Correlation between essential fatty acids and parameters of bone formation and degradation

MC Kruger PhD, N Claassen PhD, CM Smuts[1] PhD and HC Potgieter[2] PhD

Dept of Physiology, [2]Chemical Pathology, University of Pretoria, PO Box 2034, Pretoria, [1]MRC National Research Programme for Nutritional Intervention, Tygerberg, South Africa.

There are two types of essential fatty acids (EFAs), the n-6 derived from linoleic acid (LA) and the n-3, derived from alpha-linolenic acid (ALA). Most of the functions of the EFAs require the conversion of LA and ALA to their metabolites including, gammalinolenic (GLA), dihomogammalinolenic (DGLA), arachidonic (AA) (n-6) and eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (n-3). Supplementing specific GLA:EPA ratios has effects on bone formation and degradation. A study was designed to investigate the effect of various dietary ratios of n-6:n-3 on calcium homeostasis. Female Sprague Dawley rats were ovariectomised (OVX) at age =11 weeks, and were supplemented from age 12 weeks for six weeks with different ratios (9:1; 3:1; 1:3; 1:9) of GLA:EPA. Bone parameters and red blood cell (RBC) fatty acid profiles were measured at age=18 weeks. RBC GLA and DGLA increased in groups 9:1 and 3:1($p<0.05$). EPA and DGLA increased in 1:3 and 1:9 while AA decreased ($p<0.05$). Correlations were calculated between bone calcium, deoxypyridinoline (Dpyd) and specific fatty acids. DGLA was positively correlated with femur calcium and negatively with Dpyd excretion while DHA and EPA were correlated with femur calcium.

Keywords: essential fatty acids, dihomogammalinolenic acid (DGLA), essential fatty acid (EFA), docosahexaenoic acid (DHA), bone calcium, deoxypyridinolines, rats

Introduction

There are two types of essential fatty acids (EFAs), the n-6 derived from linoleic acid (C18:2n-6; LA) and the n-3, derived from alpha-linolenic acid (C18:3n-3; ALA) (Fig. 1). Most of the functions of the EFAs require the conversion of LA and ALA to their metabolites including gamma-linolenic acid (C18:3n-6; GLA), dihomogamma-linolenic acid (C20:3n-6; DGLA), arachidonic acid (C20:4n-6; AA) and eicosapentaenoic acid (C20:5n-3; EPA) and docosahexaenoic acid (C22:6n-3; DHA)[1].

The first papers with evidence that there is a link between EFAs and calcium appeared in the 1950s[2,3]. These papers mentioned loss of normal cartilage, osteoporosis, and bone weakness together with ectopic calcification, especially in the kidneys; all coinciding with EFA deficiency. A relationship between EFA and vitamin D status was established by Rasmussen and co-workers in several papers[4-6]. EFAs are required for the normal effects of vitamin D on the gut such as promotion of calcium absorption and active calcium transport, and the administration of either GLA- or EPA-rich oils enhances calcium transport in rat intestine[7].

Papers reporting a change in calcium balance and bone calcium in response to EFA manipulation, appeared in 1995[8-10]. Administration of GLA and EPA, either alone or preferably combined, was shown to increase calcium absorption, decrease calcium excretion, increase calcium retention in the body and increase bone calcium. A significant decrease in the excretion of urinary hydroxyproline and pyridinium cross-links, markers of bone collagen degradation was found[9]. Administration of EPA alone was also shown to increase bone calcium and bone strength in the ovariectomised rat[11].

In the current study, using the model of the ovariectomised (OVX) rat, the relationship between the EFAs of the n-3 and n-6 series to bone calcium and deoxypyridinolines (Dpyd) was investigated.

Methods

Female Sprague-Dawley rats were ovariectomised at age 11 weeks and were supplemented from age 12 weeks for a six week period with a semi-synthetic diet containing different ratios of GLA:EPA+DHA (9:1; 3:1; 1:3; 1:9) added to the diet (8% by weight). LA:ALA (3:1) was used as control in a sham and OVX group (n=7 per group). Animals were housed in hanger cages in a temperature and day-night controlled room with free access to demineralised water. Food was restricted to 5g/100g animal to prevent OVX-induced weight gain.

