# The biological importance and mechanisms of long-chain polyunsaturated fatty acids on bone growth in diet-induced obesity: Investigation in children with obesity and rodents

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## Abstract

**Background:** The majority of bone mass is acquired during childhood and adolescence. Individuals with the highest bone mass are best protected against bone loss with aging. However, excess adiposity could limit bone acquisition. Children with obesity are at greater risk of skeletal fractures, due in part to the combination of poor dietary patterns, sedentary lifestyle, greater propensity to fall, and force generated on impact through falls. In animal models of diet-induced obesity, bone size is enlarged yet strength compromised due to thinner cortices and trabeculae. Long-chain polyunsaturated fatty acids (LCPUFA) may benefit bone mass accrual and/or protect against age-related bone loss; as well as modulate adipocyte metabolism, with opposing effects of omega (n)-3 and n-6 LCPUFA on lipid accumulation, adipokine secretion and gene expression. It is uncertain whether LCPUFA dually influence bone metabolism and adiposity during growth. The global aim of the present thesis is to advance understanding of the role of LCPUFA *in vivo* on bone mass and architecture in children with obesity through: (1) assessment of dietary LCPUFA intake and testing the relationships with biological markers and adiposity; (2) examining the relationship between LCPUFA status and bone health outcomes; and (3) monitor longitudinal changes in bone mass accrual in an animal model of diet-induced obesity supplemented with n-6 arachidonic acid (AA, C20:4 n-6).

**Methods:** *(Aims 1 and 2)* Baseline data were obtained from the McGill Youth Lifestyle Intervention with Food and Exercise study. Healthy children (6-12 y) with obesity (n=108) were classified as overweight or obese as per the World Health Organization cut-offs for body mass index-for-age and -sex Z-scores. Whole body composition and bone mass was assessed with dual-energy X-ray absorptiometry and complemented with non-dominant distal forearm measurements with peripheral quantitative computed tomography for bone geometry and soft-

tissue analyses. Red blood cell (RBC) fatty acid profile was quantified by gas chromatography. Diet was assessed with 3-d food diaries. Children, after stratification by sex and Tanner stages were divided into tertiles based on % body fat (*Aim 1*) or AA proportions in RBC (*Aim 2)* and compared using a MIXED model ANOVA. *(Aim 3*): Male Sprague-Dawley rats (n=42, 4-week) were randomized into groups fed a control diet (CTRL, AIN-93G), high-fat diet (HFD, 35% kcal fat) or HFD+AA (1% w/w diet) for 6 weeks. Body composition, bone mineral density and microarchitecture were measured using dual-energy X-ray absorptiometry and micro-computed tomography. RBC fatty acid profile was measured with gas chromatography, and blood samples collected to measure biomarkers of bone turnover and adipokines. Bone strength was assessed with 3-point bending. 3T3-L1 adipocytes were treated with LCPUFA (10-100 μM) to assess lipid accumulation and gene expression.

**Results:** *(Aim 1)*: Children (n=63) were 9.0 ± 0.2 y of age with a BMI Z-score 3.1 ± 0.2. Dietary fat intake averaged to 30.1 ± 0.6 % for all children; intakes of LCPUFA did not differ among tertiles of % body fat. Moderate correlations were observed between RBC eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) proportions, dietary EPA and DHA ($r=0.39$, $p<0.05$) as well as fish servings ($r=0.33$, $p<0.05$). RBC proportions of α-linolenic acid and EPA+DHA adjusted for age, Tanner stages and race were 40% and 15% lower ($p<0.05$), respectively, in children in the highest tertile of % body fat (tertile 3) compared to those in tertile 1. *(Aim 2*): Ultra-distal, mid- and total- distal forearm bone mineral content, adjusted for sex, age, % body fat, race and forearm length were 10 to 13% greater ($p<0.05$) in children in the first tertile of RBC AA proportions relative to the third. Muscle cross-sectional area was 15% lower in the third tertile compared to the first, along with higher fasting insulin concentrations (27%) and the homeostatic model assessment of insulin resistance (31%). *(Aim 3*): Rats in the HFD+AA group

had significantly greater body fat % (12%), body weight (6%) and serum leptin concentrations (125%) than CTRL at end of study, whereas visceral fat (mass and %) was increased in both HFD and HFD+AA groups. HFD+AA showed reduced whole body bone mineral content and femur mid-diaphyseal cortical bone cross-sectional area than HFD and CTRL, without impairment in bone strength. Contrarily, HFD+AA had greater femur metaphyseal trabecular volumetric bone mineral density (29%) and bone volume fraction (20%) compared to CTRL. AA treatment of 3T3-L1 adipocytes resulted in greater leptin secretion and mRNA levels relative to controls, with the greatest effect observed at 25 μM. Nile red staining showed a greater proportion of cells containing small, multiple lipid droplets in AA-treated cells compared to single droplets in adipocytes with n-3 LCPUFA treatment.

**Conclusion:** This research enhanced the understanding of bone biology and mechanisms by providing a link between the effects of obesity on LCPUFA and bone. Children with obesity do not consume excess fat as a percentage of total energy. However, greater adiposity is associated with lower n-3 LCPUFA status and is consistent with suboptimal dietary intakes of n-3 LCPUFA and fish. Higher AA status in children aligned with deficits in forearm bone mass, geometry, muscle mass, and early signs of insulin resistance. While AA exacerbated adiposity development in the animal model, the impact on bone mass and bending strength was variable depending on skeletal site, showing both beneficial and detrimental effects on the appendicular and axial skeleton respectively. It remains to be determined if correction of low n-3 LCPUFA status in children with obesity through encouraging fish/seafood intake will modulate the relationship between AA and bone growth.

