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Regulation of osteoarthritis by omega-3 (n-3) polyunsaturated fatty acids in a naturally occurring model of disease

L. Knott †, N.C. Avery †, A.P. Hollander ‡, J.F. Tarlton †*

† Matrix Biology, Div. VPII, University of Bristol, Veterinary School, Langford, Bristol BS40 5DU, UK
‡ Stem Cell Biology, Cellular and Molecular Medicine, University of Bristol, Bristol BS8 1TD, UK

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SUMMARY

Objective: To examine effects of high omega-3 (n-3) polyunsaturated fatty acid (PUFA) diets on development of osteoarthritis (OA) in a spontaneous guinea pig model, and to further characterise pathogenesis in this model. Modern diets low in n-3 PUFAs have been linked with increases in inflammatory disorders, possibly including OA. However, n-3 is also thought to increases bone density, which is a possible contributing factor in OA. Therefore we aim to determine the net influence of n-3 in disease development.

Method: OA-prone Dunkin-Hartley (DH) Guinea pigs were compared with OA-resistant Bristol Strain-2s (BS2) each fed a standard or an n-3 diet from 10 to 30 weeks (10/group). We examined cartilage and subchondral bone pathology by histology, and biochemistry, including collagen cross-links, matrix metalloproteinases (MMPs), alkaline phosphatase, glycosaminoglycan (GAG), and denatured type II collagen.

Results: Dietary n-3 reduced disease in OA-prone animals. Most cartilage parameters were modified by n-3 diet towards those seen in the non-pathological BS2 strain – significantly active MMP-2, lysyl-pyridinoline and total collagen cross-links – the only exception being pro MMP-9 which was lower in the BS2, yet increased with n-3. GAG content was higher and denatured type II lower in the n-3 group. Subchondral bone parameters in the DH n-3 group also changed towards those seen in the non-pathological strain, significantly calcium:phosphate ratios and epiphyseal bone density.

Conclusion: Dietary n-3 PUFA reduced OA in the prone strain, and most disease markers were modified towards those of the non-OA strain, though not all significantly so. Omega-3 did not increase markers of pathology in either strain.

Introduction

In an ageing population, osteoarthritis (OA) is set to become the fourth leading cause of disability by 2020[1], so with the current lack of effective treatments there is an urgent need for preventative measures.

The high omega-6 (n-6) polyunsaturated fatty acid (PUFA) content in Western diets has been associated with a number of inflammatory disorders such as heart disease, rheumatoid arthritis and colitis. Similarly, elevated levels of n-6 have been linked to OA in bone and cartilage[2,3]. "Western" diets are cited as having an n-6:n-3 ratio of between 15 and 30:1, whereas diets for which we are naturally adapted, such as those for Palaeolithic and modern hunter-gatherers, contain roughly equal levels of n-6 and n-3 (1:1–2:1)[4]. Thus, dietary supplementation with n-3 PUFA has been recommended to redress this imbalance.

PUFAs are essential fatty acids and precursors to a number of important factors called eicosanoids, such as prostaglandins, thromboxanes, leukotrienes and resolvins. These mediate various processes including inflammation and bone metabolism. Conversion of PUFAs to eicosanoids is achieved by the action of cyclo-oxygenases (COX) and lipoxygenases (LOX). Omega-3 and n-6 generate different series of eicosanoids, those from n-6 are generally highly pro-inflammatory and those from n-3 somewhat less so. In particular, PGE_2, derived from n-6 PUFAs, has been associated with inhibition of anabolic processes and increased proteolytic degradation of cartilage[5–7]. Therefore, manipulation of the precursors of prostaglandins is likely to have an effect on the initiation and progression of OA. There is some evidence that n-3 PUFA can be beneficial in humans[8] and animals[9,10], and *in vitro*

* Address correspondence and reprint requests to: J.F. Tarlton, Matrix Biology, Div. VPII, University of Bristol, Veterinary School, Langford, Bristol BS40 5DU, UK. Tel: 44-117-9289266; Fax: 44-117-9289505.
E-mail address: john.tarlton@bristol.ac.uk (J.F. Tarlton).

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studies have also demonstrated positive outcomes[11,12]. On the other hand, there is considerable evidence indicating that n-3 PUFAs promote bone formation and increase bone density[13,14]. OA is characterised by increased subchondral bone density, and therefore n-3 induced bone formation may exacerbate OA progression[15–19]. With the potential for both beneficial and detrimental effects, it is essential that the net influence of n-3 PUFAs on OA is determined before being recommended to sufferers and those at risk.

Previous studies have established the use of the Dunkin-Hartley (DH) guinea pig as a naturally occurring model of OA[20–22], with the Bristol Strain-2 (BS2) as an OA-resistant control[17,23]. Studies have also examined bone and cartilage changes in DH guinea pigs compared with other OA-resistant strains[15,24]. The specific aims of this study are (1) to determine how dietary supplementation with n-3 fatty acids influences the progression of OA in the disease-prone DH guinea pigs and the OA-resistant BS2; and (2) to further characterise the DH/BS2 spontaneous guinea pig model of human OA.

