Figure 3: Prostaglandin biosynthetic pathway



(Adapted from Smith et al., 1996)

Table 4: Agonists and antagonists of PGE_2 receptors and system of signal transduction

Receptor Subtype	Agonists	Antagonists	Transduction System
EP_1	Iloprost 17-phenyl PGE_2 Sulprostone	AH6809 SC-19220	↑ Intracellular Ca^{+2}
EP_2	Butaprost AH3205 Misoprostol	None	↑ cAMP
EP_3	Enprostil GR63799 Sulprostone Misorostol M & B 28767	None	↓ cAMP ↑ Intracellular Ca^{+2}
EP_4	None	AH22921 AH23848	↑ cAMP

(Adapted from Coleman et al., 1994)

by the age of one year. Although this indicates a difference between the piglet and human, the large body size and rapid growth of the piglet not only provides ample amounts of tissue for analyzing the effect of diet on bone metabolism, but also allows investigation over a short period of time. Thus, the piglet model was used in conducting the research for this thesis.

- SECTION II -

METHODOLOGY

II. METHODOLOGY

Animals and care

Thirty-six male Cotswold piglets were removed from the sow at three days of age and transported from the Glenlea Research Unit to the University of Manitoba animal holding facility. Piglets were randomized within litters (n=4 per litter X 9 litters) to one of four treatments (n=9 per treatment) for fifteen days: Fatty acid supplemented formula (FA; 0.8 % of total fatty acids as AA and 0.1 % of total fatty acids as DHA) plus PGE_2 (Cayman Chemical, Ann Arbor, MI) injections at 0.1 mg/kg/day, FA plus saline injections, standard formula (STD; n-6:n-3 of 8:1) plus PGE_2 injections or STD plus saline injections. No more than one piglet from anyone litter was assigned to a single diet and drug combination. Only two litters or eight piglets could be studied at any one time due to limited housing capacity.

A target sample size of thirty-six was calculated based on a previous study conducted in the same laboratory (Weiler, 2000) that found a mean difference in whole body BMC of 25 g with a standard deviation of 13 with the addition of 0.5 % AA and 0.1 % DHA into milk formula. On this premise, nine piglets per diet and drug combination were required to detect this mean difference with a power of 0.80 and 0.05 level of significance. Eight piglets from two litters were excluded from the total sample size due to illness from infection, and differences in growth, BMC and BMD in comparison to the remaining twenty-eight healthy pigs (presented in section 3 of thesis).

The piglets were housed individually in stainless steel metabolic cages in a

temperature (28-30 °C) controlled room. Prior to each feed time, piglets were permitted fifteen to twenty minutes of exercise in a large pen located outside of their cages. The animal care procedures were in accordance with the Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993) and were approved by the Fort Garry Campus Protocol Management and Review Committee.

Description of diet and drug treatment

Piglets began their respective diet and drug treatment on day five of life. Prior to commencing the study, three-day old piglets followed a two-day adaptation period to milk formula designed to support the rapid growth and development of the newborn piglet (National Research Council, 1998). The formula used provided 961-1050 Kcal/L of energy and contained 60.5 g/L protein, 58.5-65.5 g/L carbohydrate, 57.2 g/L fat, 2.4 g/L calcium and 1.8 g/L phosphorus (Ross Products Division Abbot Laboratories, Columbus, Ohio). The manufacturer did not provide information on the fatty acid composition of this formula and thus the fatty acid content was measured for this thesis using gas chromatography. The formula contained 22 % of total fat as LA, 2.4 % α LNA, no AA or DHA and a n-6 to n-3 fatty acid ratio of 8.9:1.0.

The standard diet consisted of a blend of coconut, soybean and safflower oils, provided in-kind by Ross Products Division Abbot Laboratories, Columbus, Ohio. The fatty acid supplemented diet contained the standard diet oil blend plus 0.8% wt/wt of the oil as AA (RBD-ARASCO: 40.6% AA) and 0.1% wt/wt of the oil as DHA (RBD-DHASCO: 40.0% DHA; Martek Biosciences Corp., Columbia, ML). Prior to feeding,

the diets were prepared by blending the specific oil mixtures into the milk formula using an electric hand mixer. Table 5 shows the fatty acid composition (in % wt/wt of total fatty acids) of the fatty acid supplemented (FA) and standard (STD) formulas fed to the piglets. The n-6 to n-3 fatty acid ratio (9:1) and the amount of LA and α LNA were comparable between the two diets studied. The formula was fed at 350 ml/kg/day and divided into three equal volumes and the oil mixtures were provided at 1.1 ml/kg/feed. For the duration of the study period, feeding times were scheduled at 0900, 1500 and 2100 hour.

PGE_2 (dinoprostone) powder was reconstituted in its original vial with 4 X 5 ml aliquots of absolute alcohol 100% v/v (Sabex, Dehydrated ethyl alcohol 7.89 g/10ml; Lot # 299506A, Exp. JN 98). The final concentration of the stock solution was 25 mg/ml. The stock solution (0.5 ml) was further diluted in sterile deionized water (8.4 ml). The solution was stored in glass vials at –20 °C until required for the study period. An intra-muscular injection of PGE_2 (0.1 mg/kg/d) was administered daily at 2100 hour, prior to feeding to piglets randomized to receive the treatment, whereas piglets not receiving the drug were given an intra-muscular injection of saline at an equal volume. Prior research administered higher doses of PGE_2 (1, 3 and 6 mg/kg/day) to rats subcutaneously (Jee et al., 1987; Mori et al., 1990; Akamine et al., 1992; Ke et al., 1992; Chen et al., 1998). Piglet skin is not very elastic and therefore injecting PGE_2 subcutaneously would be difficult. In addition, newborn piglets have very low fat mass and therefore distinguishing between subcutaneous and intramuscular is often inaccurate. As a result, PGE_2 was delivered to piglets intramuscularly.

The original dose of PGE_2 to be used in this study was 1 mg/kg/day. Two piglets from the first litter were given this dose on one occasion. Immediately following injection the piglets suffered from severe gastrointestinal distress that persisted for approximately two hours. Administration of 1, 3 and 6 mg PGE_2/kg/d to rats depresses body weight gain (Jee et al., 1991; Akamine et al., 1992; Ito et al., 1993) after only three weeks of treatment (Jee et al., 1985). The dosage of PGE_2 was then set at a low amount (0.1 mg/kg/d) to ensure that piglet whole body weight and length would not be affected.

Growth

On day five of life, baseline (day 1) body weight of piglets was measured using a digital scale equipped with an animal-weighing program (Mettler Toledo; SB32000, Columbus, Ohio). Each morning at 0900 hr, piglets were weighed prior to feeding. Daily body weight was used to adjust formula and oil intake, as well as PGE_2 and saline dosages given to piglets. The volume of formula consumed at each feed time was monitored and recorded. After each meal, any formula remaining in the tray was measured to calculate intake.

The following equation was used to calculate average weight gain over fifteen days of the study:

Average weight gain (g) = (Day 2 - Day 1 weight (g)) + (Day 3 - Day 2 weight (g)) +...(Day 16 - Day 15 weight (g))/15

Average weight gain (g) was corrected to average piglet body weight (kg) and expressed as g/kg/d. Average weight gain was corrected to average piglet body weight to account for differences in baseline body weight of piglets. On day sixteen, body weight and

length were measured while piglets were anaesthetized. Piglet body length was measured from the tip of the snout to the base of the tail using a plastic measuring tape. Body weight and length were expressed to the nearest gram and centimetre, respectively.

Collection of plasma, urine and tissues

On day one of study (0900 hr), blood was collected in heparin using an internal jugular blind stab technique from animals in the unfed state while piglets were restrained in a V-shaped holder. After fifteen days of study, twenty-day old piglets were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (Somnotol; 65mg/ml) at 30mg/kg. Formula feeding was withheld for twelve hours prior to anaesthesia. Blood was collected via cardiac puncture into heparinized syringes and centrifuged at 3000 RPM for fifteen minutes at 4 °C (Beckman TJ-6R table top centrifuge; Palo Alto, CA). Plasma was separated from red blood cells and then centrifuged again to remove any remaining red blood cells. The plasma samples were flushed with nitrogen and stored in glass vials at –20 °C prior to analysis of calcium, phosphorus, osteocalcin and fatty acids.

After blood was collected, the piglets were killed with an intracardiac injection of sodium pentobarbital (1 ml/2.3 kg). Liver was excised, frozen in liquid nitrogen and stored at –80 °C until analyzed for fatty acids. Urine was collected via bladder puncture and stored at –20 °C until measurement of calcium, phosphorus and N-telopeptide. A 1.0 g sample of tibia diaphysis was cut from each piglet using a bone saw. Periosteum was removed prior to rinsing the bone piece with saline solution to remove bone marrow.

TABLE 5: Fatty acid composition of standard (STD) or fatty acid supplemented (FA) formulas[1]

Fatty Acid (% wt/wt)	STD Formula	FA Formula
Saturated		
16:0	8.60 ± 0.01	8.68 ± 0.05
18:0	3.94 ± 0.01	4.05 ± 0.01
Monounsaturated		
16:1 n-9	0.01 ± 0.02	0.03 ± 0.00
18:1 n-9	39.69 ± 0.05	38.87 ± 0.26
n-6 PUFA		
18:2 n-6	22.14 ± 0.03	21.72 ± 0.13
20:4 n-6	0.00 ± 0.01	0.82 ± 0.12
Total n-6	22.15 ± 0.04	22.70 ± 0.27
n-3 PUFA		
18:3 n-3	2.45 ± 0.00	2.38 ± 0.02
20:5 n-3	ND	ND
22:6 n-3	0.04 ± 0.03	0.09 ± 0.01
Total n-3	2.49 ± 0.04	2.49 ± 0.03
n-6 : n-3 ratio	8.9:1.0	9.1:1.0

[1] Data are mean ± SD for three separate samples measured in duplicate.
ND indicates not detected.
PUFA- polyunsaturated fatty acids.

followed by a two-hour incubation in Hank's Balanced Salt Solution (Sigma Diagnostics; St. Louis, MO) in a 37 °C shaking water bath. After incubation, the bone was removed from solution for measurement of calcium and phosphorus and the fluid was stored at –20 °C until analyzed for PGE_2

Fatty acid analysis

Samples of liver, plasma, semi-purified oils (ARASCO and DHASCO) and formulas were extracted according to a modified method of Folch et al. 1956.

a) Liver tissue: Extraction and methylation

Duplicate 1.0 g samples of liver were extracted in 10 ml of 2:1 chloroform:methanol (Optima and pesticide grade; Fisher Scientific, Nepean, Ont.) containing 0.01% butylated hydroxytoluene (BHT) and 100 µl of heptadecanoic acid in chloroform (C17:0; 16 mg/ml) as the internal standard. The mixture was blended in a homogenizer at room temperature and the rotor was rinsed with 3 ml of methanol. The homogenate was vortexed and then centrifuged at 2000 RPM (International equipment company CS centrifuge; Boston, Mass.) for ten minutes. The pellet of liver tissue was removed and discarded and the solvent layer was re-extracted with 5 ml of sodium chloride (NaCl) solution (0.73%) and 6 ml of chloroform. The samples were vortexed and recentrifuged for ten minutes. After centrifugation the top layer, which contains the NaCl, methanol, water and water-soluble contaminants, was discarded. The walls of the tube were rinsed twice with 2 ml of theoretical upper phase (TUP; 3:48:47 chloroform:

methanol: water) and then the top layer was removed. The bottom layer was evaporated to dryness under nitrogen gas (N-EVAP, Associates Organomation, Inc, Berlin, MA) using a 30 °C water bath. After evaporation, the lipid was dissolved in 2 ml of chloroform, transferred to glass screw top vials and flushed with nitrogen gas prior to storage at –20 °C.