## Résumé

**Contexte:** La majorité de la masse osseuse est acquise pendant l'enfance et l'adolescence. Les personnes ayant une masse osseuse plus élevée sont mieux protégées contre la perte osseuse en vieillissant. Cependant, un excès d'adiposité pourrait limiter l'acquisition d'os. Les enfants obèses ont un risque plus élevés de fractures du squelette notamment à cause d'une mauvaise alimentation, d'un style de vie sédentaire, d'une plus grande propension à chuter et de la force générée par ces chutes. Selon certains modèles d'animaux d'obésité d'origine alimentaire, la taille des os est agrandie, mais leur force est compromise à cause de la réduction de l'épaisseur des corticales et trabécules osseuses. Les acides gras polyinsaturés à longue chaîne (AGPLC) peuvent être bénéfiques pour la croissance de la masse osseuse et / ou protéger contre la perte osseuse liée au vieillissement. Les AGPLC peuvent également moduler le métabolisme des adipocytes, avec des effets opposés d'AGPLC oméga (n) -3 et n-6 sur l'accumulation de lipides, la sécrétion d'adipokine et l'expression génique. Il est incertain que les AGPLC puissent à la fois influencer le métabolisme osseux et l'adiposité pendant la croissance. L'objectif global de la présente thèse est d'accroître la compréhension du rôle des AGPLC *in vivo* sur la masse et l'architecture osseuses chez les enfants obèses en: (1) décrivant l'apport alimentaire en AGPLC et sa relation avec certains marqueurs biologiques et l'adiposité, (2) examiner la relation entre le statut d'AGPLC et les résultats de la santé des os, et (3) surveiller les modifications longitudinales de l'accumulation de la masse osseuse dans un modèle animal d'obésité d'origine alimentaire enrichi d'acide n-6 arachidonique (AA, C20: 4 n-6).

**Méthodes:** (*Objectifs 1 et 2*): Les données de base ont été obtenues par l'étude sur l'intervention du mode de vie des jeunes avec alimentation et exercices de McGill. Les enfants en bonne santé (6-12 ans) souffrant d'obésité (n = 108) ont été classés en surpoids ou obèses selon les seuils

établis par Organisation Mondiale de la Santé pour les scores-Z d'indice de masse corporelle par âge et de sexe. La composition corporelle et la masse osseuse ont été évaluées à l'aide d'absorptiométrie à rayons X à double énergie (DXA), et complétée par des mesures de l'avant-bras distal non-dominant avec un tomodensitomètre quantitatif, pour os périphériques pour la géométrie de l'os et les analyses des tissus mous. Le profil en acides gras des globules rouges a été quantifié par chromatographie en phase gazeuse. Le régime alimentaire a été évalué avec des journaux quotidiens de 3 jours. Après stratification selon le sexe et les stades de Tanner, les enfants ont été divisés en tertiles basés sur le pourcentage de graisse corporelle (objectif 1) ou en AA dans les globules rouges (objectif 2) et comparés à une ANOVA modèle MIXTE. (*Objectif 3*): Des rats Sprague-Dawley mâles (n = 42, 4 semaines) ont été randomisés en groupes recevant un régime témoin (CTRL, AIN-93G), un régime riche en gras (HFD, 35% de kcal) ou HFD + AA (régime 1% p / p) pendant 6 semaines. La composition corporelle, la densité minérale osseuse et la microarchitecture ont été mesurées par DXA et par micro-tomodensitométrie respectivement. Le profil en acides gras des globules rouges a été mesuré par chromatographie en phase gazeuse et des échantillons de sang ont été recueillis pour mesurer les biomarqueurs du remodelage osseux et des adipokines. La force des os a été évaluée avec une flexion en 3 points. Les adipocytes 3T3-L1 ont été traités avec AGPLC (10-100 μM) pour évaluer l'accumulation de lipides et l'expression génique.

**Résultats:** (*Objectif 1*): Les enfants (n = 63) étaient âgés de 9,0 ± 0,2 ans avec un indice Z de BMI de 3,1 ± 0,2. L'apport en graisses alimentaires a été en moyenne de 30,1 ± 0,6% pour tous les enfants. Les AGPLC alimentaires ne différaient pas entre les tertiles. Des corrélations modérées ont été observées entre les proportions d'acide eicosapentaénoïque (EPA) dans les globules rouges et d'acide docosahexaénoïque (DHA), d'EPA et de DHA alimentaires (r = 0,39, p

<0,05) ainsi que les de portions de poisson (r = 0,33, p <0,05). Les proportions d'acide alpha-linolénique et d'EPA + DHA dans les globules rouges ajustées en fonction de l'âge, du stade de Tanner et de la race étaient respectivement inférieures de 40% et 15% chez les enfants du tertile le plus élevé du pourcentage de graisse corporelle (tertile 3) par rapport à ceux du tertile 1. (*Objectif 2*): La teneur en minéraux de l'os de l'avant-bras ultra-distale, totale et distale, ajustée en fonction du sexe, de l'âge, du pourcentage de graisse corporelle, de la race et de la longueur de l'avant-bras était 10 à 13% supérieure chez les enfants du premier tertile des proportions d'AA dans les glubules rouges par rapport à la troisième. La section transversale musculaire était inférieure de 15% dans le troisième tertile par rapport au premier, ainsi qu'une concentration plus élevée d'insuline à jeun (27%) et de l'évaluation du modèle homéostatique de résistance à l'insuline (31%). (*Objectif 3*): À la fin de l'étude, les rats du groupe HFD + AA présentait des taux de graisse corporelle (12%), de poids corporel (6%) et de la leptine sérique (125%) significativement supérieurs à ceux du groupe CTRL. La graisse viscérale (masse et pourcentage) a augmenté dans les groupes HFD et HFD + AA. Le groupe HFD + AA a montré une réduction de la teneur en minéraux de l'os du corps entier et de la section transversale de l'os cortical médian-diaphysaire du fémur par rapport aux HFD et CTRL sans affaiblissement de la résistance des os. À l'inverse, HFD + AA présentait une densité minérale osseuse volumétrique trabéculaire métaphysaire du fémur supérieure (29%) et une fraction de volume osseux (20%) également supérieure à celle de la CTRL. Le traitement à l'AA des adipocytes 3T3-L1 a entraîné une plus grande sécrétion de leptine et des niveaux de l'ARNm par rapport aux témoins, l'effet le plus marqué étant observé à 25 μM. La coloration au rouge du Nil a démontré une plus grande proportion de cellules contenant de petites gouttelettes lipidiques multiples dans les cellules traitées à l'AA par rapport aux gouttelettes simples dans les adipocytes traités avec n-3 AGPLC.

**Conclusion**: Cette recherche a permis de mieux comprendre la biologie et les mécanismes osseux en établissant un lien entre les effets de l'obésité sur les AGPLC et les os. Les enfants obèses ne consomment pas un excès de graisse sous forme de pourcentage de l'énergie totale. Cependant, une plus grande adiposité est associée à un statut inférieur en AGPLC n-3 et est compatible avec des apports alimentaires sous-optimaux en AGPLC n-3 et du poisson. Un statut AA plus élevé chez les enfants est aligné aux déficits de la masse osseuse de l'avant-bras, de la géométrie, de la masse musculaire et des premiers signes de résistance à l'insuline. Alors que les l'AA exacerbe le développement de l'adiposité dans le modèle animal, l'impact sur la masse osseuse et la résistance à la flexion était variable selon le site du squelette, montrant des effets bénéfiques et néfastes sur le squelette appendiculaire et axial respectivement. Il reste à déterminer si l'amélioration du faible statut d'AGPLC n-3 chez les enfants obèses modulera la relation entre AA et croissance osseuse en encourageant la consommation de poisson / fruits de mer.