Materials and methods

Animals and diets

We used the established DH guinea pig model of spontaneous OA (Harlan UK, Oxon, UK), with the BS2 as an OA-resistant control (ASU, University of Bristol, UK). Animals were given a standard commercial diet until 10 weeks old, and then 10 animals from each strain were randomly allocated to either a defined control feed with n-6:n-3 ratio of 22:1 (typical of a "Western" diet), or a 'high n-3' diet with n-6:n-3 of 1.5:1 (see Table I; Teklad Custom Research Diets, UK). Each diet had 5% fat contents, contained the anti-oxidant tert-butylhydroquinone, and was vacuum packed in nitrogen at −20°C to minimise oxidation. Animals underwent no intervention other than dietary modification, and were reared with free access to food and water. After euthanasia (in accordance with UK regulations) at 30 weeks old, the hind legs were removed. All samples were stored at −80°C prior to analysis.

Histological disease assessment

The right tibial plateau was removed, divided coronally through the insertion point of the collateral ligament, and the full width anterior section was fixed in neutral buffered formalin, decalcified and processed for wax histology using standard procedures. Sections were stained with toluidine blue or Safranin-O, and scored semi-quantitatively for OA severity, using a combination of the adapted Mankin scoring system and the Osteoarthritis Research Society International (OARSI) OA histopathology grading system which includes elements of subchondral bone changes[23,25]. Three sections were "blind" scored per animal, and scores were summed for each animal to give an overall histological score, with a possible range of 0–20.

Table I
Composition of diets

g/kg	Control (high n-6)	High n-3
Alfalfa meal	380	380
Wheat	204	204
Oats	146	146
Soybean meal	140	140
Soybean hulls	50	50
Safflower oil	18	10
Canola oil	12	8
Corn oil	10	–
Fish oil	–	17
Coconut oil, hydrogenated	10	7
Mineral mix	0.5	0.5
Dicalcium phosphate	15	15
Sodium chloride	7	7
Magnesium oxide	1	1
Vitamin mix	5	5
Stay-C35 (35% ascorbic acid)	1	1
DL-Alpha-Tocopheryl acetate (500 IU/g)	0.3	0.3
Choline chloride	1	1
Folic acid	0.004	0.004
Vitamin A palmitate (500,000 U/g)	0.04	0.04
Vitamin D_3, cholecalciferol (500,000 U/g)	0.004	0.004
TBHQ (antioxidant)	0.04	0.04
n-6:n-3	22:1	1.5:1

Cartilage biochemistry

Cartilage from the posterior section of each tibial plateau was removed for analysis. Tissue taken from the left knee was analysed for glycosaminoglycan (GAG), and denatured and native type II collagen, whilst matrix metalloproteinase (MMP)-2 and MMP-9 activity and collagen cross-link analysis were undertaken on the right. All chemicals were purchased from Sigma unless otherwise stated.

Cartilage extract preparation

Freeze-dried samples were digested with tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine pancreatic α-Chymotrypsin (1 mg/ml) in 50 mM Tris buffer pH 7.5 containing inhibitors (1 mM iodoacetamide, 1 mM EDTA, 10 μg/ml pepstatin A). Digestion of denatured collagen was continued for 18 h at 34°C and the reaction stopped by the addition of tosyl-L-phenylalanine chloromethyl ketone (TPCK). The samples were centrifuged, supernatants removed and the pellets (remaining native collagen) digested with Proteinase K (1 mg/ml) in 50 mM Tris buffer pH7.5 at 56°C, with TLCK and TPCK. After 18 h, digestion was stopped by heating samples at 100°C for 15 min. Undigested material was removed, freeze-dried and weighed.

Type II collagen

The chymotrypsin and proteinase K extracts were assayed for type II collagen (% dry weight) and denatured type II (% type II) by inhibition ELISA using a mouse IgG monoclonal antibody to denatured type II collagen, as previously described[26].

GAG

The extracts were assayed for sulphated GAGs (% dry weight) by a colorimetric assay, using dimethylmethylene blue (DMMB) as previously reported[27].

MMPs

Tissue was freeze-dried, and extracted (0.1% Brij 35 in 20 mM triethanolamine; 20 μl/mg) at 4°C for 18 h. Extracts were assayed for MMP-2 and MMP-9 activity (% standard MMP-2) by gelatin zymography as previously described[28].

Collagen analyses

Collagen content (% dry weight) was determined by hydroxyproline analysis of a tissue hydrolysate using a continuous-flow autoanalyser (Burkard Scientific, Uxbridge, UK) as previously described[29,30]. Mature [hydroxylysyl-pyridinoline (HL-Pyr) and lysyl-pyridinoline (L-Pyr)] and immature hydroxyketonorleucine (HLKNL) collagen cross-links were quantified (M/M collagen) using modified amino acid analysis as described previously[31]. Lysine hydroxylation was determined using a modified amino acid analysis program as previously described[29].