A 500 µl aliquot of extracted lipid was evaporated to dryness under nitrogen gas in a 30 °C water bath (N-EVAP, Associates Organomation, Inc, Berlin, MA). Following evaporation, the lipid was dissolved in 1 ml of toluene (Optima; Fisher Scientific, Nepean, Ont.) and 1.2 ml of methanolic hydrochloric acid (Supelco; Bellefonte, PA). The samples were vortexed, put into screw capped vials and then placed in an oven preheated to 80 °C for one hour. The samples were then left to cool at room temperature for ten minutes. Deionized water (1 ml) was added and the samples were vortexed and then centrifuged for five minutes. After centrifugation, the top layer was transferred to a clean tube and 1 ml of petroleum ether (Optima; Fisher Scientific, Nepean, Ont.) was added to the remaining bottom layer. The bottom layer was then vortexed and recentrifuged. The top layer was combined with the previously removed layer and separated with 2 ml of deionized water. The samples were vortexed and centrifuged for five minutes and the top layer was transferred to a gas chromatograph vial and evaporated under nitrogen gas in a dry bath (N-EVAP, Associates Organomation, Inc, Berlin, MA). Once the solvent had evaporated, the samples were dissolved in 200 µl of hexane (Optima; Fisher Scientific, Nepean, Ont) and stored at –20 °C until analysis.

b) Plasma: Extraction and methylation

Duplicate 0.5 ml samples of plasma were extracted in 10 ml of 2:1 chloroform:methanol (Optima and pesticide grade; Fisher Scientific, Nepean, Ont.) containing 0.01% BHT and 100 µl C:17:0 (1mg/ml). The mixture was vortexed and 2 ml of methanol was added followed by ten minutes of centrifuging at 2000 RPM (International equipment company CS centrifuge; Boston, Mass.). The solvent layer was transferred to a clean tube and vortexed with 4 ml of chloroform and 3 ml of sodium chloride (0.73%). After centrifugation, the top layer was removed and discarded. The walls of the tube were rinsed twice with 2 ml of TUP followed by removing the top layer. The bottom layer was then flushed with nitrogen gas and stored at –20 °C prior to methylation. The entire lipid extracted was evaporated and then methylated as per liver tissue with the exception that samples were dissolved in 100 µl of hexane.

c) Semi-purified oils (ARASCO and DHASCO): Methylation

Duplicate 0.11 g samples of ARASCO and DHASCO oils were diluted in 1 ml of hexane (Optima; Fisher Scientific, Nepean, Ont). A 0.1 ml sample of the diluted oil was transferred into a clean tube containing 100 µl of C17:0 (16 mg/ml). The samples were evaporated under nitrogen gas (N-EVAP, Associates Organomation, Inc, Berlin, MA) in a 30 °C water bath. Once the solvent had evaporated, 1 ml of toluene (Optima; Fisher Scientific, Nepean, Ont) and 1 ml of methanolic hydrochloric acid (Supelco; Bellefonte, PA) were added and the samples were vortexed and put into screw capped vials before being placed in an oven (80 °C) for one hour. After cooling, deionized water (1 ml) and

hexane (1 ml) were added and the samples were vortexed and then centrifuged for three minutes at 2000 RPM. The top layer was transferred to a clean tube, deionized water (1 ml) was added and the samples were vortexed and recentrifuged for three minutes. A portion of the top layer was transferred into a gas chromatograph vial for analysis.

d) Formula: Extraction and methylation

Triplicate samples of formula combined with either standard or fatty acid diet oil blend was mixed in a blender as was fed to piglets at one feed. Duplicate 1.0 g samples of each mixture were extracted as per liver tissue except that no C17:0 was added prior to extraction. After extraction, the lipid was dissolved in 5 ml of chloroform from which a 500 µl aliquot was combined with 100 µl of C17:0 (10 mg/ml) prior to methylation as per oils.

e) Gas chromatography analysis

Fatty acids were analyzed as methyl esters by gas chromatography (Varian Star 3400; Varian Canada Inc., Mississauga, Ontario) equipped with a 30 meter (0.25 mm I.D.) DB-225 column with 0.25 µm film thickness (J & W Scientific, Folsom, California). The injection volume varied from 0.5 to 4.0 µl depending on sample concentration. The following temperature program was used to run liver and plasma samples: Five minutes at 180 °C followed by increasing the temperature at a rate of 3 °C per minute until 220 °C was reached. The temperature was held for thirteen minutes,

increased at a rate of 10 °C per minute to 240 °C and then held at this temperature for 9.6 minutes. The total run time was forty-three minutes.

The temperature program used to run oil blends and formula was as follows: one minute at 70 °C and then raised to 180 °C at a rate of 20 °C per minute. The temperature was then increased at a rate of 3 °C per minute until 220 °C was reached. The temperature was held at 220 °C for fifteen minutes, then increased to 240 °C at a rate of 20 °C per minute and held at this temperature for 2.5 minutes for a total run time of thirty-eight minutes. Fatty acids ranging from carbon twelve to twenty-two were identified by comparison to retention times of standards and expressed as a percent of total fatty acids weight/weight (wt/wt). The principal fatty acids discussed in this thesis are LA (n-6 precursor of AA), α LNA (n-3 precursor of DHA and EPA), AA, DHA and EPA. All other fatty acids measured in formulas, plasma and liver tissue are provided in Appendix A, B and C, respectively.

Biochemical analysis of bone metabolism

a) Osteocalcin

Osteocalcin as a marker of osteoblast activity was measured in plasma collected on day one and day sixteen of study using a iodine^{125} (I^{125}) radioimmunoassay kit (INC STAR Corp., Stillwater, MN). All samples were analyzed in duplicate. Plasma (20 µl) was diluted in 180 µl of osteocalcin 0 standard. Rabbit anti-bovine osteocalcin antibody (200 µl) and I^{125} labelled bovine osteocalcin (200 µl) were added to 50 µl of diluted plasma. The samples were vortexed and then incubated at 4 °C for twenty-four hours.

After incubation, 500 µl of precipitating complex (normal rabbit serum pre-precipitated with goat anti-rabbit serum and polyethylene glycol) was added and the samples were vortexed and re-incubated for two hours at 4 °C. Following incubation, the samples were centrifuged for forty minutes at 1300 RPM (Beckman TJ-6R tabletop centrifuge; Palo Alto, CA). The supernatant was decanted and the precipitate was counted for ten minutes per sample using a gamma scintillation counter (Packard Cobra, D5002 Gamma Counter). Concentration of plasma osteocalcin was expressed as nmol/L. Serial dilutions (24.0 ng/ml to 0.75 ng/ml) were made to generate a standard curve. The standard curve (cubic spline/lowess) was generated using Graph Pad Prism Software (San Diego, CA) and the concentration of osteocalcin was interpolated from the curve.

Lot #:103266
Osteocalcin Control: 3.8-6.0 ng/ml (mean- 5.2 ng/ml)
Measured: 5.44 ng/ml
Recovery/Accuracy: 104 %

Lot #: 100360A
Osteocalcin Control: 3.6 – 5.8 ng/ml
Measured: 4.185 ng/ml

Recovery/Accuracy:

$$\left[\frac{4.185\ \text{ng/ml}}{(3.6 + 5.8\ \text{ng/ml}/2)}\right] \text{X } 100 = 89\ \%$$

b) Cross-linked N-telopeptide of type I collagen

Cross-linked N-telopeptide of type I collagen (NTX) in spot urine samples collected on day sixteen of study was measured in duplicate by competitive-inhibition

enzyme linked immunosorbent assay (Osteomark, Seattle, WA). The assay provides a quantitative measure of NTX excretion as a marker of osteoclast activity. Urine (25 µl) was added to micro wells pre-coated with NTX antigen and allowed to incubate for ninety minutes at room temperature in the presence of 200 µl horseradish peroxidase labelled mouse monoclonal antibody. During incubation, NTX in the urine and NTX on the micro wells compete for mouse monoclonal antibody binding sites. The concentration of NTX in the urine sample is therefore inversely proportional to the amount of antibody bound to NTX on the micro plate. Following incubation, the plate was rinsed five times with wash solution (350 µl) to remove unbound material. Hydrogen peroxide (200 µl) was added as substrate for horseradish peroxidase and the plate was then incubated for fifteen minutes at room temperature. The enzyme reaction was stopped with the addition of sulfuric acid (100 µl) and absorbence was read at 450 nm using a micro plate spectrophotometer (SPECTRA MAX 340; Molecular Devices, Sunnyvale, CA). Urinary NTX was corrected to urinary creatinine and expressed as µM NTX/mM creatinine. A calibration curve (quadratic) was obtained from assay calibrators, which are purified with NTX antigen in buffered diluent (1, 30, 100, 300, 1000, 3000 nM BCE) using Soft Max Pro software (Version 1.1, Molecular Devices Corporation, Sunnyvale, California).

Lot #: 208G01
Level 1 Control: 281-377 nM BCE (Mean 329 nM BCE)
Measured: 329.255 nM BCE
Recovery/Accuracy: 100 %

Level 2 Control: 1159-1397 nM BCE (Mean 1278 nM BCE)
Measured: 1156.822 nM BCE
Recovery/Accuracy: 91 %

Lot #: 056H02
Level 1 Control: 307-403 nM BCE (Mean 355 nM BCE)
Measured: 372.502 nM BCE
Recovery/Accuracy: 105 %

Level 2 Control: 1220-1458 nM BCE (Mean 1339 nM BCE)
Measured: 1271.207 nM BCE
Recovery/Accuracy: 95 %

C) Calcium and phosphorus

Tibia (0.1 – 0.5 g), urine (0.25 ml) and plasma (0.25 ml) collected on day sixteen of study were wet ashed in 0.5-1.0 ml of concentrated nitric acid for twenty-four hours followed by dilution with 9.5-20 ml of deionized water (5% nitric acid). Plasma samples required an additional twenty-four incubation and two-hours of heating prior to dilution. Total calcium and phosphorus concentration was determined by inductively coupled plasma optical emission spectroscopy (ICPOES; Varian Liberty 200, Varian Canada, Mississauga). The average coefficient of variation (CV %) for plasma was 19% and 48% for calcium and phosphorus, respectively. The average CV% for urine was 24% for calcium and 8% for phosphorus.

Urinary creatinine analysis

Urine samples must be normalized to accommodate differences in urine concentration and piglet body size. In this study, creatinine was used to normalize urine measurements. Creatinine concentration was determined colorimetrically (procedure no. 555-A; SIGMA DIAGNOSTICS, INC., St. Louis, MO) in spot urine samples collected at

the end of the study. All samples were analyzed in triplicate. Urine was incubated in alkaline picarate solution (5:1 picric acid: sodium hydroxide) at room temperature for twelve minutes. Following incubation, the absorbence was read at 500 nm using a micro plate spectrophotometer (SPECTRA MAX 340; Molecular Devices, Sunnyvale, CA). An acid reagent (mixture of sulfuric acid and acetic acid) was added to destroy the color derived from creatinine. The plate was re-incubated for five minutes and then absorbence was read again at 500 nm. Creatinine concentration is proportional to the difference in color intensity measured before and after acidification. The average CV% for all samples measured in triplicate was less than 8%.

Ex vivo prostaglandin E_2 release

PGE_2 in Hank's Balanced Salt Solution that had been cultured with tibia (preparation outlined on page 54) was measured in duplicate by competitive enzyme linked immunosorbent assay (R & D Systems, Inc., Minneapolis, MN). Hank's Balanced Salt Solution (100 µl), alkaline phosphatase labelled PGE_2 (50 µl) and mouse monoclonal antibody (50 µl) were incubated for two hours at room temperature in micro wells pre-coated with goat anti-mouse polyclonal antibody. During incubation, PGE_2 in the sample competes with alkaline phosphatase-labelled PGE_2 for mouse monoclonal antibody binding sites. The amount of antibody that binds to alkaline phosphatase-labelled PGE_2 is therefore inversely proportional to the concentration of PGE_2 in the sample. After incubation, the plate was rinsed three times with wash solution (200 µl) to remove excess alkaline phosphatase-labelled PGE_2 and unbound sample. P-nitrophenyl phosphate (200 µl), substrate for alkaline phosphatase was added followed by a one-hour incubation at

room temperature. Stop solution (50 µl of trisodium phosphate) was added and absorbance was read at 405 nm (SPECTRA MAX 340, Molecular Devices, Sunnyvale, CA). The concentration of PGE_2 is expressed as ng/g bone. A standard curve was generated using 50,000 pg/ml PGE_2 standard stock and assay buffer to produce a dilution series. 5000 pg/ml standard served as the high standard and the assay buffer served as the zero standard (0 pg/ml). A quadratic curve was constructed using Soft Max Pro soft ware (Version 1.1, Molecular Devices Corporation, Sunnyvale, California).

Bone mineral status

Piglet carcasses were frozen in the anterior-posterior position with limbs extended and transported to the dual energy x-ray absorptiometer (QDR4500W; Hologic Inc., Waltham, MA) located in the Manitoba Clinic. Single scans were performed to determine whole body, femur and lumbar spine (L1-L4) BMC, BMD and bone area. Analysis of whole body, lumbar spine and femur were conducted using whole body, low-density spine and sub-region array hip software programs (V8.20a:5), respectively. Whole body BMC was corrected to day sixteen body weight and length and expressed in g/kg and g/cm, respectively. Femurs were excised and freed of soft tissue for determination of weight (g) and length (cm) and then placed in a water bath for measurement of BMC, BMD and bone area. Femur BMC was corrected to day sixteen femur length (g/cm) and weight (g/kg) to account for differences in bone size.