At age = 18 weeks animals were sacrificed and blood collected for erythrocyte membrane (EMB) fatty acid analysis. Right femurs were dissected out to analyse bone calcium. 24-hour urine samples were collected for three consecutive days before sacrifice to measure urinary Dpyds.

Calcium/femur

Femurs were ashed at 660°C overnight. The bones were weighed and measured, dissolved in 2ml concentrated HCl and then diluted 400 times for analysis using absorption spectroscopy.

Correspondence address: Prof MC Kruger, Dept of Physiology, BMW 9.23, University of Pretoria, PO Box 2034, Pretoria 0001, South Africa. Tel: +2712 3192423; Fax: +2712 3192238
Email: MKRUGER@medic.up.ac.za

Table 1. Mean ± standard deviation erythrocyte membrane essential fatty acid concentrations (mg/mg protein) in response to a six-week supplementation period with different EFA ratio diets.

Fatty	Sham	OVX				
acids	Control	Control	9:1	3:1	1:3	1:9
C18:2 n-6	38.7±4.1	39.4±5.9	35.8±5.7	31.3±8.6^{*+}	32.8±6.4	26.2±5.0^{*+}
C18:3 n-6	0.2±0.3	0.3±0.2	1.0±0.5^{*+}	0.9±0.3^{*+}	0.7±0.2^{*+}	0.6±0.1^{*+}
C18:3 n-3	0.6±0.4	0.7±0.1	0.2±0.1^{+}	0.2±0.1^{+}	0.2±0.2^{+}	0.3±0.1
C20:3 n-6	2.1±0.3	2.2±0.8	3.2±0.4^{*+}	3.0±0.8^{*+}	3.4±0.6^{*+}	2.9±0.7^{*+}
C20:4 n-6	119.9±15.1	119.5±15.1	123.5±13.0	107.4±30.8	102.5±13.1	88.0±13.5^{*+}
C20:5 n-3	2.5±0.3	2.6±1.1	1.6±1.5	1.5±0.5	10.4±1.5	21.1±5.4^{+}
C22:6 n-3	18.9±3.2	16.6±3.7	14.7±1.8	16.2±4.5	25.4±3.7^{*+}	29.4±5.5^{*+}

Ratios are GLA: EPA + DHA. Controls were LA : ALA 3:1. Significance is indicated by: * $p < 0.05$ vs Sham; + $p < 0.05$ vs OVX control.

Deoxypyridinolines

Dpyds were measured using HPLC according to a published method[9].

EMB Fatty Acid Analysis

EMBs were prepared by hemolysing erythrocytes with different phosphate buffers[12,13]. Lipids were extracted from EMBs with chloroform/methanol (2:1, v/v)[14]. An aliquot of the extract was transmethylated with 2.5ml methanol-18M sulphuric acid (95:5, v/v) at 70°C for 2 hrs. The resultant fatty acid methyl esters were analysed on a Varian model 3700 Gas Liquid Chromatograph using fused silica megabore DB-225 columns (J&W Scientific, Folsom, CA, USA, cat. no. 125-2232) as described by Smuts *et al* (1992)[15]. The individual FA methyl esters were identified by comparison of the retention times with those of a standard mixture of free FA C14:0 to C22:6. Heptadecanoic acid (C17:0) was used as internal standard to quantify EMB total fatty acid composition. The EMB total protein concentrations were measured by a modified Lowry procedure[16]. The EMB total fatty acid concentrations were expressed as mg/mg protein.

Statistical Analyses

Data are represented as means ± standard deviations. The degree of linear association between variables was determined by Pearson correlation. A linear association between two variables is considered to be significant if $p<0.05$. For the purpose of the correlations only OVX groups were used, five groups of 7 each.

Results

The erythrocyte fatty acid compositions in the various treatment groups are shown in Table 1. GLA increased significantly in all groups compared to sham ($p<0.05$). As expected, as GLA is rapidly elongated to DGLA, the DGLA levels in all ratio groups also increased significantly compared to sham and OVX control. There was a significant reduction in AA in the groups fed n-6/n-3 ratios of 1:3 and 1:9, compared to sham and OVX control.