Subchondral bone biochemistry

Subchondral bone was sampled from the posterior medial tibial plateau after removal of the articular cartilage, by cryosectioning to

a depth of 450 μm, and collecting the sections. Sectioning in this manner powders the subchondral bone with minimal losses or contamination. The resulting samples were extracted for soluble proteins and the extracts assayed for MMP-2 and MMP-9 as described earlier. In addition extracts were analysed for alkaline phosphatase (ALP) activity (mU/L) by conversion of *p*-nitrophenylphosphate, to *p*-nitrophenol and monitored at 405 nm using a Konelab analyser[32].

Collagen quantification and cross-link analysis were undertaken on the insoluble pellet as described earlier. Mineral calcium and phosphate were quantified from aliquots of hydrolysate, using a Konelab analyser, whereby inorganic phosphate forms a complex with ammonium molybdate, measured at 340 nm[33], and calcium ions with arsenazo III, measured at 660 nm[34]. These values are used to give a mineral calcium:phosphate ratio.

Dual-energy X-ray absorptiometry (DXA)

The remaining left proximal tibial epiphysis adjacent to the subchondral was analysed by DXA using a PIXImis 1.44 (Lunar, Maddison, WI) fitted with small animal software. The bone mineral density (BMD, mg/cm^2) of an area (7 mm × 7 mm) immediately distal to the growth plate was measured[35].

Statistical analysis

Results were analysed using PASW Statistics v17 (SPSS.com). Data was tested for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using Levene's test of equality of variances. Assuming normality and equal variances, a one-way ANOVA was used to test for significant differences specific for each aim: (1) model characterisation, BS2 compared to DH for n-6 control diet; (2) OA progression, n-3 compared to n-6 diet for DH strain; (3) OA promotion, n-3 compared to n-6 diet in control BS2 strain. Where data was not normal and variances not equal, log transformations were used, otherwise a Kruskal–Wallis test with Dunn's multiple comparison tests. To evaluate multiple outcomes in the model, binomial discrete probability distribution and Chi-square tests were performed. Graphical data is presented as mean ± S.E.M.

Results

Characterisation of the DH/BS2 model

OA pathology

The pathology scores were significantly lower in the BS2 strain compared to the DH strain ($P = 0.001$), in line with previous studies verifying the BS2 strain as an OA-resistant guinea pig strain[23]. When the separate scores were considered, there was a strain effect overall ($P < 0.0001$), with toluidine blue ($P = 0.0008$), cartilage structure ($P = 0.0057$) and OARSI criteria ($P = 0.0007$) each being lower in the BS2 (Fig. 1).

Pathogenic markers

Cartilage. The GAG and type II collagen (total or denatured) contents of the cartilage were similar between strains [Fig. 2(A and B)]. However the post-translational modifications of the collagen were markedly different between OA-prone DH and non-prone BS2 strains, with significantly lower levels of lysyl hydroxylation and higher levels of the cross-link L-Pyr, associated with a mineralising matrix, in the DH strain [$P = 0.01$ and $P < 0.0001$ respectively; Fig. 3(A and B)].

Differences were also notable in mediators of matrix metabolism – the DH strain having significantly higher cartilage pro MMP-9 and pro MMP-2 levels than the control BS2 Strain [$P = 0.012$ and $P < 0.0001$ respectively, Fig. 3(C and D)], and the levels of pro MMP-2 were positively correlated with the total histological changes of the joint surface ($r = 0.576$; $P = 0.003$) and for each individual parameter and overall ($P < 0.05$). The BS2s had very low levels of pro MMP-2, with no detectable active MMP-2 [Fig. 3(E)].

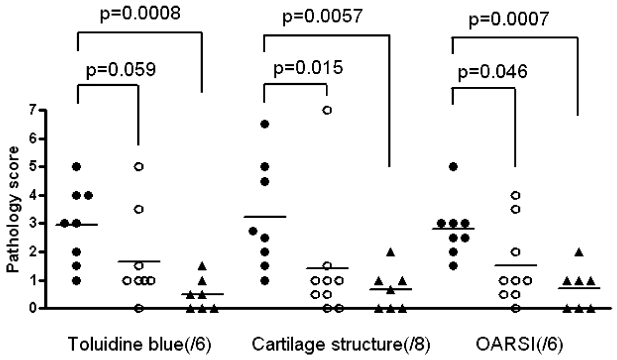

Fig. 1. Differences in individual pathology scores for each criteria as a result of diet (Std vs n-3 in the DH strain) and strain (DH vs BS2 both fed a standard diet). Closed circles, DH fed a standard diet; open circles, DH fed an n-3 diet; closed triangles, BS2 fed a standard diet. Total pathology scores for all comparisons are shown in Table II.