Statistical analysis

Data was expressed as group means ± standard deviations, which were calculated on Microsoft Excel and then analyzed using SAS v.8 (Cary, North Carolina). Main and interactive effects of diet and drug were analyzed by two-way analysis of variance within a randomized complete block design to account for the effect of litter. Post Hoc multiple comparison analysis was used to identify differences between groups. The level of significance used was $P<0.05$ unless otherwise stated. Outcome measurements with skewed distributions were transformed using $\log_{10}$ and square root scales.

- SECTION III -

RESULTS

III. RESULTS

Growth

Eight piglets from two litters were excluded from the total sample size of thirty-six due to illness, which compromised growth of these animals. Average weight gain, end study (day 16) body weight and length of these eight piglets was 63.4 g/kg, 4.8 kg and 51.9 cm compared to 80 g/kg, 6.9 kg and 56.8 cm of the twenty-eight healthy piglets. These differences in growth had a large impact on BMC. For example, average whole body BMC of the eight piglets was 43.67 g compared to 64.37 g for the twenty-eight healthy piglets. In addition, two piglets from the first litter received 1mg/kg/day PGE_2 for one day. These piglets were not outliers and therefore were included in the analysis.

The means and standard deviations of piglet body weight, length and formula intake are presented in Table 6. Over fifteen days, piglets tripled their body weight (2.2 – 6.9 kg). There were no main or interaction effects of diet and drug on baseline (day 1) body weight, average formula consumption, weight gain over fifteen days, end study body weight and length.

Fatty acids

a) Plasma

Plasma fatty acids of piglets after fifteen days of study are presented in Tables 7, 8 and 9. There was no interaction effect of diet and drug on plasma fatty acids (Table 7),

however significant main effects were observed (Table 8 and 9). Piglets fed FA had lower LA (FA: 25.8 ± 1.7 vs. STD: 27.8 ± 2.6 % wt/wt, n=14 per group, P<0.05), yet higher levels of AA (FA: 10.9 ± 1.6 vs. STD: 8.9 ± 0.9 % wt/wt, n=14 per group, P<0.05) in plasma compared to piglets fed STD (Table 8). Total n-6, n-3, α LNA, EPA, DHA and total lipid were similar between the two diet groups.

Piglets that were given PGE_2 injections had higher levels of plasma EPA (PGE_2: 0.3 ± 0.1 vs. SALINE: 0.2 ± 0.1 % wt/wt, n=14 per group, P<0.05), DHA (PGE_2: 2.6 ± 0.4 vs. SALINE: 2.3 ± 0.5 % wt/wt, n=14 per group, P<0.05), total n-3 fatty acids (PGE_2: 4.9 ± 0.6 vs. SALINE: 4.5 ± 0.7 % wt/wt, n=14 per group, P<0.05) and total lipid (PGE_2: 1.2 ± 0.2 vs. SALINE: 1.0 ± 0.2 mg/ml, n=14 per group, P<0.05) compared to piglets given saline injections (table 9). Plasma levels of LA, AA, α LNA and total n-6 fatty acids were similar between PGE_2 and saline treated piglets.

b) Liver

Fatty acids measured in liver are presented in Tables 10, 11 and 12. There was no interaction affect (Table 10), however main effects of diet and drug were observed (Table 11 and 12). FA fed piglets had lower LA (FA: 15.7 ± 0.9 % wt/wt vs. STD: 16.9 ± 1.0, n=14 per group, P<0.05) and EPA (FA: 0.2 ± 0.1 % wt/wt vs. STD: 0.3 ± 0.1, n=14 per group, P<0.05) in liver compared to piglets fed STD (Table 11). There were no significant differences between diet groups in levels of AA, α LNA, DHA, total n-6 and n-3 fatty acids and total lipid.

Piglets treated with PGE_2 had higher levels of EPA in liver (PGE_2: 0.3 ± 0.1 vs. SALINE: 0.2 ± 0.1 % wt/wt, n=14 per group, P<0.05) compared to piglets given saline (Table 12). The levels of all other fatty in liver were similar between PGE_2 and saline treated piglets.

BONE METABOLISM

Results for biochemical markers of bone metabolism, plasma osteocalcin and urinary-N-telopeptide (NTX) and ex vivo PGE_2 release in bone organ culture are presented in Table 13. There was no interaction affect (Table13), however a main effect of drug was observed such that piglets given PGE_2 injections had significantly higher plasma osteocalcin concentration (PGE_2: 16.8 ± 4.8 vs. SALINE: 11.9 ± 3.3 nmol/L, n=14 per group, P<0.05) compared to saline treated piglets (Figure 4). Delta plasma osteocalcin, the difference between day one and day sixteen osteocalcin was greater in PGE_2 treated (PGE_2: 9.6 ± 4.7 vs. SALINE: 4.3 ± 4.4 nmol/L, n=14 per group, P<0.05) compared to saline treated piglets (Figure 5). There was no effect of diet on plasma osteocalcin concentration.

There was no interaction affect on urinary NTX and PGE_2 concentration (Table 13), however the distributions of these measurements were skewed. NTX and PGE_2 data were transformed using $\log_{10}$ and square root scales, respectively revealing a significant main effect of diet. Piglets fed FA had lower urinary NTX (Figure 6) (FA: 1.1 ± 0.2 vs. STD: 1.3 ± 0.2 umol/mmol, n=14 per group, P<0.05) and PGE_2 (Figure 7) (FA: 2.7 ± 0.5

vs. STD: 3.0 ± 0.5 ng/g tibia, n=14 per group, P<0.05) compared to piglets fed STD. There was no effect of drug on urinary NTX and PGE_2 concentration.

Calcium and phosphorus content in urine, plasma and tibia diaphysis are reported in Table 14. There was no interaction affect, however the urinary ratio of calcium to creatinine was lower in piglets given PGE_2 injections (PGE_2: 0.11 ± 0.08 vs. SALINE: 0.49 ± 0.50 mol:mol, n=14 per group P<0.05) compared to saline (Figure 8). There was no main effect of diet on urinary calcium concentration.

BONE MINERAL STATUS

Femur, whole body and lumbar (L1-L4) BMC, BMD and bone area are presented in Tables 15, 16 and 17, respectively. There was no interaction affect on femur (Table 15) and whole body (Table16) BMC and BMD. However after fifteen days, a significant diet and drug interaction effect was observed in lumbar spine such that piglets fed FA combined with PGE_2 injections had higher BMC (FA + PGE_2: 2.0 ± 0.5 vs. FA + SALINE: 1.7 ± 0.7 g, n=7 per group, P=0.059) and BMD (FA + PGE_2: 226 ± 34 vs. FA + SALINE: 195 ± 65 mg/cm^2, n=7 per group, P=0.057) compared to piglets fed the same diet but given saline (Table 17). In contrast, piglets fed standard formula combined with PGE_2 treatment had lower lumbar BMC (STD + PGE_2: 1.7 ± 0.4 g vs. STD + SALINE: 2.1 ± 0.5, n=7 per group, P=0.059) and BMD (STD + PGE_2: 188 ± 56 mg/cm^2 vs. STD + SALINE: 223 ± 27, n=7 per group, P=0.057) compared to STD diet, saline treated piglets (Table 17). There were no significant main effects of diet or drug on femur, whole body and lumbar BMC, BMD and bone area.

TABLE 6: Piglet body weight, length and formula intake[1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Day 1 Weight (kg)	2.245 ± 0.347	2.172 ± 0.367	2.230 ± 0.403	2.206 ± 0.275
Day 16 Weight (kg)	7.030 ± 1.577	6.705 ± 1.061	7.052 ± 1.498	6.642 ± 1.213
Weight Gain (g/kg/d)	80.7 ± 12.2	80.1 ± 10.4	82.2 ± 13.1	76.7 ± 10.2
Day 16 Length (cm)	56.9 ± 2.9	56.8 ± 2.5	58.0 ± 3.9	55.4 ± 2.8

[1] Data are mean ● SD, n=7 per group.

TABLE 7: Selected long-chain fatty acids in plasma of piglets fed either standard (STD) or fatty acid supplemented (FA) formula and treated with either saline or PGE_2 [1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
18:2 n-6	27.53 ± 3.48	28.00 ± 1.65	25.78 ± 1.76	25.73 ● 1.76
20:4 n-6	8.62 ● 0.92	9.19 ± 0.93	10.36 ± 2.14	11.37 ± 0.77
Total n-6	37.76 ● 3.54	39.04 ● 1.33	37.81 ± 3.53	38.88 ± 1.06
18:3 n-3	0.90 ± 0.23	0.93 ± 0.08	0.97 ± 0.19	0.98 ± 0.10
20:5 n-3	0.25 ± 0.11	0.35 ± 0.06	0.24 ± 0.06	0.32 ● 0.05
22:6 n-3	2.19 ± 0.30	2.53 ● 0.50	2.39 ± 0.70	2.73 ± 0.39
Total n-3	4.32 ● 0.66	4.84 ● 0.69	4.69 ± 0.645	5.02 ± 0.48
Total lipid (mg/ml plasma)	0.95 ± 0.24	1.25 ± 0.24	0.99 ± 0.25	1.14 ± 0.09

[1] Data are mean ± SD, n=7 per group.

TABLE 8: Main effect of diet on plasma fatty acids[1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula	FA Formula
18:2 n-6	27.76 ± 2.63 [a]	25.76 ± 1.69 [b]
20:4 n-6	8.90 ± 0.94 [a]	10.87 ± 1.63 [b]
Total n-6	38.40 ± 2.65	38.34 ± 2.57
18:3 n-3	0.91 ± 0.17	0.97 ± 0.14
20:5 n-3	0.30 ± 0.10	0.28 ± 0.07
22:6 n-3	2.36 ± 0.43	2.56 ± 0.58
Total n-3	4.58 ± 0.70	4.85 ± 0.57
Total lipid (mg/ml plasma)	1.10 ± 0.28	1.07 ± 0.19

[1] Data are mean ± SD, n=14 per group.
Values with different superscript letters ([a] and [b]) within rows are significantly different from each other by two-way ANOVA, $P<0.05$.

TABLE 9: Main effect of drug on plasma fatty acids[1]

Fatty Acid (% wt/wt of fatty acids)	SALINE	PGE_2
18:2 n-6	26.66 ± 2.80	26.86 ± 2.02
20:4 n-6	9.49 ± 1.82	10.28 ± 1.40
Total n-6	37.78 ± 3.40	38.96 ± 1.16
18:3 n-3	0.93 ± 0.21	0.95 ± 0.09
20:5 n-3	0.25 ± 0.08[a]	0.34 ± 0.06[b]
22:6 n-3	2.29 ± 0.53[a]	2.63 ± 0.44[b]
Total n-3	4.50 ± 0.66[a]	4.93 ± 0.58[b]
Total lipid (mg/ml plasma)	0.97 ± 0.24[a]	1.19 ± 0.18[b]

[1] Data are mean ± SD, n=14 per group.
Values with different superscript letters ([a] and [b]) within rows are significantly different from each other by two-way ANOVA, $P<0.05$.

TABLE 10: Selected long-chain fatty acids in liver of piglets fed either standard (STD) or fatty acid supplemented (FA) formula and treated with either saline or PGE_2 [1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
18:2 n-6	17.32 ± 1.01	16.44 ± 0.72	15.61 ± 0.98	15.70 ± 0.94
20:4 n-6	16.52 ± 1.57	16.28 ± 1.80	17.87 ± 1.62	16.89 ± 1.05
Total n-6	35.67 ± 1.37	34.59 ± 2.02	35.24 ± 0.83	34.30 ± 1.45
18:3 n-3	0.40 ± 0.17	0.43 ± 0.09	0.38 ± 0.09	0.49 ± 0.13
20:5 n-3	0.24 ± 0.04	0.31 ± 0.06	0.17 ± 0.08	0.25 ± 0.06
22:6 n-3	4.70 ± 0.49	4.89 ± 1.24	5.44 ± 0.74	5.04 ± 0.67
Total n-3	6.57 ± 0.46	6.89 ± 1.36	7.22 ± 0.77	6.99 ± 0.58
Total lipid (mg/g liver)	94.08 ± 11.62	81.89 ± 12.18	90.70 ± 15.64	91.22 ± 14.01

[1] Data are mean ± SD, n=7 per group.