Correlations were calculated between bone calcium, deoxypyridinoline and fatty acids, specifically, 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3. Correlations are quoted in Table 2. DGLA (20:3n-6) correlated significantly with both calcium/femur ($r=0.54$; $p=0.007$) and with Dpyd ($r=-0.605$; $p=0.002$) (Figure 2). DHA (22:6n-3) showed a strong correlation with calcium/femur ($r=0.65$; $p=0.008$) (Figure 3). EPA correlated with calcium/femur but not with Dpyd (Figure 4).

Figure 1. Outline of the metabolism of the n-6 and n-3 essential fatty acids.

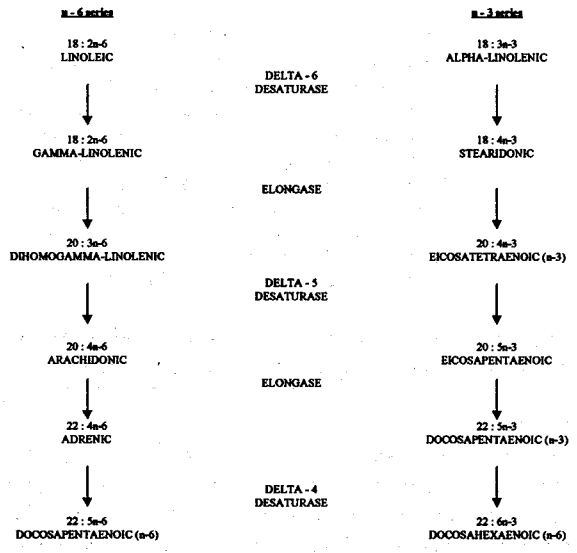


Table 2. Correlations between fatty acids and parameters of bone formation and degradation.

Fatty acid	Calcium/femur	P value	Dpyd	P value
18:2n-6	-0.22	0.30	-0.14	0.51
18:3n-6	0.021	0.92	-0.31	0.153
20:3n-6	0.54	0.007**	-0.61	0.002**
20:4n-6	-0.01	0.96	0.07	0.73
18:3n-3	-0.223	0.31	0.15	0.48
20:5n-3	0.59	0.003**	-0.21	0.33
22:6n-3	0.65	0.008**	-0.21	0.34

Significance is indicated by ** $P<0.01$

Discussion

In this study, the correlations between the fatty acids of the erythrocyte membranes and parameters of bone formation and degradation, were assessed. Different ratios of n-3 and n-6 EFAs were provided as it has been shown that appropriate n-6/n-3 EFA ratios are important for various physiological and biological functions[1,17]. EFAs have major roles in the structure of all biological membranes and as components and regulators of second messenger systems. Certain fatty acids are precursors of prostaglandins (PGs) such as DGLA for PGE_1, AA for PGE_2 and EPA, precursor for DHA, for PGE_3. Dietary modulation using EFAs in different ratios can modulate PG synthesis[18]. Recent research has indicated that both EFAs and PGs exert a modulatory effect on bone metabolism, mostly anabolic, in animals and humans[10,19].

Figure 2. Correlation between DGLA and calcium/femur and Dpyd.