Subchondral bone. The collagen content was significantly higher in the DH animals compared to BS2's ($P = 0.04$; Table II), and was also positively correlated with histological score of the cartilage ($r = 0.403$, $P = 0.034$). Again the levels of lysyl hydroxylation were low in DH animals on the standard n-6 diet compared to control BS2s, although this was not significant [Fig. 4(A)]. DH subchondral bone had significantly more total cross-links [$P = 0.006$, Fig. 4(B)] compared to BS2s.

The Ca:P was lower in the DH than the control guinea pig subchondral bone [$P = 0.002$, Fig. 4(C)]. Although we did not directly measure the BMD of the subchondral bone region, there was significantly greater BMD in the adjacent proximal tibial epiphysis in the DH ($P = 0.0001$; Table II).

Levels of ALP, a marker of osteoblast activity, were significantly higher in DH bone extracts compared to the BS2 strain [$P = 0.04$; Fig. 4(D)]. Pro MMP-9 levels were more than 2-fold lower in the DH animals [$P = 0.001$; Fig. 4(E)] and were negatively correlated with histological score ($r = -0.540$, $P = 0.004$). There was no detectable pro or active MMP-2 in the BS2 extracts, and below accurately quantifiable levels of active MMP-2 in the DH animals only.

Effect of n-3 on disease progression in OA-developing DH strain

OA pathology

The n-3 diet halved the average histological scores in the DH animals ($P = 0.048$) by comparison with the standard diet. When

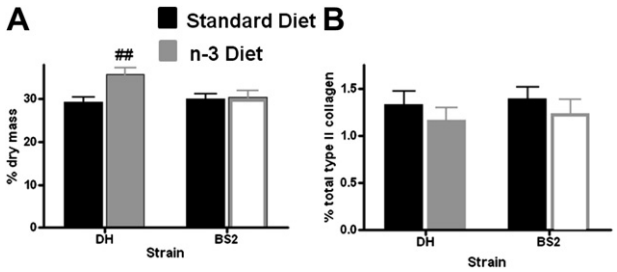

Fig. 2. Differences in cartilage composition as a result of Strain (DH vs BS2) or Diet (standard diet black bars, n-3 diet grey bars, BS2/n-3 unfilled grey bars). (A) GAG (DH std $n = 9$, DH n-3 $n = 10$, BS2 std $n = 9$, BS2 n-3 $n = 8$); (B) Denatured type II collagen (DH std $n = 9$, DH n-3 $n = 9$, BS2 std $n = 10$, BS2 n-3 $n = 10$). For Diet ##$P = 0.003$.

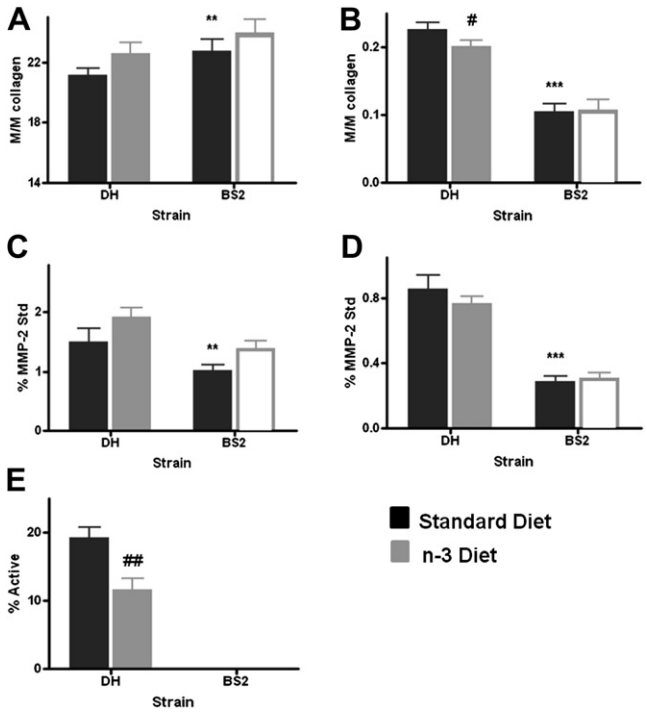

Fig. 3. Differences in biochemical parameters of cartilage as a result of Strain (DH vs BS2) or Diet (standard diet black bars, n-3 diet grey bars, BS2/n-3 unfilled grey bars). (A) Lysyl hydroxylation of collagen (DH std $n=9$, DH n-3 $n=8$, BS2 std $n=9$, BS2 n-3 $n=9$); (B) Lysyl-pyridinoline (DH std $n=9$, DH n-3 $n=8$, BS2 std $n=8$, BS2 n-3 $n=7$): (C) Pro MMP-9 (DH std $n=9$, DH n-3 $n=10$, BS2 std $n=10$, BS2 n-3 $n=10$); (D) Pro MMP-2 (DH std $n=9$, DH n-3 $n=10$, BS2 std $n=10$, BS2 n-3 $n=10$); (E) MMP-2 activation (DH std $n=9$, DH n-3 $n=10$, BS2 std $n=10$, BS2 n-3 $n=10$). For diet #$P<0.05$, ##$P<0.01$. For strain **$P<0.01$, ***$P<0.001$.

the separate scores were considered, there was a diet effect overall ($P=0.0083$), with toluidine blue ($P=0.059$), cartilage structure ($P=0.015$) and OARSI criteria ($P=0.046$) each being lower in the n-3 (Fig. 1).