TABLE 11: Main effect of diet on liver fatty acids [1]

Fatty Acid (% wt/wt of fatty acids)	STD formula	FA formula
18:2 n-6	16.88 ± 0.96 [a]	15.66 ± 0.92 [b]
20:4 n-6	16.40 ± 1.63	17.38 ± 1.41
Total n-6	35.77 ± 2.75	34.77 ± 1.24
18:3 n-3	0.42 ± 0.13	0.43 ± 0.12
20:5 n-3	0.28 ± 0.06 [a]	0.21 ± 0.08 [b]
22:6 n-3	4.79 ± 0.91	5.24 ± 0.71
Total n-3	6.73 ± 0.99	7.10 ± 0.67
Total lipid (mg/g liver)	87.98 ± 13.07	90.96 ± 14.27

[1] Data are mean ● SD, n=14 per group.
Values with different superscript letters ([a] and [b]) within rows are significantly different from each other by two-way ANOVA, $P<0.05$.

Table 12: Main effect of drug on liver fatty acids[1]

Fatty Acid (% wt/wt of fatty acids)	SALINE	PGE_2
18:2 n-6	16.47 ± 1.31	16.07 ± 0.89
20:4 n-6	17.19 ± 1.69	16.58 ± 1.45
Total n-6	35.45 ± 1.11	35.09 ± 2.89
18:3 n-3	0.39 ± 0.13	0.46 ± 0.11
20:5 n-3	0.20 ± 0.07 [a]	0.28 ± 0.07 [b]
22:6 n-3	5.07 ± 0.71	4.97 ± 0.96
Total n-3	6.90 ± 0.70	6.94 ± 1.01
Total lipid (mg/g liver)	92.39 ± 13.35	86.56 ± 13.51

[1] Data are mean ± SD, n=14 per group.
Values with different superscript letters ([a] and [b]) within rows are significantly different from each other by two-way ANOVA, $P<0.05$.

TABLE 13: Plasma, urine and bone biochemistry after fifteen days of diet and PGE_2 treatment[1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Day 1 Plasma Osteocalcin (nmol/L)	8.046 ± 3.741*	7.755 ± 1.913	7.403 ± 2.651	7.618 ± 4.013*
Day 16 Plasma Osteocalcin (nmol/L)	11.967 ± 3.094	17.430 ± 7.733	11.899 ± 3.767	16.102 ± 5.922
Delta Plasma Osteocalcin (nmol/L)	4.147 ± 3.247*	9.675 ± 3.123	4.496 ± 5.524	9.537 ± 6.363*
Urinary NTX/Creatinine (umol/mmol)	24.367 ± 15.941	19.970 ± 4.234	20.169 ± 12.508	11.700 ± 2.965
Ex vivo PGE_2 (ng/g bone)	9.832 ± 2.225	8.839 ± 3.569	8.486 ± 2.928	6.263 ± 2.324

[1] Data are mean ● SD, n=7 per group.
* Not enough day 1 blood collected from one pig (n=6).

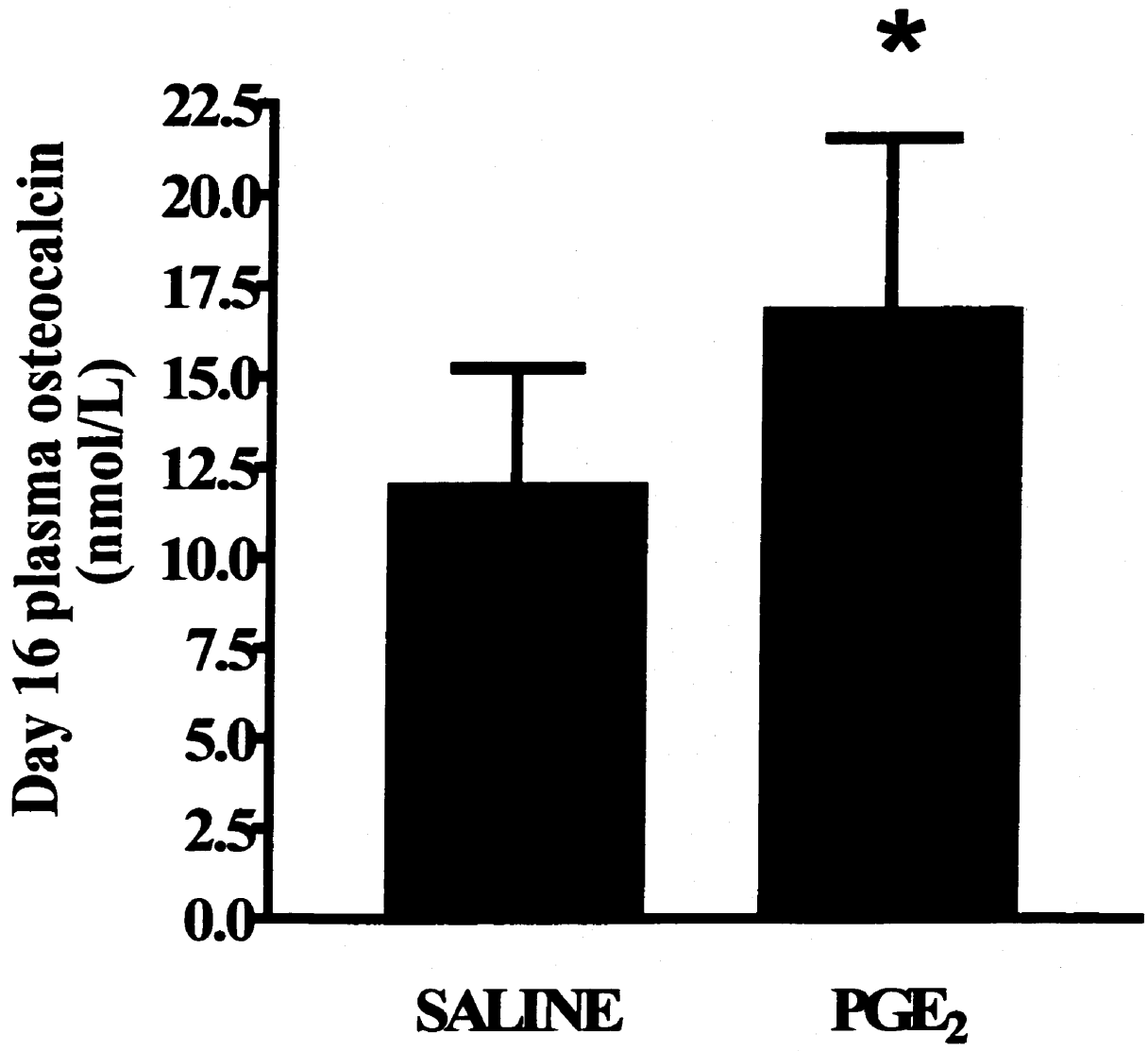


FIGURE 4: Plasma osteocalcin in piglets after fifteen days of treatment with either saline or PGE_2.

***indicates a significant difference using two-way ANOVA, n=14 per group, $P<0.05$.**

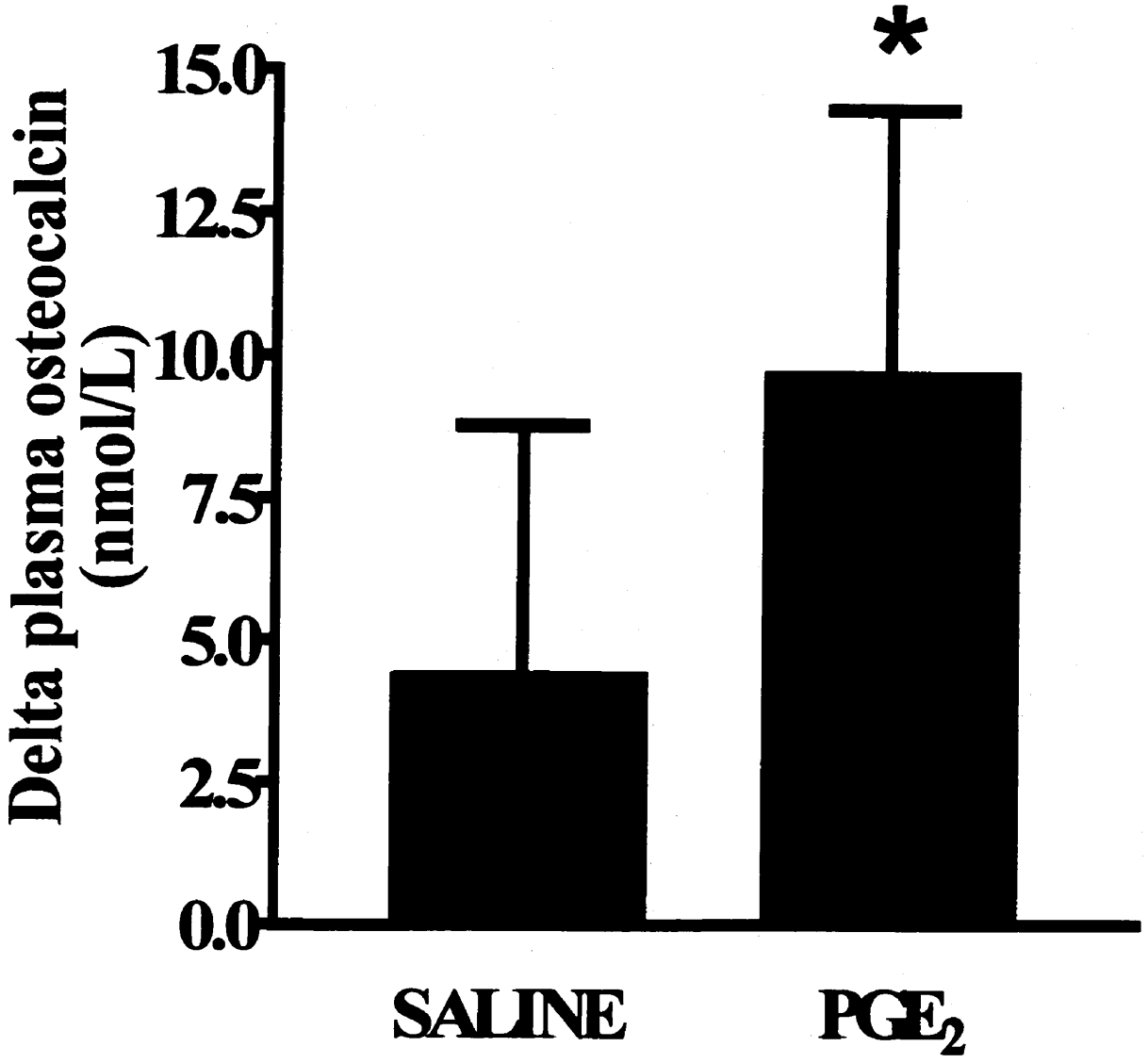


FIGURE 5: Delta plasma osteocalcin in piglets given either saline or PGE_2.

***indicates a significant difference using two-way ANOVA, n=14 per group, P<0.05.**

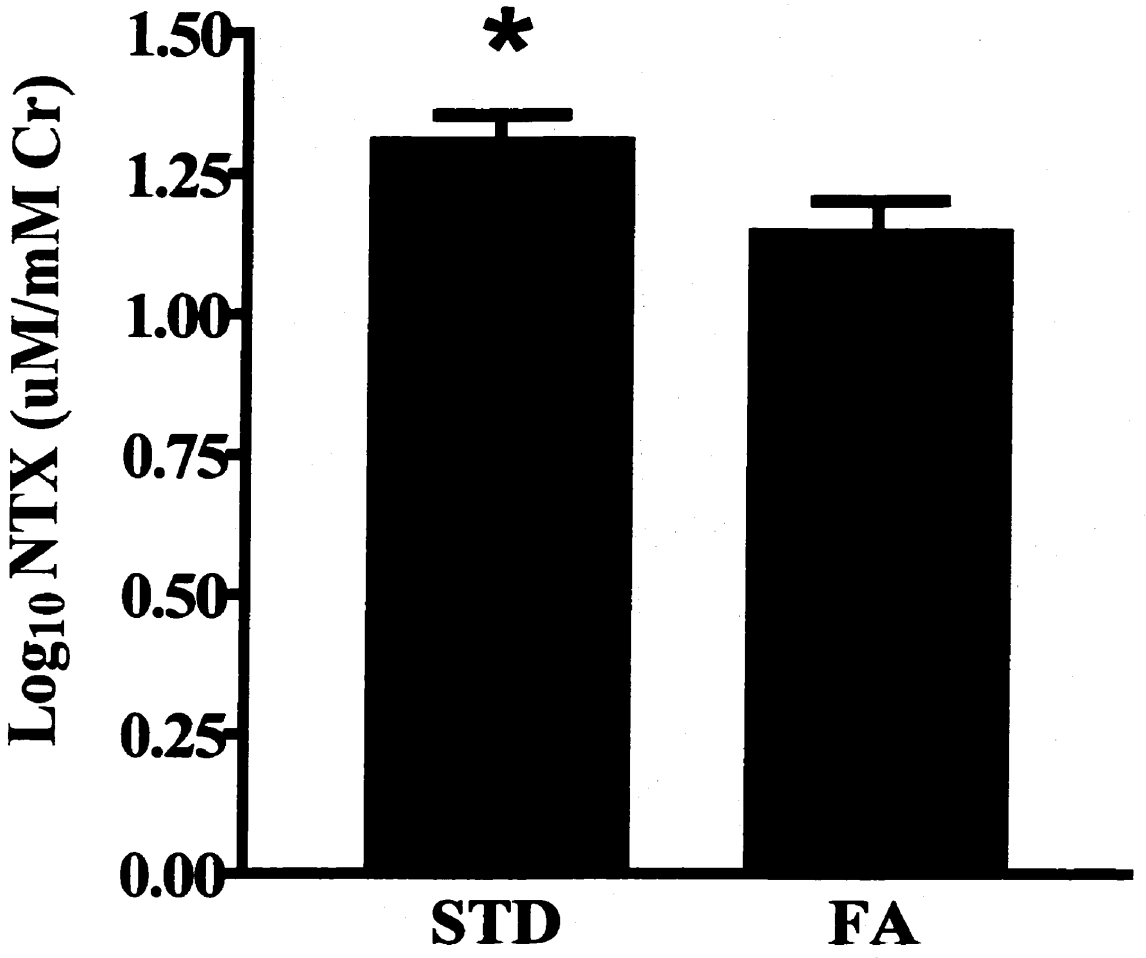


FIGURE 6: NTX in spot urine samples of piglets fed either standard (STD) or fatty acid supplemented (FA) formula for fifteen days.