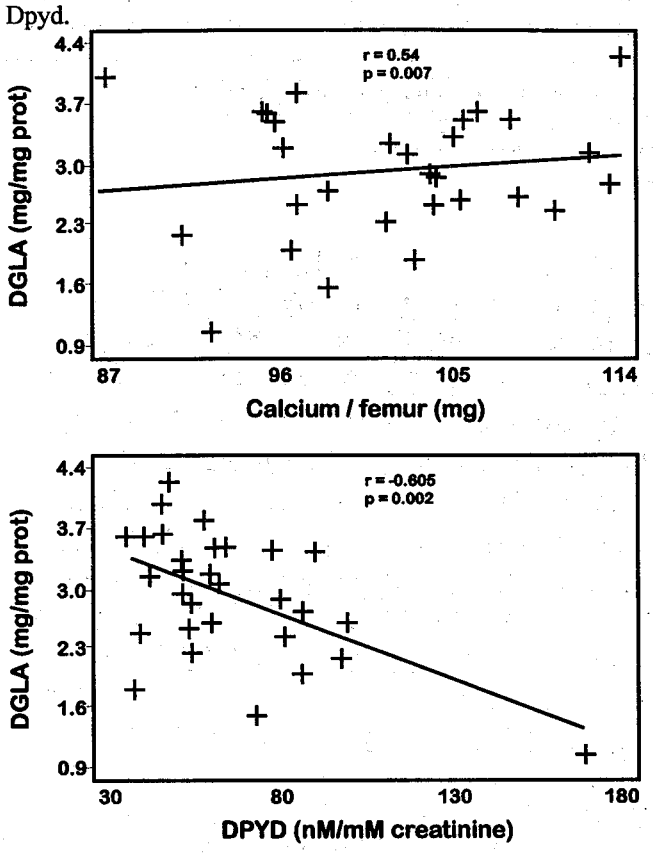
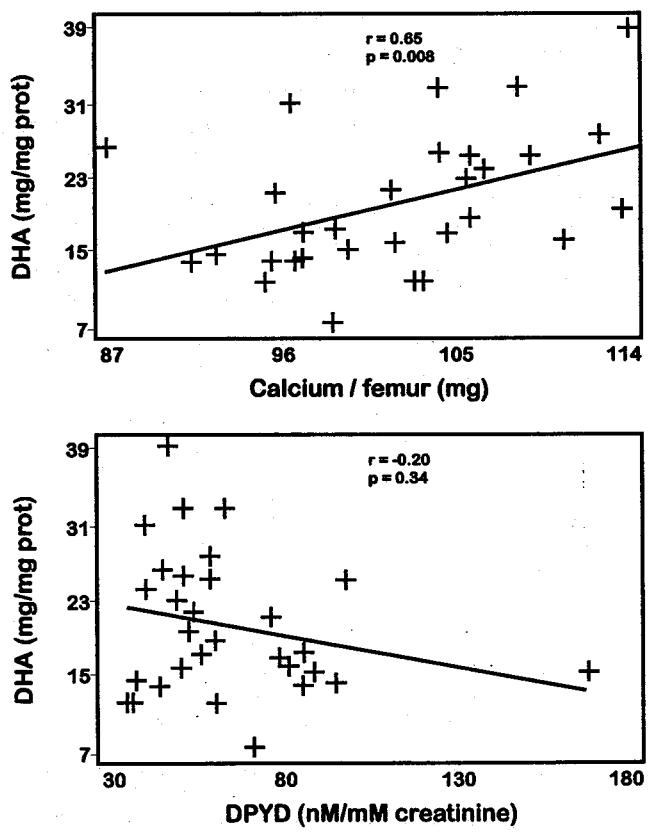


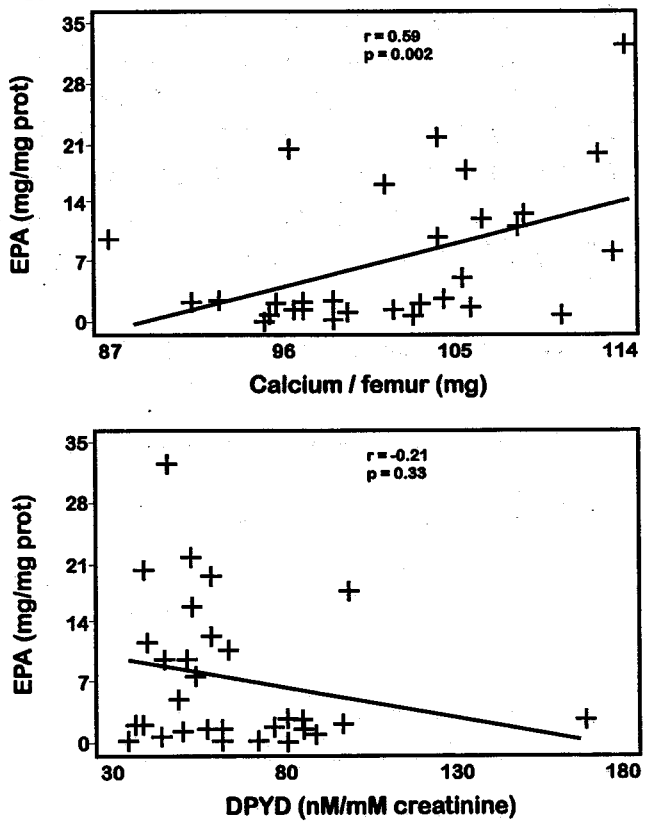
Figure 3. Correlation between DHA and calcium/femur and Dpyd.