Pathogenic markers

Cartilage. The GAG content of the cartilage significantly increased ($P=0.003$) with n-3 compared to the control diet in the DH strain [Fig. 2(A)]. Levels of denatured type II collagen were reduced slightly with the n-3 diet in both strains [Fig. 2(B)], although comparison between strains showed no clear association with pathology. High n-3 diet increased lysyl hydroxylation in the OA-prone DH towards those seen in the non-prone control BS2 [$P=0.1$, Fig. 3(A)], and this was reflected in significantly decreased levels of L-Pyr [$P=0.05$, Fig. 3(B)]. The total level of cross-links was also lower in the n-3 fed DH ($P=0.008$, Table II). Levels of pro MMP-9 were elevated, although not significantly [Fig. 3(C)], whilst total MMP-2 (NS) and the percentage active MMP-2 ($P<0.002$) levels were decreased [Fig. 3(D and E)] for the n-3 diet group.

Subchondral bone. The collagen biochemistry parameters measured in the OA-prone strain all changed towards control BS2 levels as a result of the n-3 diet. However, most of these failed to reach significance in this study (Table II). The Ca:P ratio increased in the DH with the n-3 diet [$P=0.015$, Fig. 4(C)].The BMD of the adjacent proximal epiphyseal area also decreased with n-3 ($P=0.03$, Table II). MMP-9 expression increased [$P=0.07$, Fig. 4(E)], and there was a trend towards increased levels of collagen lysine hydroxylation [$P=0.1$, Fig. 4(A)], decreased HLKNL ($P=0.12$) and decreased total collagen cross-links ($P=0.17$) with the n-3 diet (Table II).

Examining the overall data, of the measures of cartilage and bone pathogenic markers examined in the DH on a high n-3 or a standard diet, 16 changed in the direction of the OA-resistant BS2 as a result of the n-3 diet (significant in 7). Of the remainder, two (denatured type II and total cross-links) showed no difference for breed, and two (cartilage pro MMP-9 and HL-Pyr) showed a non-significant change

Table II
Summary of results

	DH		BS2	
	Standard diet	n-3 diet	Standard diet	n-3 diet
Total path. Score†	8.97 (6.17, 11.8)	4.56 (0.83, 8.28)#	2.20 (0.05, 4.34)***	1.78 (0.67, 4.24)
Cartilage				
Type II collagen†	55.7 (47.9, 63.4)	58.7 (50.7, 66.6)	59.2 (53.7, 64.8)	56.9 (46.4, 67.4)
Denatured type II	1.33 (1.01, 1.65)	1.16 (0.85, 1.48)	1.39 (1.11, 1.68)	1.23 (0.88, 1.58)
GAG†	29.3 (27.0, 31.7)	35.7 (32.3, 39.1)##	30.1 (27.4, 32.7)	30.3 (26.5, 34.1)
Pro MMP-2†	0.858 (0.671, 1.04)	0.769 (0.670, 0.865)	0.289 (0.212, 0.366)***	0.312 (0.245, 0.37)
Activated MMP-2†	19.3 (16.1, 22.6)	11.6 (8.0, 15.2)##	0	0
ProMMP-9	1.50 (0.98, 2.02)	1.93 (1.5, 2.25)	1.03 (0.85, 1.2)**	1.38 (1.08, 1.68)
Hydroxylysine†	21.2 (20.1, 22.2)	22.6 (20.9, 24.3)	24.0 (22.0, 26.0)**	22.8 (21.0, 24.5)
HL-Pyr	1.1 (1.0, 1.27)	1.03 (0.96, 1.1)	1.28 (1.09, 1.46)	1.32 (1.26, 1.49)
L-Pyr†	0.228 (0.203, 0.251)	0.200 (0.178, 0.221)#	0.103 (0.076, 0.131)***	0.107 (0.070, 0.144)
Total cross-links	1.92 (1.75, 2.08)	1.68 (1.57, 1.78)##	2.05 (1.84, 2.26)	2.13 (1.96, 2.30)
Subchondral bone				
Collagen†	13.4 (12.3, 14.6)	13.0 (12.49, 13.48)	11.7 (10.4, 13.1)*	12.7 (11.6, 13.7)
Ca:P†	2.73 (2.37, 3.09)	3.36 (2.97, 3.75)#	4.39 (3.43, 5.35)**	4.95 (4.11, 5.79)
ALP†	1.10 (0.85, 1.35)	0.952 (0.873, 1.03)	0.779 (0.58, 0.98)*	0.800 (0.600, 1.00)
Pro MMP-9†	3.07 (2.28, 3.86)	7.22 (2.14, 12.3)	9.83 (5.65, 14.01)***	12.4 (8.86, 15.9)
Hydroxylysine†	14.9 (13.7, 16.1)	16.8 (14.1, 19.4)	16.1 (14.7, 17.6)	16.9 (14.6, 19.2)
HLKNL†	1.08 (0.959, 1.21)	0.976 (0.889, 1.06)	0.927 (0.831, 1.02)*	0.955 (0.840, 1.07)
HL-Pyr†	0.453 (0.391, 0.514)	0.425 (0.366, 0.484)	0.356 (0.312, 0.4)**	0.393 (0.361, 0.425)
L-Pyr†	0.097 (0.070, 0.124)	0.091 (0.075, 0.107)	0.086 (0.065, 0.106)	0.087 (0.058, 0.117)
Total cross-links†	1.82 (1.64, 1.99)	1.67 (1.52, 1.82)	1.52 (1.40, 1.64)**	1.63 (1.52, 1.75)
Adjacent BMD†	519 (507, 530)	495 (479, 520)#	465 (448, 483)***	461.1 (440, 482)