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## Preface and advancement of scholarly knowledge

This thesis is based on (1) cross-sectional analyses of baseline data obtained from the McGill Youth Lifestyle Intervention with Food and Exercise study; a study that enrolled healthy children classified with overweight condition or obesity from Montreal, QC, Canada; and (2) an animal model of diet-induced obesity designed based on the observations in human children. It aims to fill current knowledge gaps in the understanding of biological responses of bone to long-chain polyunsaturated fatty acids (LCPUFA) under the constraints of childhood obesity.

This thesis is presented in a manuscript-based format for submission to peer-reviewed scientific journals. The health benefits associated with dietary LCPUFA across life stages extend beyond the well-documented outcomes (e.g. brain and cognitive development, modulating inflammatory and immune responses and cardiovascular diseases risks). Appendix A has been published as a book chapter that aims to summarize the effects and selected mechanisms by which docosahexaenoic acid supports optimal bone growth. The first manuscript aims to describe the dietary intakes of children with obesity, with particular focus on omega-3 long-chain polyunsaturated fatty acids (LCPUFA) and its relationship with a common biological marker. The first study utilized comprehensive assessment methods including dietary recalls, gas chromatography and absorptiometry-based (i.e. DXA) measures of adiposity. The data presented in this manuscript are unique as they include assessment of body composition, representing a more sensitive approach to estimate adiposity in children compared to body weight and/or the body mass index. The inclusion of a biomarker of dietary intake was used to compensate for errors related to recall bias associated with routine dietary assessment methods. The second manuscript examined the relationship between LCPUFA status, particularly that of arachidonic acid (AA) with bone health outcomes in children with obesity. To our knowledge, this is the first

study that reported on the association between AA status and bone in human children during growth. The 3D imaging technique peripheral quantitative computed tomography complemented DXA measurements and provided additional information on forearm bone geometry and local tissue composition, which have important implications for bone strength. The third manuscript, based on the animal model, investigated the effect of AA addition to high-fat diet in growing male rodents. To date, a paucity of animal studies has examined the influence of omega-6 LCPUFA on growth in the post-natal period, and few are conducted in the context of diet-induced obesity. Possible mechanisms and cellular mediators are identified but require characterization of exact pathways involved are required. Together, these manuscripts will contribute to the literature related to the biological roles of LCPUFA in the bone-fat relationship.

The recognition of the ability of LCPUFA to enhance intellectual and visual development in young mammals has led to widespread supplementation that has extended beyond human consumption to LCPUFA-enriched pet foods, due in part to the potent anti-oxidative and anti-inflammatory properties of LCPUFA. For instance, feeding omega-3 LCPUFA have shown efficacy in improving arthritic conditions in pet dogs affected by osteoarthritis. This work is not intended for translation to set recommendations for human supplementation; the lack of intra-cortical remodeling and a gallbladder in rats signifies the dissimilarity between humans and rodents in terms of bone and fatty acid metabolism. This thus restricts the applicability of this research's outcome to humans unless further tests in larger animal models (e.g. sheep, pigs, non-human primates) are performed. Nonetheless, this work remains highly applicable to understanding mammalian bone biology; devising nutrition regimens in veterinary practices, as well as the food industry in light of the prevailing fortification of omega fats either as a semi-purified food ingredient or as fish oil to common foods.

**Invited book chapter:**

- **Mak IL** and Weiler HA. Docosahexaenoic acid (DHA) and properties, function and health effects: DHA and growing bone. *Docosahexaenoic acid (DHA): Properties, Function and Health Effects*. 2016. Nova Science Publishers, Incorporated (Appendix A)

**Research articles currently submitted or under review in peer-reviewed journals:**

- **Mak IL,** Cohen TR, Vanstone CA and Weiler HA. Increased adiposity in children with obesity is associated with low red blood cell omega-3 fatty acid status and inadequate LCPUFA dietary intake (Chapter 3: Under peer-review in *Pediatric Obesity*)
- **Mak IL,** Cohen TR, Vanstone CA and Weiler HA. Arachidonic acid status negatively associates with forearm bone outcomes and glucose homeostasis in children with overweight condition or obesity (Chapter 4: Published online in *Applied Physiology, Nutrition and Metabolism*)
- **Mak IL**, Lavery P, Agellon S, Rauch F, Murshed M and Weiler HA. Arachidonic acid exacerbates diet-induced obesity and reduces bone mineral content without impacting bone strength in growing male rats (Chapter 5: Published online in *The Journal of Nutritional Biochemistry*)

**Abstracts at professional meetings:**

- **Mak IL**, Cohen TR, Vanstone CA, Loiselle SE, Hazell TJ and Weiler HA. Arachidonic acid status is associated with forearm bone outcomes and glucose homeostasis in overweight and obese children. The American Society of Nutrition Annual Meeting, 2018. Boston, MA. USA
- **Mak IL**, Wang K, Lavery P, Agellon S and Weiler HA. Arachidonic acid reduces bone mass without influencing visceral adiposity in growing obese rats. The Reseau de

Recherche en Sante Buccodentaire et Osseuse (RSBO) Scientific Day, 2016. Montreal, QC, Canada

- **Mak IL**, Wang K, Lavery P, Agellon S and Weiler HA. Arachidonic acid reduces bone mass without influencing visceral adiposity in growing obese rats. The American Society for Bone Mineral Research Annual Meeting, 2015. Seattle, WA, USA
- **Mak IL**, Cohen TR, Loiselle SE, Vanstone CA, Hazell TJ and Weiler HA. Long-chain polyunsaturated fatty acid status associated with bone mass in overweight and obese children. Experimental Biology, 2015. Boston, MA, USA
- **Mak IL**, Wang K, Lavery P, Agellon S and Weiler HA. Elevated arachidonic acid intakes reduce bone mineral content and trabecular volume in growing obese rats, 2015. Experimental Biology, 2015. Boston, MA, USA