There is usually an abundance of AA present in the plasma and cell membranes of the body. PGE_2 derived from AA, can induce changes in bone[19] but is also associated with harmful pro-inflammatory effects in the body[1]. In contrast PGE_1, derived from DGLA, has an anti-inflammatory effect and as recently shown, is also associated with long-term anabolic effects on bone[19]. When n-3 EFAs are administered, AA falls in various lipid fractions and PG synthesis may be directed towards the 3-series from EPA and away from the 2-series from AA.

In this study easily harvested erythrocyte membranes were used to indicate effects of the diets on membrane composition. Fatty acid analyses showed significant increases in DGLA levels, a decrease in AA and increases in EPA and DHA varying in relation to the n-6/n-3 ratio fed. Increases in DGLA and EPA are likely to be associated with increases in 1- and 3-series PGs with a decline in PGs of the 2-series. DGLA is closely positively correlated with bone calcium and negatively correlated with Dpyd, indicating an anabolic effect on bone. These correlations may possibly be due to the change in balance of the PGs or possibly to other direct effects of the fatty acids.

Figure 4. Correlation between EPA and calcium/femur and Dpyd.



At present it is uncertain whether these relationships can be attributed to PGs , to the fatty acids themselves or to other fatty acid derivatives. Fatty acids have also been associated with certain second messenger systems which may cause a decrease in cAMP (anti-resorptive effect) or increase in inositol triphosphate or calcium-calmodulin system, both inducing calcium flux[20].

As far as we are aware this is the first time that such relationships between specific fatty acids and bone metabolism have been demonstrated. These observations need to be supported by further evidence from other models. However, such correlations in the OVX model may indicate

a new approach to increasing bone mass in the absence of oestrogen, or alternatively amplifying the effects of oestrogen and vitamin D using simple dietary manipulation.

Acknowledgements. Martelle Marais and Helene Coetzer for technical assistance; Medical Research Council (SA); Research Committee (University of Pretoria) and Scotia Pharmaceuticals (SA and UK) for financial assistance.

Correlation between essential fatty acids and parameters of bone formation and degradation
MC Kruger, N Claassen, CM Smuts and HC Potgieter
Asia Pacific Journal of Clinical Nutrition (1997) Volume 6, Number 4: 235-238

摘要

有两类基本脂肪酸，亚油酸(LA) n-6 的衍生物和 α - 亚麻酸 n-3 的衍生物．基本脂肪酸的大多数功能都需要亚油酸(LA) 和 α - 亚麻酸(ALA) 转变成它们的代谢产物，包括γ - 亚麻酸(GLA)，双高亚麻酸(DGLA)，花生四烯酸(AA)(n-6)；二十碳五烯酸(EPA)和二十二碳六烯酸(DHA)(n-3)．补充特定比例的γ- 亚麻酸与二十碳五烯酸对骨的形成和降解有作用．本研究设计为调查饮食中各种不同的n-6与n-3之比对钙体内平衡的作用．雌性Sprague Dawley小鼠在十一周龄时行卵巢切出，从十二周龄起开始补充不同比例的γ- 亚麻酸和二十碳五烯酸(9:1, 3:1, 1:3, 1:9)，持续六周．骨参数及红细胞脂肪酸的分布在十八周龄时进行测量．红细胞γ-亚麻酸和双高亚麻酸的量在 9:1 比例组和 3:1 比例组增加 ($p<0.05$)． 二十碳五烯酸和双高亚麻酸的含量当花生四烯酸下降时在 1:3 和 1:9 组增加 ($p<0.05$)． 骨钙，去氧吡啶酮啉(Dpyd)和特殊脂肪酸之间的相关性进行了计算．当二十二碳六烯酸和二十碳五烯酸与股骨钙相关时，双高亚麻酸与股骨钙成正相关，与去氧吡啶酮啉的分泌成负相关．