Mean (95% CI); #$P<0.05$, ##$P<0.01$, ###$P<0.001$ comparing diet for DH; *$P<0.05$, *$P<0.01$, ***$P<0.001$ comparing strain (n-6 diet).
† Those parameters for which the n-3 diet modified DH values towards those of the non-OA strain.

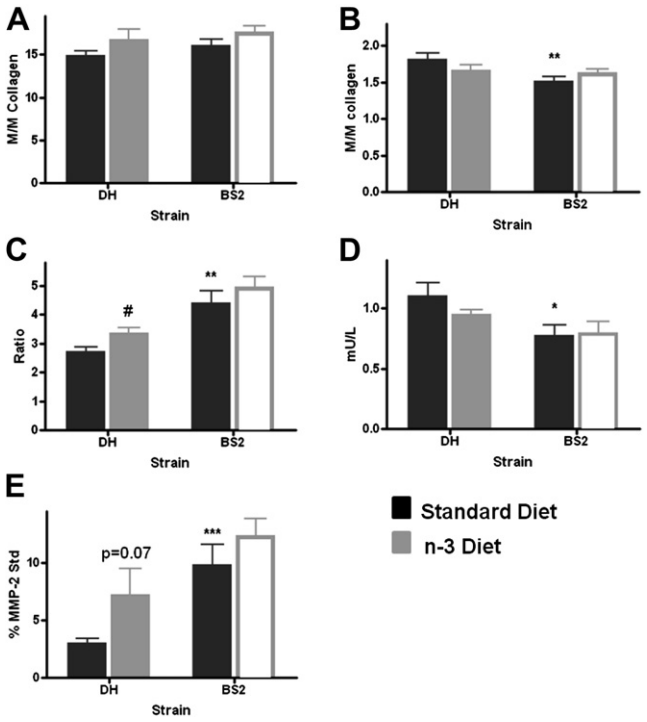

Fig. 4. Differences in biochemical parameters of subchondral bone as a result of Strain (DH vs BS2) or Diet (standard diet black bars, n-3 diet grey bars, BS2/n-3 unfilled grey bars). (A) Lysyl hydroxylation of collagen (DH std $n = 10$, DH n-3 $n = 10$, BS2 std $n = 10$, BS2 n-3 $n = 8$); (B) Total collagen cross-links: (C) Ca:PO_4 ratio ($n = 10$); (D) Alkaline phosphatise (DH std $n = 10$, DH n-3 $n = 10$, BS2 std $n = 8$, BS2 n-3 $n = 10$); (E) Pro MMP-9 (DH std $n = 10$, DH n-3 $n = 10$, BS2 std $n = 8$, BS2 n-3 $n = 8$). For diet #$P < 0.05$. For strain *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

in the opposite direction (Table II). Comparing the direction of dietary changes with respect to the model, a binomial discrete probability distribution demonstrated a probability of $P = 0.0046$ that these occurred by chance, and Chi-square test showed a probability of $P = 0.001$ that the data distribution was equal.

Effect of n-3 in promoting OA in control BS2 strain

The n-3 diet had little apparent effect on control BS2 animals in histopathological OA scores, and fewer effects, compared with DH, on biochemical parameters investigated in this study. This is in line with n-3 modulating both disease parameters in OA and baseline physiological levels of bone and cartilage mediators. The only significant change with the high n-3 diet in the BS2 was raised levels of pro MMP-9 in cartilage [$P = 0.033$, Fig. 3(C)].