*** indicates a significant difference using two way-ANOVA, n=14 per group, $P<0.05$.**

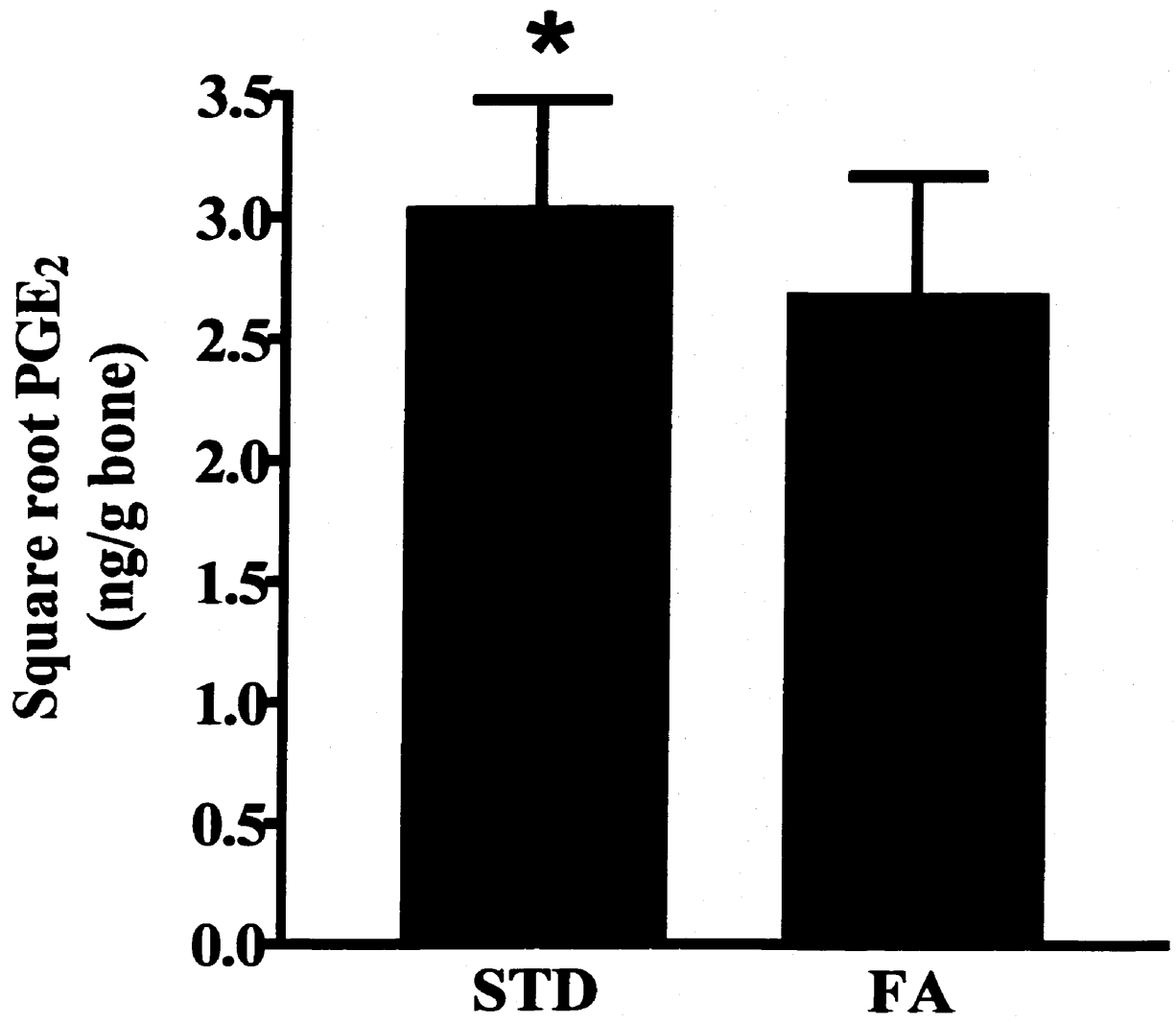


FIGURE 7: Ex vivo PGE_2 release in bone organ culture of piglets fed either standard (STD) or fatty acid supplemented (FA) fomula.

***indicates a significant difference using two-way ANOVA, n=14 per group, $P<0.05$.**

TABLE 14: Calcium and phosphorus concentration in urine, plasma and tibia samples after fifteen days of study[1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Day 16 Urinary Ca:Cr (mol:mol)	0.55 ± 0.54	0.12 ± 0.08	0.43 ± 0.50	0.09 ± 0.08
Day 16 Urinary P:Cr (mol:mol)	11.48 ± 8.19	9.24 ± 5.89	8.06 ± 6.86	6.30 ± 3.87
Day 16 Plasma Ca (mmol/L)	2.22 ± 0.40	2.24 ± 0.42	2.27 ± 0.55	2.14 ± 0.28
Day 16 Plasma P (mmol/L)	4.63 ± 2.61	3.88 ± 2.02	5.65 ± 3.52	7.32 ± 3.10
Tibia Diaphysis Ca (mg/g bone)	122.39 ± 28.37	126.32 ± 64.97	104.03 ± 36.25	145.61 ± 66.55
Tibia Diaphysis P (mg/g bone)	64.81 ± 18.16	65.03 ± 32.36	54.27 ± 18.74	74.85 ± 34.63

[1] Data are mean ± SD, n=7 per group; cr: creatinine

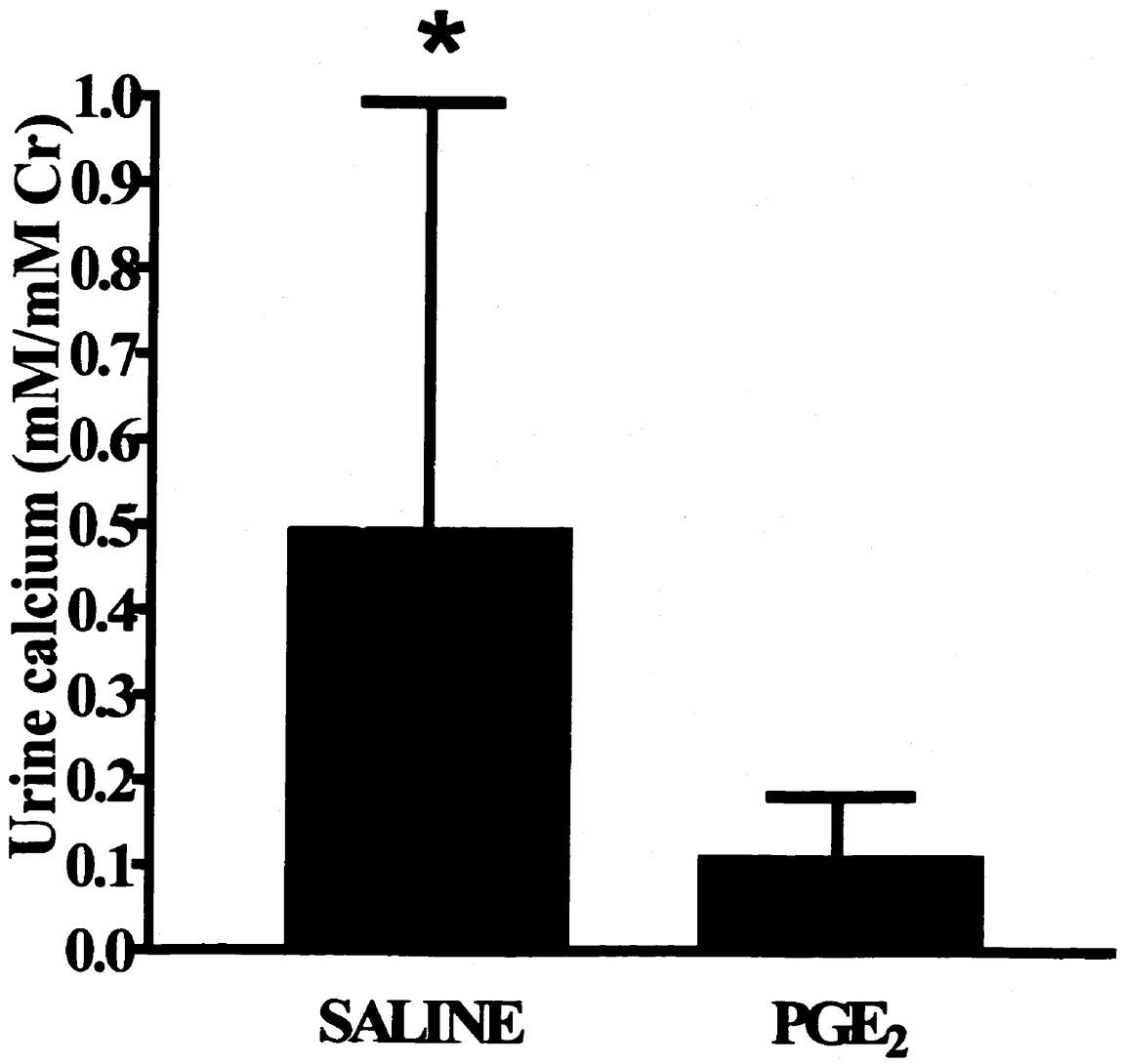


FIGURE 8: Calcium in spot urine samples of piglets after 15 days of treatment with either saline or PGE_2.

*** indicates a significant difference by two-way ANOVA, n=14 per group, $P<0.05$.**

TABLE 15: Femur BMC, BMD and bone area in piglets fed either standard (STD) or fatty acid supplemented (FA) formula and treated with either saline or PGE_2 [1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Femur BMC (g)	2.43 ± 0.80	2.67 ± 0.78	2.63 ± 0.97	2.24 ± 0.68
Femur BMC (g/kg)*	92.89 ± 8.88	100.09 ± 15.71	95.98 ± 18.58	91.10 ± 20.70
Femur BMC (g/cm)*	0.31 ± 0.08	0.33 ± 0.09	0.32 ± 0.10	0.28 ± 0.08
Femur BMD (mg/cm^2)	270 ± 31	282 ± 31	271 ± 38	258 ± 37
Femur Bone Area (cm^2)	8.84 ± 1.86	9.34 ± 1.81	9.52 ± 2.48	8.54 ± 1.54

[1] Data are mean ● SD, n=7 per group.
* BMC corrected to weight (kg) and length (cm) of femur bone.

TABLE 16: Whole body BMC, BMD and bone area in piglets fed either standard (STD) or fatty acid supplemented (FA) formula and treated with either saline or PGE_2 [1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Whole Body BMC (g)	68.84 ± 15.07	61.92 ± 14.25	63.20 ± 23.74	63.53 ± 19.44
Whole Body BMC (g/kg)*	9.90 ± 1.32	9.17 ± 0.99	8.81 ± 1.89	9.44 ± 1.56
Whole Body BMC (g/cm)*	1.20 ± 0.22	1.08 ± 0.21	1.08 ± 0.35	1.14 ± 0.31
Whole Body BMD (mg/cm^2)	468 ± 29	472 ± 57	465 ± 73	441 ± 41
Whole Body Bone Area (cm^2)	147.87 ± 34.14	131.17 ± 26.64	135.72 ± 44.52	143.62 ± 41.31

[1] Data are mean ± SD, n=7 per group.
* BMC corrected to body weight (kg) and length (cm) of piglet.