**Oral conference presentations:**

- **Mak IL**, Murshed MM and Weiler HA. Differential effects of long-chain polyunsaturated fatty acids on lipid accumulation and adipokine secretion in mature 3T3-L1 adipocytes. The International Society of the Study of Fatty Acids 2018. Las Vegas, NV, USA

## Contributions of authors

**Manuscripts 1 and 2:** The candidate was the primary author, responsible for posing the research question, conducted all statistical analyses, wrote the first draft of the manuscript, and edited all subsequent versions. Dr. T. Cohen and C. Vanstone aided with revisions to the manuscript. Dr. H. Weiler made contributions to the manuscript, aided with data interpretation and statistical analysis. All authors have read and approved the manuscript prior to submission for publication. The McGill Youth Lifestyle Intervention with Food and Exercise (MYLIFE) study was funded by the Dairy Research Cluster Initiative (Dairy Farmers of Canada, Agriculture and Agri-Food Canada, and the Canadian Dairy Commission) (operating grant), and the Canadian Foundation for Innovation (infrastructure funding).

Author study involvement for Manuscripts 1 and 2:

The candidate conducted all the gas chromatography analysis to assess fatty acid composition in red blood cells, participated in the analysis and auditing of dietary data.

Dr. T. Cohen actively participated in participant recruitment and study visits, performed anthropometric assessments, data entry and auditing.

C. Vanstone prepared the ethics submissions, actively participated in recruitment, collected blood samples, and when necessary performed DXA assessments and anthropometry.

Dr. H. Weiler is the primary investigator of the MYLIFE study, was involved in the development and grant proposal stages of the project and supervised all aspects of the study.

**Manuscripts 3:** The candidate was the primary author, responsible for posing the research question, conducted all statistical analyses, wrote the first draft of the manuscript, and edited all subsequent versions. P. Lavery, S. Agellon, Dr. F. Rauch and Dr. M. Murshed aided with revisions to the manuscript. Dr. H. Weiler made contributions to the manuscript, aided with data

interpretation and statistical analysis. All authors have read and approved the manuscript prior to submission for publication. The study was funded by the Natural Resource and Engineering Council of Canada and the Canadian Foundation for Innovation (infrastructure funding).

Author study involvement for Manuscript 3:

The candidate was responsible for developing the research project, helping with ethics approval, welfare of animals, blood sample collection and processing, bone and body composition analysis (DXA, *in vivo* and *ex vivo* μCT), bone strength testing, biochemistry, fatty acid analysis and cell culture studies.

P. Lavery actively participated in sample collection during the animal study, involved in the welfare of animals, and assisted with bone imaging and strength testing analysis.

S. Agellon was involved in blood sample processing, biochemistry and gas chromatography analysis.

Dr. F. Rauch helped to guide the project and provided feedback on the manuscript and assisted in the design of the *in vitro* experiments.

Dr. M. Murshed assisted in the conception of the *in vitro* work, supervised the experiments and ensured proper techniques were used. All cell culture work was performed in his laboratory.

Dr. H. Weiler, the senior author, was involved in the development and grant proposal stages of the project and supervised all aspects of the study.

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## List of abbreviations

| | |
|---|---|
| 25(OH)D | 25-hydroxyvitamin D |
| α-MSH | α-melanocyte stimulating hormone |
| μCT | Micro-computed tomography |
| AA | Arachidonic acid, C20:4 n-6 |
| aBMD | Areal bone mineral density |
| AI | Adequate Intake |
| ALA | α-linolenic acid, C18:3 n-3 |
| ALP | Alkaline phosphatase |
| AMDR | Acceptable Macronutrient Distribution Range |
| AMPK | AMP-activated protein kinase |
| ANOVA | Analysis of variance |
| aP2 | Fatty acid binding protein |
| ARASCO | Arachidonic Acid-rich Single Cell Oil |
| BMC | Bone mineral content |
| BMD | Bone mineral density |
| BMI | Body mass index |
| BS/TV | Bone surface density |
| BSA | Bovine serum albumin |
| BV/TV | Bone volume fraction |
| cAMP | Cyclic adenosine monophosphate |
| CART | Cocaine- and amphetamine-regulated transcript |
| CCHS | Canadian Community Health Survey |
| C/EBP | CCAAT/enhancer-binding proteins |
| CE | Cholesterol esters |
| CFG | Canada's Food Guide |
| CI | Confidence interval |
| CNS | Central nervous system |
| Conn.D | Connectivity density |
| COX | Cyclooxygenase |
| CSA | Cross sectional area |
| Ct.Ar | Cortical area |
| CT | Computed tomography |
| CTRL | Control |
| CTx | Carboxy-terminal telopeptide of type I collagen |
| CV% | Coefficient of variation |
| CVD | Cardiovascular diseases |
| d | Day (s) |

| | |
|---|---|
| D6D | Δ6 desaturase |
| DHA | Docosahexaenoic acid, C22:6 n-3 |
| DHGLA | Dihomo-γ-linolenic acid, C20:3 n-6 |
| DIO | Diet-induced obesity |
| DMEM | Dulbecco's Modified Eagle's medium |
| DPA | Docosapentaenoic acid, C22:5 n-3 |
| DRI | Dietary Reference Intake |
| DXA | Dual-energy X-ray absorptiometry |
| EAR | Estimated Average Requirement |
| ELISA | Enzyme-linked immunosorbent assay |
| EPA | Eicosapentaenoic acid, C20:5 n-3 |
| FAME | Fatty acid methyl esters |
| FBS | Fetal bovine serum |
| FFMI | Fat-free mass index |
| FFQ | Food frequency questionnaire |
| FiSK | Fish, children, health and cognition |
| FMI | Fat mass index |
| FOXO | Forkhead box O |
| g | Gram (s) |
| GC | Gas chromatography |
| GLA | γ-linolenic acid, C18:3 n-6 |
| h | Hour (s) |
| HFD | High fat diet |
| HOMA-IR | Homeostasis Model Assessment for Insulin Resistance |
| ICV | Intracerebroventricular |
| IGF-1 | Insulin-like growth factor-I |
| IL | Interleukin |
| i.p. | Intraperitoneal |
| IP-R | Prostacyclin receptor |
| IQR | Interquartile range |
| IR | Insulin resistance |
| IU | International units |
| kcal | Kilocalorie |
| kg | Kilogram (s) |
| LA | Linoleic acid, C18:2 n-6 |
| LCPUFA | Long chain polyunsaturated fatty acids |
| LOX | Lipoxygenase |
| LV | Lumbar vertebrae |
| m | Meter (s) |