关键词：脂肪酸，骨钙，去氧吡啶酮啉

References

1. Horrobin DF. Gammalinolenic Acid. Rev Contep Pharmacother 1990; 1: 1-14.
2. Alfin-Slater R, Bernick S. Changes in tissue lipids and tissue histology resulting from essential fatty acid deficiency in rats. AMJ Clin Nutr 1958; 6:613-624.
3. Sinclair HM. Deficiency of essential fatty acids in lower animals. pp 249-256 in Essential Fatty Acids, Butterworths, London. 1957.
4. Goodman DBP; Haussler MR, Rasmussen H. Vitamin D_3 induced alteration of microvillar membrane lipid composition. Biochem Biophys Res Comm 1972; 46:80-86.
5. Matsumoto T, Fontaine O, Rasmussen H. Effect of 1,25-dihydroxy vitamin D_3 on phospholipid metabolism in chick duodenal mucosal cells. J Biol Chem 1981;256:3354-3360.
6. Rasmussen H, Matsumoto T, Fontaine O, Goodman DBP. Role of changes in membrane lipid structure in the action of 1;25 dihydroxy vitamin D_3. Fed Proc 1982;41:72-77.
7. Coetzer H, Claassen N, van Papendorp D, Kruger MC. Calcium transport by isolated brush border and basolateral membrane vesicles: role of essential fatty acid supplementation. Prostaglandins Leukot Essent Fatty Acids 1994; 50:257-266.
8. Kruger MC, Coetzer H, de Winter R, Claassen N. Eicosapentaenoic acid and docosahexaenoic acid supplementation increases calcium balance. Nutr Res 1995; 15:211-219.
9. Claassen N, Potgieter HC, Seppa M, Vermaak WJ, Coetzer H, van Papendorp DH, Kruger MC. Supplemented gamma-linolenic acid and eicosapentaenoic acid influence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyproline and bone calcium content. Bone 1995; 16:385S-392S.
10. Claassen N, Coetzer H, Steinmann CM, Kruger MC. The effect of different N6/N3 essential fatty acid ratios on calcium balance and bone in rats. Prostaglandin Leukot Essent Fatty Acids 1995; 53:13-19.
11. Sakaguchi K, Morita I, Murota S. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. Prostaglandin Leukot Essent Fatty Acids 1994; 50:81-84.
12. Steck TL, Kant JA. Preparation of impermeable ghosts and inside-out vesicles of human erythrocyte membranes. In: Methods in Enzymology. Academic Press, New York, 1974 pp 172-173.
13. Burton GW, Ingold KU, Thompson KE. An improved procedure for the isolation of ghost membranes from human red blood cells. Lipids 1981; 16: 946.
14. Folch J, Lees M, Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957; 226: 497-509.
15. Smuts CM, Kruger M, Van Jaarsveld PJ, Fincham JE, Schall R, Van der Merwe KJ, Benadé AJS. The influence of fish oil supplementation on plasma lipoproteins and arterial lipids in vervet monkeys with established atherosclerosis. Prostaglandins Leukot and Essent Fatty Acids 1992; 47: 129-138.
16. Markwell MAK, Haas SM, Bieber LL and Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87: 206-210.
17. Uauy R. Are ω-3 fatty acids required for normal eye and brain development in the human? J Pediatr Gastroenterol Nutr 1990; 11:296-300.
18. Huang YS, Wainwright PE, Redden PR, Mills DE, Bulman-Fleming B, Horrobin DH. Effect of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in suckling mice. Lipids 1992; 27:104-110.
19. Jee WSS, Ke HZ, Li XJ. Longterm anabolic effects of prostaglandin E_2 on tibial diaphyseal bone in male rats. Bone Min 1991; 15:33-55.
20. Graber R, Sumida C, Nunez EA. Fatty acids and signal transduction. J Lipid Mediaters Cell Signalling 1994; 9: 91-116.