TABLE 17: Total lumbar BMC, BMD and bone area in piglets fed either standard (STD) or fatty acid supplemented (FA) formula and treated with either saline or PGE_2[1]

Parameter	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
Lumbar BMC (g)*	2.121 ± 0.499	1.727 ± 0.398	1.747 ± 0.655	2.016 ± 0.526
Lumbar BMD (mg/cm^2)*	223 ± 27	188 ± 56	195 ± 65	226 ± 34
Lumbar Bone Area (cm^2)	9.50 ± 1.70	9.41 ± 1.16	9.09 ± 1.92	8.87 ± 1.48

[1] Data are mean ± SD, n=7 per group.
* Effect of diet and drug interaction (P=0.059 and P=0.057 for BMC and BMD, respectively) by two-way ANOVA.

- SECTION IV -

DISCUSSION

IV. DISCUSSION

The fatty acid composition of plasma and soft tissues as influenced by dietary PUFA has been extensively investigated, however very little information is available on the effects of feeding formula supplemented with both AA and DHA on bone metabolism and mineral mass during early infancy. Both dietary AA and long-term administration of PGE_2 have been shown to stimulate bone formation and mineral density using rodent (Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Jee et al., 1991; Ke et al., 1992; Akamine et al., 1992; Ito et al., 1993) and piglet models (Weiler, 2000).

The purpose of this thesis was not to study PGE_2 as a possible therapy for use in humans or piglets, but rather to begin to clarify the mechanisms by which long-chain PUFA, particularly dietary AA, might affect bone metabolism and subsequent mineralization. Whether or not dietary AA and PGE_2 share the same mechanism is also unknown. No study has investigated the combined effects of diet and PGE_2 on bone during periods of rapid growth and development to see if the response is similar. It was hypothesized that dietary AA would support greater PGE_2 synthesis and elevate BMC and BMD similar to that observed with low dose PGE_2 injection. The primary objective of this thesis was to compare the effects of dietary AA versus PGE_2 on 1) the fatty acid composition of plasma and liver tissue, 2) bone cell metabolism, 3) ex vivo PGE_2 release, and 4) bone mineral content and density of the new born piglet.

Growth

Feeding formula with 0.8 % AA and/or administration of 0.1 mg/kg/d PGE_2 for fifteen days did not limit nor elevate growth of piglets as indicated by no differences between groups in weight gain or weight and length by the end of study. This contrasts other reports where administration of 1, 3 and 6 mg/kg/d PGE_2 depressed body weight gain of rats after sixty days (Jee et al., 1991; Ke et al., 1992; Ito et al., 1993) and after three weeks of treatment (Jee et al., 1985). In the present study, although piglets treated with 0.1 mg/kg/d PGE_2 had mild diarrhea that persisted for approximately twenty to thirty minutes after injection, they gained weight similar to pigs given saline. In contrast to Weiler (2000), there were no beneficial effects shown with supplementing AA and DHA into formula on piglet growth. Weiler (2000) showed elevated body weight of piglets fed formula supplemented with 0.5 % AA and 0.1 % DHA for fourteen days compared to those fed unsupplemented diet. However, Weiler studied older piglets (ten days of age) and the formula contained a lower amount of dietary fat (37g/L) that could have resulted in a greater effect of the supplement on growth.

Fatty acids in plasma and liver tissue

There were no differences in total n-6 and n-3 fatty acids, α LNA, EPA, DHA or total lipid in plasma between piglets fed the fatty acid supplemented or standard diets. Supplementing formula with 0.8 % AA and 0.1 % DHA resulted in lower LA, yet higher levels of AA in plasma compared to feeding standard formula containing LA and α LNA.

These observations are consistent with previous studies conducted by others (Whelan et al., 1993; Boyle et al., 1998; Weiler, 2000).

Feeding diets supplemented with AA to rats for fifteen days (Boyle et al., 1998) and hamsters for three weeks (Whelan et al., 1993) resulted in higher levels of AA in plasma and liver (Boyle et al., 1998) and in liver, lung, heart, spleen, kidney, testes, macrophages and platelets (Whelan et al., 1993) compared to those fed diets with only LA. Weiler (2000) showed that piglets fed formula for fourteen days with 0.5 % AA and 0.1 % DHA had lower levels of LA and higher AA in liver compared to those fed unsupplemented diet. In addition, bone AA content was elevated in piglets fed fatty acid supplemented formula.

The fatty acid composition of bone was not measured in the present study. Since AA is a precursor to PGE_2, a significant elevation in its concentration in bone (Alam et al., 1993; Weiler, 2000) may result in increased levels of PGE_2 in bone (Alam et al., 1993; Watkins et al., 1996; Watkins et al., 1997; Watkins et al., 2000), which in turn could affect metabolism.

The lower levels of plasma LA observed in the present study suggest that the enrichment of AA in plasma was at the expense of LA (Whelan et al., 1993). The interpretation regarding the importance of the reduction in plasma LA as a result of feeding AA is unknown. Tissue AA content is derived from dietary LA when LA is the sole n-6 PUFA in the diet. As dietary AA content increases, it becomes the predominant source of tissue AA by inhibiting the conversion of LA to AA. Since the formula studied contained 0.8 % AA, it is possible that the contribution of dietary LA as a precursor for tissue AA may have decreased (Figure 9, Whelan et al., 1993).

Liver content of AA, α LNA, DHA, total n-6 and n-3 fatty acids and total lipid were similar between the two dietary groups. As observed in plasma, feeding piglets formula with 0.8 % AA and 0.1 % DHA resulted in lower levels of LA in liver. Dietary AA and DHA did not increase AA levels in liver, but rather decreased EPA content with no change to DHA. Similarly, Dela Presa-Owens et al., 1998 demonstrated that feeding piglets from birth to eighteen days of life a formula with (in % of total fatty acids) 20% LA, 2% α LNA and 0.8% AA, with no DHA resulted in higher AA content in plasma, liver, heart and kidney tissue, however lower plasma DHA and reduced levels of EPA in liver, heart, and kidney. In addition, the decrease in liver EPA rather than DHA in the present study after feeding formula with 0.8 % AA and 0.1 % DHA suggests preferential formation of DHA from α LNA or conservation of tissue DHA during inhibition of DHA synthesis (De la Presa-Owens et al., 1998).

There were no differences between PGE_2 and saline treated piglets in plasma LA, AA, total n-6 fatty acids or α LNA. Administration of 0.1 mg/kg/d PGE_2 for fifteen days resulted in elevated total n-3 fatty acids, EPA, DHA and total lipid in plasma of piglets compared to those given saline. Piglets that received PGE_2 at 0.1 mg/kg/d also had higher levels of EPA in liver compared to those that were given saline. Elevated n-3 fatty acids in plasma and liver after administration of PGE_2 is difficult to interpret since no other study has investigated the effects of exogenous PGE_2 on plasma and tissue fatty acid content.

The present study demonstrated that dietary AA and DHA and PGE_2 treatment affected plasma and liver fatty acids differently. Feeding formula with 0.8 % AA and

0.1% DHA elevated AA in plasma and decreased EPA in liver, whereas PGE_2 increased n-3 fatty acids.

The observed main effects of diet and drug on plasma and liver fatty acids were small. For example, the mean value of plasma LA of piglets fed FA formula was 25.76 % compared to 27.76 % in those fed STD diet (Table 8), a 7 % reduction. Another example is the small difference observed in EPA content of liver in piglets fed FA or STD formula. The mean value of liver EPA of piglets fed FA formula was 0.21 % compared to 0.28 % in those fed STD diet (Table 11), a 25 % reduction. Although statistically significant, the small difference in the means of these two diet groups questions whether the effects are biologically significant. It is quite possible that the main effects of diet and drug observed in plasma and liver fatty acids are not biologically significant. However, the main purpose for measuring the fatty acid composition of plasma and liver tissue was in ensuring that piglets were responsive to dietary fat supplementation.

Bone metabolism

a) Osteocalcin

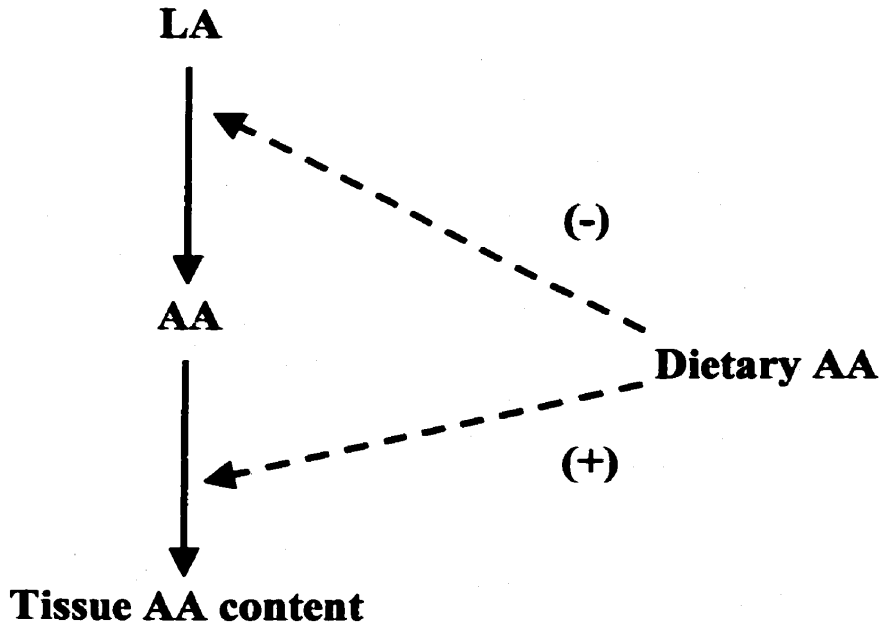
Administration of PGE_2 at 0.1 mg/kg/d for fifteen days resulted in elevated bone formation and/or bone turnover, as indicated by increased plasma osteocalcin of piglets treated with PGE_2. This is consistent with other studies that have described the anabolic effects of PGE_2 on bone formation (Akamine et al., 1992; Ito et al., 1993). Akamine et al. (1992) and Ito et al. (1993) demonstrated that rats treated with 1,3 and 6 mg

PGE_2/kg/day had elevated plasma osteocalcin after two and six weeks (Akamine et al., 1992) and after 60, 120 and 180 days of treatment (Ito et al., 1993) compared to untreated rats. In the present study it was not possible to determine how soon after the administration of PGE_2 that bone formation was elevated. This response may have been detected sooner if earlier time periods were studied. Feeding formula with 0.8 % AA and 0.1 % DHA did not affect bone formation since there were no differences in plasma osteocalcin concentration between the two dietary groups. It is clear from these observations that the diet studied and PGE_2 do not affect bone metabolism in the same manner.

b) Cross-linked N-telopeptide of type I collagen

The supplementation of formula with 0.8 % AA and 0.1 % DHA suppressed bone resorption, as indicated by the lower concentration of NTX in urine of piglets fed the fatty acid supplemented diet. Kruger et al. (1997) used eleven week old ovariectomized female rats to study the relationship between feeding varying dietary ratios of GLA:EPA + DHA (9:1, 3:1, 1:3, 1:9) on bone turnover and bone calcium. After six weeks of feeding, red blood cell levels of GLA and DGLA increased in rats fed the 9:1 and 3:1 diet. EPA and DGLA red blood cell levels increased in rats fed 1:3 and 1:9 diets, however AA decreased. DGLA was positively correlated with calcium concentration in the femur and negatively correlated with deoxypyridinoline, marker of bone collagen degradation, indicating an anabolic effect of this n-6 fatty acid on bone. On the basis of the findings from Kruger and colleagues (1997) as well as the observations from this

FIGURE 9: The effects of dietary arachidonic acid (AA) on linoleic acid (LA) metabolism and tissue AA content



(Adapted from Whelan et al., 1993)

thesis, it appears that while PGE_2 elevates formation, dietary PUFA effect bone through alterations in bone resorption rather than formation. In contrast, Watkins and colleagues demonstrated elevated plasma alkaline phosphatase activity, a non-specific marker of bone metabolism that is used to detect metabolic bone disorders in four day old chicks (1996) and twenty-one day old rats (2000) fed diets high in n-3 fatty acids or a low ratio of n-6:n-3 (1.98 and 1.19, respectively) compared to animals fed a high n-6 diet or high ratio of n-6:n-3 diet (7.19 and 23.76). The administration of 0.1 mg/kg/d PGE_2 did not affect bone resorption as there were no differences observed in NTX concentration between PGE_2 and saline treated piglets.

c) Urinary calcium excretion

The administration of 0.1 mg/kg/d PGE_2 for fifteen days reduced urinary calcium excretion. Urinary excretion is typically low during periods of rapid skeletal growth (Anderson, 2000). Lower urinary calcium may indicate increased calcium retention in bone, however there were no differences in calcium content of tibia between PGE_2 and saline treated piglets. It is possible that the 0.1 mg/kg/d dose level was to low or the experimental design was insufficient in length to observe elevated bone calcium with PGE_2 treatment. Previous studies in male rats indicated that at least three weeks of PGE_2 treatment at 3 and 6 mg/kg/d is needed to increase trabecular and hard tissue mass (Jee et al., 1987). Intestinal calcium absorption was not assessed in the present study. Elevated intestinal calcium absorption and urinary calcium occurs simultaneously with increased bone calcium levels (Kruger et al., 1995). Therefore, the reduced urinary calcium with

no differences in bone calcium content may suggest limited intestinal absorption in PGE_2 treated animals.