| | |
|---|---|
| Ma.Ar | Marrow area |
| MAPK | Mitogen-activated protein kinase |
| M-CSF | Macrophage colony stimulating factor |
| Meat & Alt | Meat and Alternatives |
| Milk & Alt | Milk and Alternatives |
| mo | Month (s) |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cells |
| MUFA | Monounsaturated fatty acids |
| MYLIFE | McGill Youth Lifestyle Intervention with Food and Exercise |
| n-3 | Omega-3 |
| n-6 | Omega-6 |
| NEFA | Non-esterified fatty acids |
| NHANES | National Health and Nutrition Examination Survey |
| NIST | National Institute of Standards and Technology |
| NPY | Neuropeptide Y |
| O3I | Omega-3 Index |
| OPG | Osteoprotegerin |
| OR | Odds ratio |
| Osx | Osterix |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PG | Prostaglandin |
| PI3K | Phosphoinositide 3-kinase |
| PKA | Protein kinase A |
| PL | Phospholipids |
| $PLA_2$ | Phospholipase $A_2$ |
| PPAR | Peroxisome proliferator-activated receptor |
| PPRE | Peroxisome proliferator-activated receptor response element |
| pQCT | Peripheral quantitative computed tomography |
| RANK | Receptor activator of nuclear factor-κB |
| RANKL | Receptor activator of nuclear factor-κB ligand |
| RBC | Red blood cells |
| RDA | Recommended Dietary Allowance |
| ROI | Region of interest |
| RT-QCR | Reverse transcription polymerase chain reaction |
| Runx | Runt-related transcription factor |
| SCD | Stearoyl-CoA desaturase |
| SD | Standard deviation |

| | |
|---|---|
| SEM | Standard error of the mean |
| SFA | Saturated fatty acids |
| SMI | Structure model index |
| SNS | Sympathetic nervous system |
| SREBP | Sterol-response element binding protein |
| $SSI_p$ | Strength-strain index (polar) |
| Tb.N | Trabecular number |
| Tb.Sp | Trabecular separation |
| Tb.Th | Trabecular thickness |
| TNF | Tumor necrosis factor |
| TRAF | Tumor necrosis factor receptor associated factor |
| TRAP | Tartrate-resistant acid phosphatase |
| Tt.Ar | Total area |
| V&F | Vegetables and Fruits |
| vBMD | Volumetric bone mineral density |
| VMH | Ventromedial nuclear of the hypothalamus |
| VOI | Volume of interest |
| WHO | World Heath Organization |
| wk | Week (s) |
| WT | Wild-type |
| y | Year (s) |

# CHAPTER 1

## Introduction and Rationale

## 1.1 Background

Childhood and adolescence represent the most critical stages for developing optimal bone strength. Approximately 90% of bone mineral acquisition occurs between 12 and 18 y [1], where genetic, hormonal and environmental influences interact to enhance skeletal mineralization, expansion, and linear growth to the greatest extent. Peak bone mass is defined as the bone mineral density (BMD) during the stable period following growth and accrual of bone mass [2]. In women, peak bone mass of the lumbar spine and proximal femur are attained in late adolescence or early adulthood [3, 4], whereas total body bone mass continue to increase slightly through the third decade of life [4]. Thereafter, bone mass declines at a rate of approximately 0.4-1.6% per year for both sexes before the age of 50 y [5-7], and could reach up to 10% per year when levels of biologically-active sex steroids decline. The variance in bone mass among individuals at completion of growth is an order of magnitude greater than the variance in rate of bone loss during aging (1SD = 10% and 1% respectively) [8]. Hence one's susceptibility to fractures later in life is strongly dependent on whether peak bone mass is maximized during growth. The full genetic potential for bone mineral accrual can be attained only if nutrition, physical activity and other lifestyle factors are optimized. Therefore, any disorders or conditions that alter bone formation and/or resorption during this maturation period may result in suboptimal skeletal development, and eventually pose greater risks of osteoporotic fractures later in life [9].

Excess adiposity due to compromised nutrition, activity and lifestyle has traditionally been considered to positively influence BMD because of the anabolic osteogenic response from additional mechanical loading. However, recent findings suggest that bone quality is compromised. Furthermore, persistent weight-bearing associated with obesity during growth

may inhibit normal movement patterns, predisposing children with obesity to musculoskeletal pain and injuries [10, 11]. Children and adolescents with obesity (n=227, 12.6 ± 2.7 y, BMI>95$^{th}$ percentile) had significantly greater chances of skeletal fractures (OR: 4.54; 95% CI 1.6-13.2) compared to their normal weight counterparts. They are also more likely to complain of musculoskeletal pain (OR: 4.04; 95: CI 1.5-10.6) [12], and suffer from orthopedic complications including lower-limb misalignment [12], Blount's disease (adolescent bowing of the legs) [13] and slipped capital femoral epiphyses [14].

About 40–50% of children sustain at least one fracture by the age of 18 y [15], proportionately more compared to the fracture rates of 12-17% in elderly >50 y [16, 17]. The incidence rate is most pronounced among boys [18, 19], and peaks during the pubertal growth spurt [20]. Additionally, whereas fractures usually occur at the hip and spine of older adults, the forearm is the most common site of fractures in children, showing nearly four times greater incidence rates than the lower limbs [21]. Children (n=401, 3.5-17.3 y) with forearm fractures are also more likely to be overweight or obese compared to those who suffered an upper arm or hand fracture [22]. In addition, children with obesity mostly tend to fall from lower heights (as they avoid rapid-movement activities and climbing activities), yet they still exhibit a greater risk for fractures [23, 24]. It is possible that specific skeletal sites as well as skeletal compartments are differentially affected by excess adiposity, underlying the deficits in bone strength observed in children with obesity.

The impact of overweight and obesity on bone metabolism manifests as a result of direct and indirect factors. In addition to the excess weight and adiposity associated with obesity and disproportionate weight relative to bone strength, complications of childhood obesity include acceleration of thelarche and menarche timing in girls [25], and pubertal advancement in boys

[26] that can result in adverse effects on maturing bones in both sexes [27]. Obesity also affects pubertal timing through nutrition-related signals (e.g. insulin) on the reproductive axis [28]. Hormonal influences, such as increased conversion of androstenedione to estrogen, or secretion of bio-active hormones including leptin and adiponectin from adipose tissue may play additional roles. The regional distribution of fat may also alter bone mass independently of obesity, contributing to a low-grade inflammation state that promotes bone resorption through increasing production of pro-inflammatory cytokines expression.