Discussion

The principle outcomes from this study were (1) that n-3 PUFAs reduce OA pathology in this naturally occurring model, and (2) that a number of physiological markers of disease confirmed this at a mechanistic level. Also, there was no evidence that n-3 contributed to an increase in OA in either strain. Furthermore, we were able to confirm the utility of this model of OA and demonstrated a number of changes in metabolism and composition not previously reported which may give further insight into the model, and into early OA changes in human disease.

Characterisation of the DH/BS2 model

The pathology scores show clear and spontaneous development of OA in the DH guinea pigs, with little apparent pathology in the BS2 strain at 30 weeks. Characterisation of this model of spontaneous OA, with BS2 as a non-disease prone control, provides valuable information on changes in composition and metabolism of the cartilage and subchondral bone in early OA. However, it should be acknowledged that OA does develop in much older BS2 guinea pigs, and this strain may more accurately be described as late onset.

This is the first study to report levels of collagen cross-linking in guinea pig joints. Cartilage collagen of the DH animals was found to have a reduced lysyl hydroxylation compared with control BS2s, which was reflected in higher levels of the less hydroxylated mature collagen cross-link, L-Pyr. Pokharna *et al* reported no difference in overall Pyr levels (L-Pyr plus HL-Pyr) in a rabbit model of OA, in accordance with our findings[36]. However, Bank *et al* reported elevated levels of hydroxylysine and HL-Pyr in degenerated human articular cartilage[37]. However, these changes were associated with individuals with no apparent OA, unlike the present study.

We identified higher levels of both pro MMP-9 and pro MMP-2 in the DH cartilage compared with BS2, and that active MMP-2 was only detected in OA DH cartilage, in accordance with studies in other species[38]. Elevated catabolism/metabolism, as shown by increased levels and activation of MMPs is associated with OA progression[39–41]. MMP-9 is linked with inflammation, and MMP-2 with collagen turnover. As both are degradative proteases, their association with OA may be expected. However, as both have a role in tissue repair their relationship with OA is likely to be complex.

In subchondral bone, we identified elevated levels of ALP in DH compared with BS2, consistent with previous studies of OA *in-vitro*[42,43] and *in-vivo*[24,44,45], demonstrating increased subchondral bone metabolism in guinea pig OA. Pro and active MMP-2 were barely detectable in DH bone, and absent in the control BS2 strain. Previous studies reported elevated MMP-2 and enhanced activation in OA bone, most notably in the proximal epiphysis[46]. However MMP-9 was found to be lower in the OA-developing DH strain than the control strain. MMP-9 is produced by osteoclasts, and is elevated during active bone resorption[47,48]. Reduced MMP-9 in the OA-developing strain is in line with subchondral bone sclerosis in OA[49], and corroborates the higher levels of bone formation (ALP) seen in DH. However, other studies have shown a link between OA and raised MMP-9. This contradiction may relate to their bone sampling site being outside the subchondral compartment[41], or disease being more advanced[47].

There were elevated levels of individual and total collagen cross-links in the DH subchondral bone compared with the control strain. There have been no other reports to the author's knowledge of collagen cross-linking in the subchondral bone in any species, although some have analysed trabecular bone immediately proximal to the subchondral plate[46]. Raised levels of cross-links are indicative of a stiffer subchondral bone, in line with the previous findings indicating sclerosis in the OA-developing strain[23].

Guinea pig subchondral bone has an unusually high Ca:P ratio (mean 3.86). Although this has not been reported before in guinea pigs, in other species this is generally between 1.7 and 2, increasing with maturity of hydroxyapatite. High Ca:P ratios have been reported in OA subchondral bone[50,51] but in this study it was the control BS2 strain which exhibited the highest ratios. High Ca:P ratios could be indicative of the presence of amorphous, calcium rich deposits[52], and matrix vesicles associated with cartilage mineralisation accumulating large amounts of calcium[53,54]. The low Ca:P in the OA-prone DH strain may result from formation of new, less mature bone, in line with raised ALP.

Effect of n-3 on disease progression in OA-developing DH strain

Few experimental studies have investigated the effects of n-3 PUFAs on OA, although there is clinical evidence that increasing

dietary n-3 relative to n-6 may be beneficial in terms of symptom management in humans[11] and other species[9]. Not all studies however conclude that dietary n-3 PUFA supplementation is of benefit, in the treatment of OA[55]. It is possible that OA could be aggravated due to the potential for increased bone formation which is linked to the progression of OA. This study is the first to look at both cartilage and subchondral bone changes with increased dietary n-3 PUFAs.

In terms of gross pathology, the high n-3 diet significantly and substantially (by 50%) reduced OA associated changes in the DH strain, with no significant effect on the control strain. Having established this improvement in OA pathology, we went on to demonstrate parallel changes in a number of compositional and metabolic markers of cartilage and bone function.