Feeding formula supplemented with 0.8 % AA and 0.1 % DHA did not effect urinary calcium excretion. Similarly, Weiler (2000) demonstrated no difference in urine calcium, bone calcium content or intestinal calcium absorption between piglets fed formula with 0.5 % AA and 0.1 % DHA and those fed unsupplemented diet. The researcher suggested that the lack of difference in intestinal absorption might be due to the already high rate of calcium absorption observed in growing piglets (Weiler et al., 1995). In contrast, Kruger et al. (1995) showed in twenty-one day old rats that dietary PUFA influence intestinal absorption, urinary excretion and bone calcium content. After six weeks of feeding from weaning, rats that were fed 3:1 (GLA:EPA + DHA) diet had increased intestinal calcium absorption, urinary calcium and bone calcium content compared to those fed 1:1 and 1:3 (GLA:EPA + DHA) and control diet (LA:α LNA of 3:1).

Ex vivo prostaglandin E_2 release

AA is the precursor to PGE_2 and therefore it was hypothesized that piglets fed formula with 0.8 % AA and 0.1 % DHA would have elevated ex vivo PGE_2 synthesis compared to those fed the unsupplemented diet. In contrast to Whelan et al., 1993 and Watkins et al., 1996, 1997 and 2000, piglets fed fatty acid supplemented formula had lower ex vivo PGE_2 release in tibia diaphysis compared to piglets fed the unsupplemented diet. Whelan and colleagues (1993) showed that hamsters fed diets supplemented with AA produced the highest levels of PGE_2 in macrophages compared to

those fed oleic acid, LA and EPA diets for three weeks. Watkins showed that chicks (1996, 1997) and rats (2000) fed diets rich in n-6 fatty acids for nineteen, sixteen and forty-two days, respectively had lower EPA, yet higher AA and ex vivo PGE_2 synthesis in liver and bone.

Weiler (2000) demonstrated that although piglets that were fed formula with 0.5 % AA and 0.1 % DHA had higher AA content in liver and cortical bone, urinary PGE_2 concentration was not different between dietary groups. The researcher suggested that dietary AA and the resulting AA status did not provide sufficient competition for cyclooxygenase between AA and EPA.

There are a number of speculations that can be made in interpreting the lower PGE_2 release observed with feeding piglets AA and DHA in the present study. The reduced synthesis of PGE_2 suggests that the response of bone to dietary AA may have not been due to endogenous PGE_2 synthesis (Watkins et al., 1996). It is also possible that the fifteen-day experimental period may have not been long enough to observe elevated PGE_2 synthesis. Elevated ex vivo PGE_2 release was shown within only sixteen days of feeding one day old chicks a soybean oil diet (Watkins et al., 1997). However, the soybean oil diet studied by these researchers contained a higher n-6 fatty acid content (50 mol/100mol with a n-6:n-3 ratio of 7.6), therefore it is not surprising that this diet elevated ex vivo tibia PGE_2 release.

Formula was withheld for approximately twelve to fifteen hours prior to measuring ex vivo PGE_2 release from bone. It is possible that the AA content of bone may have decreased during the fast resulting in lower PGE_2 release. It is also possible that the amount of dietary AA used in this study (0.8 % of total fatty acids) may have

elevated the amount of PGE_2 in bone organ culture at one point in the study. For example, elevated PGE_2 on day seven of study may have down regulated prostaglandin endoperoxide H synthase enzymes by negative feedback inhibition resulting in lower PGE_2 after fifteen days. Future research is needed to measure the amount of PGE_2 synthesized endogenously by labelling AA using radioisotopes and/or by measuring varying end points.

In addition to PGE_2, AA is also the precursor of leukotrienes by the lipoxygenase pathway, and these eicosanoids may also play an important role in the local regulation of bone metabolism. It has been reported that the inhibition of cyclooxygenase activity stimulated the lipoxygenase pathway resulting in elevated leukotriene production (Bach et al., 1982; Raisz and Martin, 1993). In the present study, it is possible that lipoxygenase metabolism of AA may have exceeded cyclooxygenase activity resulting in lower ex vivo PGE_2 release after feeding formula with 0.8 % AA.

The administration of 0.1 mg/kg/d PGE_2 did not effect ex vivo PGE_2 release in tibia diaphysis. To the author's knowledge, this is the first study conducted that investigated the effect of exogenous PGE_2 on ex vivo PGE_2 release. PGE_2 was administered to piglets at 9:00 p.m. and samples were processed the next morning. Although the plasma half-life of PGE_2 is short, it is possible that dosing once daily was not enough to alter PGE_2 synthesis in bone. The above interpretations are merely speculations and therefore require future investigation.

Bone mineral status

The supplementation of formula with 0.8 % AA and 0.1 % DHA and providing 0.1 mg/kg/d PGE_2 resulted in similar BMC, BMD and bone area of lumbar spine, femur and whole body of piglets. The combination of feeding piglet's fatty acid supplemented formulas plus PGE_2 injections resulted in higher lumbar BMC and BMD compared to feeding the same formula, but with saline treatment. In contrast, feeding the unsupplemented formula combined with PGE_2 injections resulted in lower lumbar BMC and BMD compared to providing the unsupplemented diet with saline treatment.

The diet and drug interventions synergistically elevated BMC and BMD of the lumbar spine. The mechanism behind this observation is that the addition of AA and DHA in formula was shown to lower bone resorption and the administraton of PGE_2 resulted in higher bone formation (Figure 10). Numerous studies have shown that PGE_2 elevates trabecular bone mass by stimulating bone formation associated remodeling, whereby formation exceeds resorption, and by shortening the period of bone remodeling (Jee et al., 1987; Mori et al., 1990; Akamine et al., 1992; Ke et al., 1992; Chen et al., 1998). Jee et al. (1987) studied the effects of injecting rats with PGE_2 for three weeks to elucidate the role of bone cells in increasing hard tissue mass. The researchers showed that giving 3 and 6 mg PGE_2/kg/d increases trabecular and hard tissue bone mass by depressing resorption and stimulating the replication and differentiation of osteoblast precursors to form new woven bone.

Similarly, Ke and colleagues (1992) demonstrated in seven-month old rats that in vivo PGE_2 administration at 1,3 and 6 mg /kg/day for 60, 120 and 180 days shortened the entire bone remodelling cycle from 45 days in control to 35 days in treated animals.

PGE_2 stimulated bone modelling by producing new woven trabeculae and increased both bone formation and resorption, but shifted bone balance in a positive direction in favour of formation.

Summary of major findings

It was hypothesized that dietary AA and DHA would support greater PGE_2 synthesis and elevate BMC and BMD similar to that observed with low dose PGE_2 injection. Feeding formula with 0.8 % AA and 0.1 % DHA did not support greater PGE_2 synthesis. In addition, dietary AA and DHA and 0.1 mg/kg/day PGE_2 resulted in similar BMC and BMD of the lumbar spine as opposed to the hypothesized elevation.

The present study demonstrated that feeding formula with 0.8 % AA and 0.1 % DHA and administration of 0.1 mg/kg/d PGE_2 resulted in similar BMC, BMD and bone area of the lumbar spine, femur and whole body of piglets, however the mechanism by which they affected fatty acid status and the activities of bone formation and resorption were different (Figure 11).

Strengths and limitations

This was the first study to compare the effects of dietary PUFA versus PGE_2 administration on bone metabolism and mineralization. The piglet model offered a significant advantage of observing alterations in bone metabolism in a short period of time. The measurement of bone markers and fatty acids were conducted in duplicate, and recovery of measurements, such as osteocalcin (104 %) and NTX (95-105 %) were high.

In addition, piglet body weight and length, bone markers and BMC were corrected for the effects of litter.

Although the research for this thesis was carefully conducted, there are limitations, which could be addressed through subsequent research. The existence of a circadian rhythm with large fluctuations in plasma osteocalcin concentration throughout a twenty-four hour period (Nielsen et al., 1990) represents extreme difficulty in the accurate interpretation of plasma levels from a single blood sample. In the present study, plasma osteocalcin was measured only at day one and day sixteen in the morning at approximately 0900 hour. In addition, the time of blood collection (0900 hr) reflects a period when osteoblast activity is low (Nielsen et al., 1990), suggesting that the findings on bone formation obtained from this study may have been different if other time points were studied. In addition, the measurement of NTX in spot urine samples may also reflect the circadian variation in bone metabolism. For these reasons, future research should collect blood and urine samples at various times of the day for a more accurate estimation of average formation and resorption activity.

The study period of only fifteen days did result in observable alterations in bone cell metabolism, but was likely not long enough to observe alterations in mineral mass, particularly in femur bone. Ke and colleagues (1992) demonstrated that the bone modelling cycle in rats was 35 in control and 45 days in PGE_2 treated animals. Thus, it is possible that extending this study for thirty-five days may enable clarification of the effects of dietary PUFA and PGE_2 on bone mineral mass as well as metabolism. In addition, intestinal calcium absorption, and histology to measure the rate of bone growth were not assessed in this study. In contrast to histology techniques, biochemical markers