Childhood obesity is a global epidemic. In Canada alone, the prevalence of youth that are overweight or obese increased from 3% in 1978 to 31% in 2013 [29]. About 80% of adolescents with obesity grow up to be adults with overweight condition or obesity [30]. If current trend continues, up to 70% adults will be overweight or obese by 2040 [31]. With the rapid rise in childhood obesity and concerns over associated musculoskeletal pain or disorders, the obesity cycle may be perpetuated by reduced motivation to participate in physical activity in youths who are overweight, thereby partially contributing to their lower activity and higher sedentary behavior. It is therefore important to understand the effects of excess adiposity on bone health outcomes, as well as factors contributing to bone mass deficits and increased fracture risks in children with obesity to prevent progression of the cycle (**Figure 1.1**).

The role of specific nutrients, foods or food groups in the nutritional etiology of childhood overweight and obesity remains controversial [32]. While certain dietary components (e.g. excess caloric intake, fried foods and sugar-sweetened beverages) have been associated with increased adiposity in children [33, 34]; others have reported no clear associations between dietary patterns and measures of overweight and obesity in youths [35, 36]. The influence of diet on adiposity and bone mass accrual in children has also only been scarcely investigated. Only

one study has reported high intakes of dark green and deep yellow vegetables to be related to low fat mass and high bone mass in children (n=325, 3.8 – 7.8 y) [34]. Likewise, the importance of high fat intake in childhood obesity has been challenged [37, 38]. There is a lack of evidence for increased energy intake as fat, despite the striking increase in the prevalence of overweight and obesity among youths over the last decades [39]. Long-term follow-up study of children (8-10 y) of healthy weight counseled to lower total fat intake to <30% kcal, reported little or no differences in BMI at 12 mo (-0.30 kg/$m^2$, CI: -0.75 to 0.15, n=620) or at 5 years (0.0 kg/$m^2$, 95% CI: -0.63 to 0.63, n=541) when compared to usual fat intake (31.4% kcal) [40]. This brings into question the importance of the quality in the fatty acid composition of ingested fats.

Long chain polyunsaturated fatty acids (LCPUFA) have received substantial attention not only due to a central role in brain and visual development early in life, but also the association with chronic disease risks throughout life. The potential of LCPUFA in increasing bone mass and protecting against bone loss with aging are more recently explored. Most [41-46] observational studies suggest that diets high in fish and seafood are associated with benefits to bone health in postmenopausal women. Recent studies have also indicated that various types of LCPUFA intake, such as α-linolenic acid (ALA, C18:3 n-3) [44, 47] and arachidonic acid (AA, C20:4 n-6) [48], were associated with reduced hip fracture rates. Accordingly, low LCPUFA intake, particularly that of linoleic acid was considered as a risk factor of hip fractures in women [45]. Recent systematic meta-analyses examining the relationship between dietary consumption of fish (or their derivatives) reported that 11% of subjects (n=432,924) showed an increase in BMD and decreased risk of fractures. The negative effects on bone linked to fish consumption were almost null [49]. However, studies included in the analyses have been largely limited to those in adults (84%). Human trials investigating the roles of LCPUFA in periods of bone

acquisition remain scarce.

Despite the reported benefits of high LCPUFA intake, data collected from the U.S. National Health and Nutrition Examination Survey (NHANES) 2003-2008 showed more than 90% individuals (n=24,621) consumed less than 0.5 g/d of omega-3 (n-3) LCPUFA from food sources. Median daily intake of n-3 LCPUFA in children aged 1-6 y (n=3354) and 7-12 y (n=2846) was 0.06 and 0.09 g/d, respectively [50]. Among that, fish contributed to only 1% of dietary sources of n-3 LCPUFA [50]. To date, no published data exist on dietary LCPUFA intake specifically in children with obesity. Although children that are overweight or obese are more likely to consume more calories than age-matched peers and consequently more eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), it is unlikely for fish or shellfish intake to be to major contributor to the difference in energy intake. It is therefore reasonable to expect LCPUFA intakes to be similar in children with obesity, if not lower due to poorer diet quality.

LCPUFA and their derivatives play critical roles in modulating obesity-related metabolic consequences including insulin resistance and inflammation [51]. Evidence from animal and human studies supports the premise that increasing n-3 LCPUFA status decreases adipocyte differentiation [52] and benefits body composition [53]. Possible mechanisms underlying its effects include regulating mesenchymal stem cell lineage commitment, adipokine production, and alleviating inflammatory responses in obesity [54]. It is known that cross talk exists between adipocytes and osteoblasts in the bone milieu [55]. It can then be hypothesized that the amelioration of obesity will benefit bone metabolism, yet to what extent does LCPUFA participate in such relationship remains to be characterized.