Increased cartilage GAG content, reduced denatured type II collagen (NS), and reduced pro and activated MMP-2 in DH guinea pigs fed an n-3 diet are all indicative of disease attenuation. Others, such as L-Pyr and lysine hydroxylation change to be more in line with the control animals. Only cartilage MMP-9 changed in the opposite direction to that seen in the control animals, though not significantly. However, as noted, the relationship between MMP-9, cartilage degradation and regeneration is likely to be complex. Omega-3 supplemented DH guinea pigs had fewer calcifying cartilage lesions identified as part of the Mankin grading, corroborated by reduction in markers of mineralisation, collagen lysyl hydroxylation and L-Pyr cross-links, each shifting towards those seen in the BS2s. Taken together, the analyses of the biochemical parameters demonstrate an n-3 treatment effect, and provide evidence of particular mechanisms of disease, but that these should be explored further to confirm links to n-3 diet.

Evidence that n-3 modifies some elements only in the disease-prone animals, whilst others are also moderated in the control strain, supports the contention that n-3 regulates both the disease process and the underlying physiology of cartilage and subchondral bone in the absence of overt disease. As there is evidence of OA in very old BS2 guinea pigs, it could be speculated that those elements modified in the "later onset" BS2 as well as the DH represent the earliest stages of OA pathology, such that denatured type II collagen, collagen hydroxylation (in cartilage and bone), MMP-9 (also in both) and Ca:P, are early events, whereas changes in GAG content, collagen crosslinking, MMP-2 and ALP are part of established disease in th DH animals. Thus n-3 has the potential to prevent or delay the onset of disease as well as to reduce its progression.

Though no previous *in vivo* studies have examined cartilage matrix changes with n-3, *in vitro* studies using bovine chondrocytes identified reduced expression for cartilage-degrading proteinases, cyclooxygenase-2 and inflammatory cytokines in the presence of n-3, in particular eicosapentanoic acid[12].

Reports on the effect of PUFAs on bone are more numerous, with n-3 PUFAs generally reducing bone loss, by decreasing bone resorption[56], and/or increasing bone formation[57,58]. However specific effects on subchondral bone are less well documented, and extrapolation of data from other skeletal sites may be misleading as sclerotic subchondral bone present in OA is dissimilar to other bone sites and disease states[17,23,49,59]. Increase in subchondral bone formation as a result of n-3 is potentially detrimental in the progression of OA. However, in the OA-prone DH guinea pigs, n-3 acts to modify markers indicating reduced subchondral bone deposition, including increased pro MMP-9, increased Ca:P ratio, decreased collagen content and decreased ALP levels. Supplementation with n-3 also directed collagen cross-linking and lysine hydroxylation towards control BS2 levels in DH subchondral bone.

Supplementation with n-3 reduced the BMD of the adjacent proximal epiphyseal bone, again towards control levels. This is contrary to expectations as n-3 is generally reported to increase bone density[14], but as increased density of bone underlying cartilage is thought to contribute to OA[19], this may demonstrate a further protective effect of n-3.

There is circumstantial evidence for a link between human OA and a high n-6:n-3 ratio. High n-6, particularly arachidonic acid, have been reported in bone from OA patients[2], and an association between OA and subchondral osteoblastic production of PGE_2, IL-6, and COX-2 levels has been identified, as well as an elevated response to exogenous PGE_2[60].

Therefore supplementation of n-3 PUFAs returns the balance in n-3 and n-6 to a more 'normal' functional state, reducing signs of OA in both cartilage and subchondral bone.

Effect of n-3 on OA promotion in control BS2 strain

There is little evidence from this study for any detrimental effects of n-3 supplementation in the control OA-resistant strain. This suggests that there are no ill effects of n-3 on OA development in disease free individuals.

Limitations of the study

The principle limitations of this study were the small quantities of tissues available for analysis and the large number of analyses attempted, such that many measures were at the lower end of their detectable range. A group size of 10 was adequate for determining differences in pathology with diet, which was the primary outcome of this study and for detecting differences in strain. However, there were a number of biochemical measures that did not reach significance when comparing diets within the OA-developing strain. It should also be noted that the development of OA in this model is at an early age by comparison with human disease, and occurs on an accelerated timescale.

Conclusion

This study demonstrates clear benefits of n-3 supplementation in reducing the signs of OA in a naturally occurring model of disease. Furthermore, there was no sign that increased n-3 would lead to disease in the OA free strain. We have also further characterised this model, and identified fundamental differences in cartilage and bone biology associated with OA. We propose that a high n-3 diet has the potential to reduce signs of OA in both cartilage and subchondral bone. Further studies are needed to determine the influence of n-3 on established disease, and to confirm these effects in human OA.

Author contributions

We declare that all authors listed contributed to the acquisition of data, drafting, critical revision and final approval of this manuscript, in line with Osteoarthritis and Cartilage guidelines. Dr John Tarlton (john.tarlton@bristol.ac.uk) takes responsibility for the integrity of this work.

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Conflict of interest

The Authors have no conflict of interest relating to this manuscript.

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