FIGURE 10: Bone formation favoured remodelling

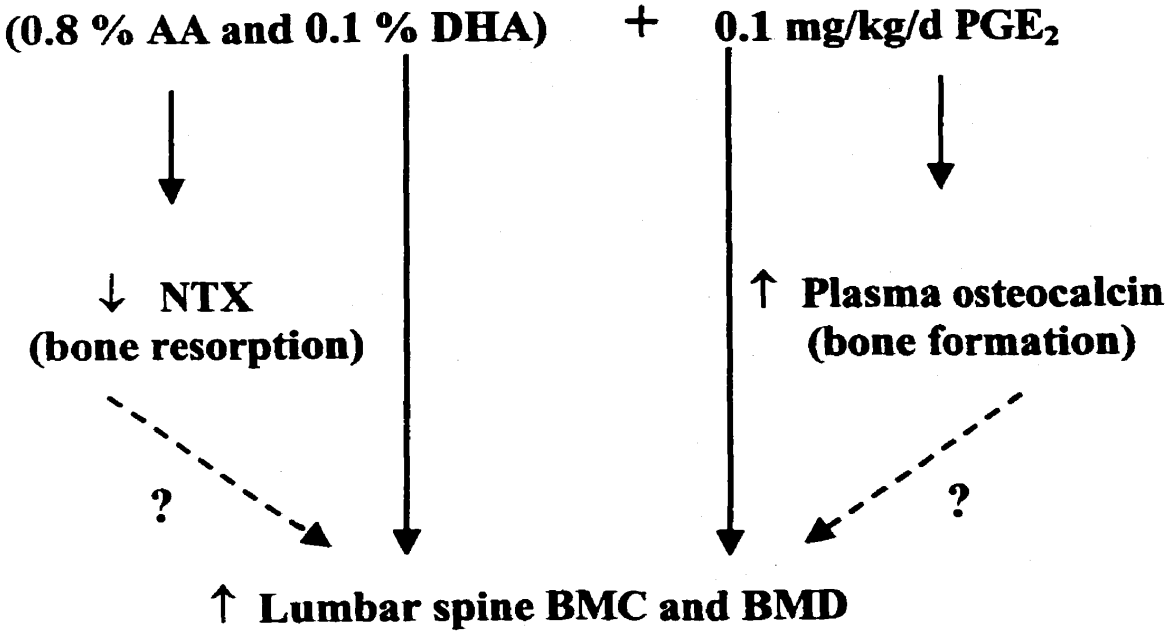
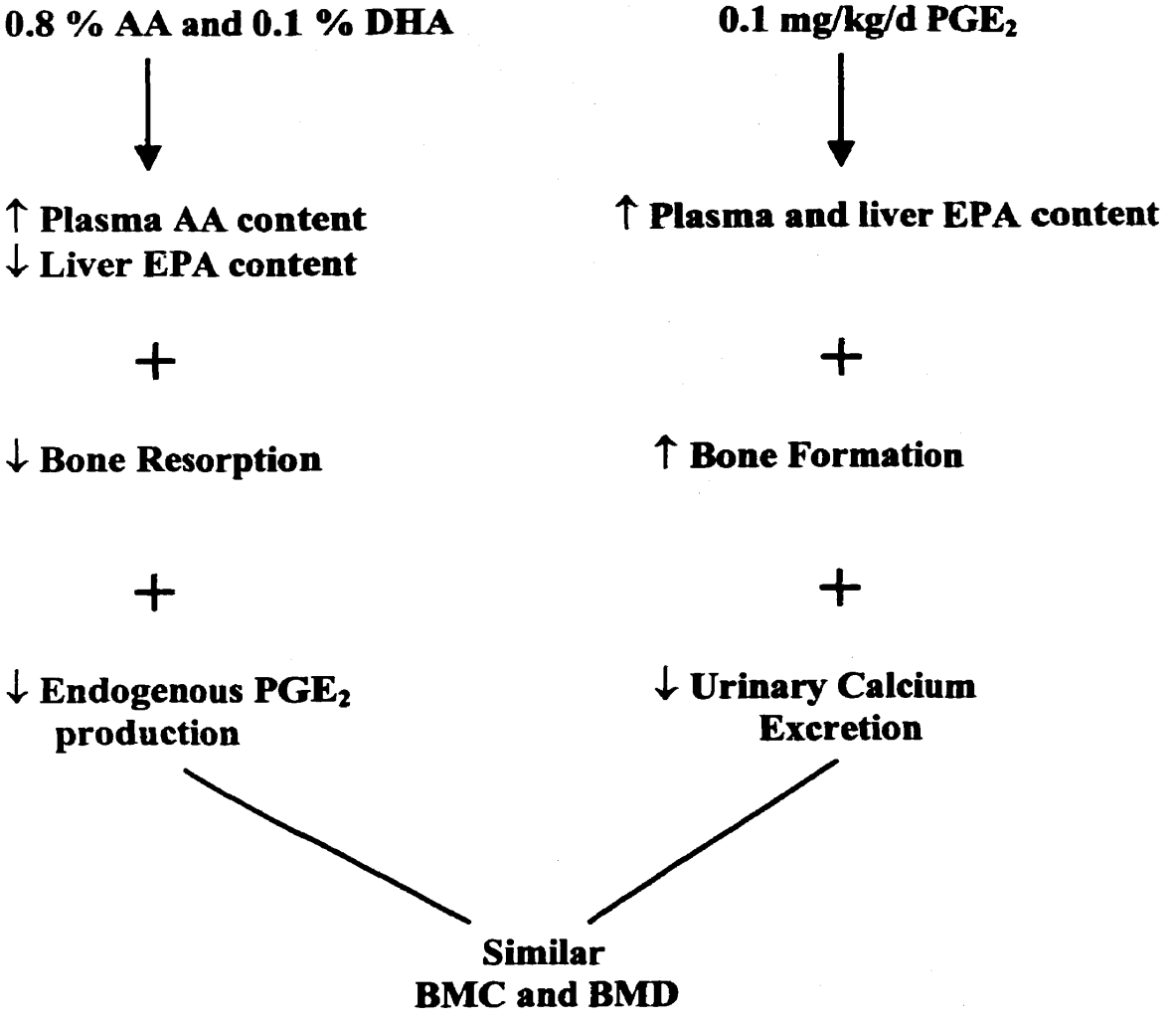


FIGURE 11: The effect of feeding formula with arachidonic acid (AA) and docosahexaenoic acid (DHA) versus administration of PGE_2 on fatty acid status and bone metabolism and mineralization



of bone metabolism assess short-term changes in the whole skeleton, but not localized areas. Histological measurements could be beneficial in clarifying the effects of dietary PUFA on bone formation.

Future research

Further research using the piglet model is required to investigate the effects of feeding formula supplemented with varying ratios of AA and DHA versus increasing dose levels of PGE_2 on:

- The length of the bone remodelling cycle
- Intestinal calcium absorption
- The fatty acid composition of cortical and trabecular bone compartments
- The enzymatic activity of phospholipase A_2 and cyclooxygenases in liver and bone
- The synthesis of other prostaglandins and eicosanoids in bone, such as PGE_3 and leukotrienes that may also be involved in bone resorption and formation activities

- SECTION V –

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V. REFERENCES

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- SECTION VI -

APPENDICES

APPENDIX A

Fatty acid composition of fatty acid supplemented (FA) and standard (STD) formulas fed to piglets [1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula	FA Formula
8:0	1.97 ± 0.05	2.04 ± 0.03
10:0	1.58 ± 0.03	1.61 ± 0.04
12:0	12.68 ± 0.16	12.55 ± 0.23
14:0	5.53 ± 0.03	5.43 ± 0.02
14:1	0.01 ± 0.01	0.01 ± 0.01
17:1	0.03 ± 0.02	0.04 ± 0.00
15:0	0.04 ± 0.03	0.05 ± 0.00
16:0	8.60 ± 0.01	8.68 ± 0.05
18:0	3.94 ± 0.01	4.05 ± 0.01
20:0	0.32 ± 0.00	0.33 ± 0.00
22:0	0.28 ± 0.01	0.31 ± 0.01
24:0	0.13 ± 0.00	0.17 ± 0.01
16:1 n-9	0.01 ± 0.02	0.03 ± 0.00
18:1 n-9	39.69 ± 0.05	38.87 ± 0.26
20:1 n-9	0.19 ± 0.00	0.19 ± 0.00
24:1 n-9	0.05 ± 0.04	0.08 ± 0.00
16:1 n-7	0.07 ± 0.05	0.10 ± 0.00
18:1 n-7	ND	ND
18:3 n-6	ND	0.07 ± 0.01
20:2 n-6	0.01 ± 0.01	0.03 ± 0.00
20:3 n-6	0.00 ± 0.01	0.08 ± 0.01
22:4 n-6	ND	ND
18:4 n-3	0.01 ± 0.02	ND
20:3 n-3	ND	0.03 ± 0.00
22:5 n-3	ND	ND

[1] Data are mean ± SD.
ND indicates not detected.

APPENDIX B

Fatty acid composition of plasma in piglets fed either fatty acid supplemented (FA) or standard (STD) formula and treated with either PGE_2 or saline[1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
12:0	0.24 ± 0.13	0.25 ± 0.06	0.28 ● 0.10	0.22 ± 0.04
14:0	1.25 ± 0.20	1.32 ± 0.19	1.30 ± 0.24	1.36 ± 0.12
14:1	0.03 ± 0.07	ND	ND	ND
15:0	0.07 ± 0.06	0.06 ± 0.06	0.03 ± 0.04	0.04 ± 0.04
16:0	15.21 ± 0.93	14.70 ± 0.58	14.80 ± 0.67	14.81 ± 0.55
17:1	0.05 ± 0.10	0.03 ± 0.04	0.17 ± 0.43	0.02 ± 0.04
18:0	13.98 ± 1.32	13.64 ± 0.99	13.35 ± 1.63	14.29 ± 0.85
20:0	0.34 ± 0.08	0.36 ± 0.05	0.35 ± 0.06	0.33 ± 0.06
22:0	0.27 ± 0.15	0.24 ± 0.03	0.32 ± 0.26	0.22 ± 0.05
24:0	0.38 ± 0.13	0.34 ± 0.03	0.35 ± 0.03	0.34 ± 0.07
16:1 n-9	0.66 ± 0.15	0.69 ± 0.10	0.64 ± 0.15	0.66 ± 0.03
18:1 n-9	22.35 ± 1.18	21.62 ± 1.40	22.39 ± 2.51	21.10 ± 0.85
20:1 n-9	0.12 ± 0.09	0.12 ± 0.04	0.16 ± 0.34	0.08 ± 0.07
24:1 n-9	0.44 ± 0.16	0.40 ± 0.03	0.40 ± 0.07	0.35 ± 0.08
16:1 n-7	0.35 ± 0.11	0.35 ± 0.05	0.46 ± 0.36	0.38 ± 0.08
18:1 n-7	1.08 ± 0.08	1.06 ± 0.09	1.08 ± 0.09	1.10 ± 0.06
18:3 n-6	0.62 ± 0.27	0.86 ± 0.24	0.64 ± 0.21	0.80 ± 0.10
20:2 n-6	0.30 ± 0.05	0.30 ± 0.03	0.30 ± 0.03	0.28 ± 0.04
20:3 n-6	0.39 ± 0.10	0.40 ± 0.07	0.35 ± 0.08	0.33 ± 0.06
22:4 n-6	0.30 ± 0.02	0.30 ± 0.03	0.38 ± 0.05	0.37 ± 0.07
18:4 n-3	0.10 ± 0.16	0.08 ± 0.09	0.19 ± 0.43	0.08 ± 0.07
20:3 n-3	0.10 ± 0.14	0.06 ± 0.06	0.18 ± 0.39	0.07 ± 0.06
22:5 n-3	0.78 ± 0.19	0.89 ± 0.18	0.71 ± 0.18	0.84 ± 0.11

[1] Data are mean ± SD, n=7 per group. Comparisons were not conducted.
ND indicates not detected.

APPENDIX C

Fatty acid composition of liver in piglets fed either fatty acid supplemented (FA) or standard (STD) formula and treated with either PGE_2 or saline[1]

Fatty Acid (% wt/wt of fatty acids)	STD Formula		FA Formula	
	SALINE	PGE_2	SALINE	PGE_2
12:0	0.08 ± 0.12	0.07 ± 0.08	0.03 ± 0.03	0.15 ± 0.13
14:0	0.72 ± 0.37	0.83 ± 0.32	0.61 ± 0.12	0.92 ± 0.41
14:1	ND	0.01 ± 0.02	ND	0.02 ± 0.04
15:0	0.01 ● 0.04	0.01 ● 0.02	ND	0.01 ± 0.02
16:0	13.43 ± 0.94	14.07 ± 1.39	13.57 ± 1.06	13.80 ± 0.66
18:0	25.23 ± 2.50	24.95 ± 2.40	26.26 ± 0.92	25.33 ± 2.38
20:0	0.33 ± 0.05	0.35 ± 0.04	0.34 ± 0.04	0.37 ± 0.06
22:0	0.38 ± 0.06	0.39 ± 0.05	0.41 ± 0.06	0.44 ± 0.08
24:0	0.80 ± 0.16	0.79 ± 0.14	0.86 ± 0.12	0.91 ±0.12
16:1 n-9	0.51 ± 0.31	0.46 ± 0.11	0.38 ± 0.05	0.42 ± 0.08
18:1 n-9	12.34 ± 2.55	12.50 ± 2.28	11.29 ± 1.15	12.45 ± 2.06
20:1 n-9	0.17 ± 0.05	0.16 ± 0.08	0.16 ± 0.04	0.17 ± 0.02
24:1 n-9	0.46 ± 0.12	0.49 ± 0.07	0.46 ± 0.08	0.49 ± 0.06
16:1 n-7	0.20 ± 0.08	0.24 ± 0.09	0.16 ± 0.06	0.24 ± 0.08
18:1 n-7	1.20 ± 0.15	1.28 ± 0.11	1.15 ± 0.10	1.17 ± 0.04
18:3 n-6	0.27 ± 0.14	0.34 ± 0.07	0.21 ± 0.11	0.34 ± 0.14
20:2 n-6	0.53 ± 0.07	0.51 ± 0.07	0.52 ± 0.05	0.49 ± 0.05
20:3 n-6	0.58 ± 0.17	0.55 ± 0.07	0.47 ± 0.07	0.40 ± 0.08
22:4 n-6	0.44 ± 0.06	0.47 ± 0.20	0.56 ± 0.07	0.49 ± 0.06
20:3 n-3	0.10 ± 0.07	0.10 ± 0.04	0.11 ± 0.07	0.15 ± 0.01
22:5 n-3	1.14 ± 0.20	1.16 ± 0.22	1.12 ± 0.14	1.05 ± 0.11

[1] Data are mean ± SD. Comparisons were not conducted.
ND indicates not detected.