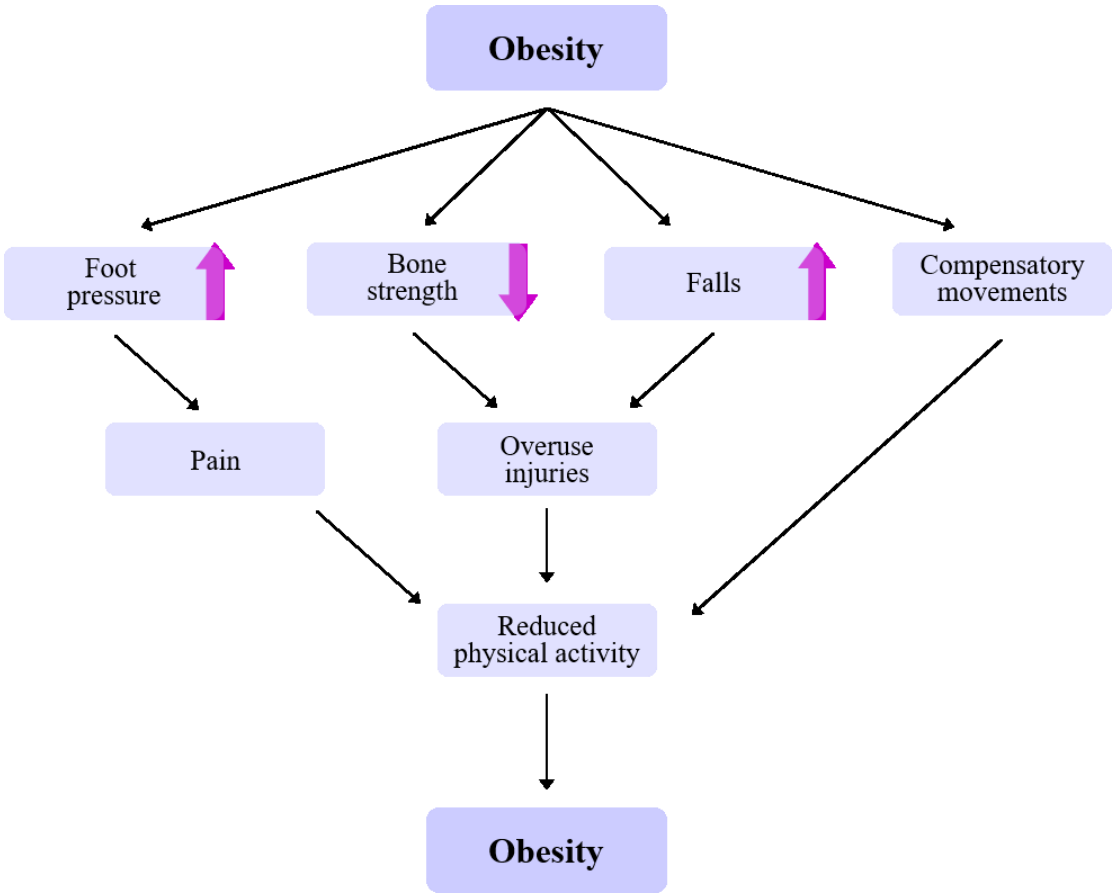
Changes in LCPUFA profile are also influenced by the metabolic alterations due to obesity.

Such relationship has been scantily investigated in younger ages and results are nonetheless controversial [56-58]. Youths that are overweight or obese exhibited lower n-3, but greater omega-6 (n-6) LCPUFA proportions in plasma [59, 60]. Alternatively, an increase in plasma levels of n-3 and n-6 LCPUFA, AA and DHA was reported in children with obesity following a 1-y nutritional-behavior intervention, along with 12% decrease in BMI Z-score [61]. The differences observed in LCPUFA profile may be contributed by alterations in desaturase activities. Since fatty acids from the n-3 and n-6 series are not equivalent in promoting adipogenesis and adipose tissue development [62], varying the proportions of these essential fatty acids would have important implications in changes in adiposity during growth.

Altogether, LCPUFA demonstrate great potential in modulating both adiposity and skeletal metabolism and may represent a useful non-pharmacological mean of ameliorating the bone mass deficits in obesity. Changes in the balance of essential LCPUFA could alter early stages of adipocyte development, not only during fetal life and infancy which show the highest adaptability and vulnerability to external factors, but also afterwards. Deviation from normal bone mass trajectory in relation to excess adiposity may be confined to specific periods during growth and development. In a study of healthy adults (n=509) between 20 and 97 y, the inverse relationships between adiposity and vBMD was negated only in men >50 y after adjustments for age, but not in younger men (20-50 y) or pre-menopausal women. This suggests that limiting fat mass accumulation may be more important in early life to allow peak bone mass accrual [63]. Hence, timely interventions are necessary for minimizing the negative impacts of adiposity on bone mass. The optimization of bone mass and body composition during growth can then reduce comorbidities and/or burdens as a result of suboptimal bone-muscle-fat development later in life (i.e. osteosarcopenia). The overarching goal of this thesis research is to determine the potential of

using LCPUFA to promote maximum accrual in bone mass and inhibit adiposity accumulation during growth. To date, limited evidence exists for the influence of LCPUFA on peak bone mass attainment in children with obesity. Hence, this project aims to fill this knowledge gap by enhancing the understanding of the physiological importance and mechanisms by which LCPUFA affects bone in children with obesity and a rodent model of diet-induced obesity during growth.

**Figure 1.1** The vicious cycle of childhood obesity, musculoskeletal discomfort and reduced physical activity. Adapted from [64]



## 1.2 Thesis rationale

The beneficial role of LCPUFA on obesity-related metabolic consequences is recognized. Many epidemiological and animal studies have also identified LCPUFA to be positively associated with bone mass and/or protective against bone loss with aging. Despite so, limited literature exists for the interaction among obesity, LCPUFA and bone metabolism, especially during periods of growth. Given those with the highest peak bone mass are best protected against age-related bone loss, this work aims to enhance understanding of the physiological importance and mechanisms by which LCPUFA affects bone in children with obesity and in pre-clinical studies using a rodent model of diet-induced obesity (DIO).

### Study objectives and hypotheses

The global objective is to advance understanding of the role of LCPUFA *in vivo* on bone mass and architecture in children with obesity, and to further characterize the important mediators in this relationship using an appropriate animal model.

*Aim 1: Dietary intakes (Manuscript 1)*

To describe the diet of children with obesity and to determine if dietary intakes relate to body composition outcomes.

*Hypothesis:* children with obesity will have suboptimal LCPUFA intake, and that omega-3 LCPUFA status measured in RBC membranes inversely relate to adiposity, due in part to lower abundance in the diet.

*Aim 2: Bone mass (Manuscript 2)*

To examine the relationships between LCPUFA status, bone mass and geometry assessed using dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) in children with overweight condition or obesity.

*Hypothesis:* based on previous studies that reported on altered fatty acid metabolism in individuals with obesity, the higher omega-6 LCPUFA status in children with overweight condition or obesity will negatively relate to bone health outcomes, particularly at sites where fractures are common.

*Aim 3: Microarchitecture and mechanisms (Manuscript 3)*

To determine the effects of a moderately high-fat diet, with or without addition of arachidonic acid on bone mass and LCPUFA status during growth in a growing rat model of diet-induced obesity.

*Hypothesis*: the adverse effects of DIO on bone mass during growth are exacerbated by addition of arachidonic acid, mediated in part through promoting adipogenesis and increasing omega-6 LCPUFA in circulation and tissue.

# CHAPTER 2

## Literature Review

There has been a significant rise in the worldwide prevalence of childhood obesity. The concomitant rise in the incidence of childhood forearm fractures [65] has led to a focus on the relationship between childhood obesity, bone mass and fracture incidence. Importantly, bone mass acquired during childhood, as well as lifestyle habits such as dietary intake, physical activity patterns, tracks into adulthood and may ultimately determine future osteoporotic risk. It has been proposed that the greater risk of fracture in children in obesity is a result of a mismatch between greater impact force generated during a fall, and suboptimal skeletal response to excess adiposity [23]. The introduction of imaging modalities that generate information about bone microarchitecture, soft tissue composition and indices of bone strength has pointed towards a differential effect of excess fat mass and/or low lean mass on various skeletal sites, as well as detailed alterations in the trabecular and cortical compartments observed in children with obesity.

## 2.1 Influence of obesity on bone health outcomes

### 2.1.1 Epidemiological and clinical studies linking obesity to bone mass

#### *2.1.1.1 Human studies*

The effects of obesity alleged to benefit bone, to date, are based mainly on the positive relationship between body weight, or mechanical loading conferred by body weight and bone mineral density (BMD) found in humans and animals. Bone is a mechanosensitive tissue that continually adapts its mass, size and architecture in response to changes in mechanical stimuli from muscle forces, as well as gravitational forces associated with body weight [66]. Growing bones are more responsive to mechanical loading than mature bones [67]. Specifically, dynamic loads induced by muscle contraction exert a greater anabolic effect on bone than do static loads induced by excess adipose tissue [68]. Therefore, the impact of obesity on bone metabolism may be more pronounced in childhood and adolescence than adulthood.

A considerable body of bone densitometry data in adults demonstrated protective effects of body weight on BMD [69-71]; at least to a certain degree. Both body weight and body mass index (BMI) explained up to 19.8% of total variance in areal BMD (aBMD) for weight-bearing sites (proximal femur and spine) in elderly men and women (n=1132, mean age 76 y) from the Framingham osteoporosis cohort [72]. In addition, postmenopausal women (n=417, 45-59 y) in the lowest tertiles of % body fat and BMI had 12% lower hip and spine aBMD and more than 2-fold greater bone loss after 2 y compared with those in the highest tertile [73]. In a meta-analysis of the relationship between BMI and fracture risk, high BMI was in general related to reduced fracture risk in men and women (n=59,644, mean age 63.2 y); yet the gradient of risk per unit BMI increase was lower beyond a BMI of 30 $kg/m^2$, suggesting that the protective effect might be reduced at higher BMI [74].

In contrast to adults, the effects of obesity on bone mass accrual during growth are mixed. Obesity (BMI $\geq 95^{th}$ percentile) was positively associated with greater vertebral aBMD for height, bone mineral content (BMC) for bone area, as well as bone mineral apparent density ($r^2$ = 0.78 – 0.96) in children aged 4-20 y (n=103, mean age 9.8 ± 2.9 y) [75]. In the same study, children with obesity also had 1.2-1.7 times greater whole body bone area and BMC for-age and for-height compared to healthy weight counterparts after correction for sexual maturation and lean mass. A strong positive relationship was reported between whole body fat mass and BMC ($r^2$ = 0.44-0.82) and area ($r^2$= 0.46-0.87) after adjustment for height and/or lean mass in children (n=3082, 9.9 y) that are overweight and obese. The adjustment for height had a minimal effect on the relationship, suggesting that fat mass acts to increase bone size by stimulating appositional rather than longitudinal bone growth, presumably by increasing the rate of periosteal apposition [76]. The duration of obesity also contributes to the influence on BMD. Children (n=59, 11.8 ±

2.7 y) with a long (> 7 years) duration of obesity had approximately 15% and 35% greater lumbar vertebrae (LV) 2-4 aBMD than those with an obesity duration of 4-7 y and those with a recent onset (<4 y), respectively [77].

Contrarily, children (n=336, 3-19 y) who are overweight and obese (≥ BMI 85$^{th}$ percentile) had lower total BMC and bone area (2.5-10.1%) for their body weight, despite their relatively higher BMC and bone area (5-30%) for chronological age [78]. Children classified as obese (BMI>99.6$^{th}$ percentile, 12.1 ± 2.9 y, n=52) who suffered prior fracture were also shown to have 0.8-3 SD reduction in whole-body LV 2-4 and aBMD of the distal radius [79]. In female adolescents of healthy weight (n=60, 10-19 y), higher % body fat was negatively associated with BMC despite wider bones [80]. Hence, the increase in growth velocity in children with obesity may have occurred at the expense of optimal mineral accrual, resulting in bigger bones of lower BMC, contributing to the reduced bone strength and/or higher fracture risks.

The conflicting relationships between adiposity and BMD reported above could in part be related to limitations of dual-energy X-ray absorptiometry (DXA) assessments. The validity of bone measuresments by DXA in individuals with obesity has been questioned due to the artifact with larger tissue depth interfering with the X-ray attenuation and over-estimation of aBMD. Magnification errors associated with fan-beam DXA also underestimates BMC, as the bone is elevated at a greater distance off the scanning table, as occurs with obesity [81]. Three-dimensional quantitative computed tomography (CT) techniques, on the other hand, allow for assessment of volumetric BMD (vBMD) and bone geometry, along with delineation of the trabecular and cortical bone compartments at appendicular sites. CT techniques therefore provide a better estimate of bone strength as small changes in bone size or shape can lead to significant changes in bone strength, independent of bone mass [82].

A cross-sectional study of pre-school children (n=329, 3-5 y) reported negative relationships between % body fat and periosteal and endosteal circumference at the 20% distal tibia [83]. Similarly, % body fat inversely related (r=-0.19 to -0.20) to bone size, BMC and the strength-strain index at the distal radius (20% site) in late adolescent females (n=115, 18.2 ± 0.4 y). Such associations became stronger (r=-0.24 to -0.28) after correction for muscle cortical surface area (CSA) and bone length [84]. Despite the greater cortical CSA in the distal radius of children with overweight condition (n=93, 7-10 y), they had a smaller increase in bone strength for a given estimated load (forearm length x body weight) [85]. Additionally, longitudinal follow-up for 16 mo children who are overweight (n=143, 9-11y) showed that increases in bone size and estimated strength at the tibial midshaft were related to increase in muscle mass only and not fat mass. This mismatch in bone size and strength during development poses greater challenge to bone stability in the event of a fall and can be expected to be more apparent in children who are overweight compared to their normal-weight peers. Although children with overweight condition appear to be at an advantage in terms of absolute bone strength, bone strength is adapted to the greater muscle area instead of excess body fat [86]. Obesity due to excess fat mass alone then is not predictive of greater bone acquisition in children, unless it is coupled with physical activity that favors lean mass accrual and confers dynamic loads to growing bone.

#### *2.1.1.2 Animal studies*

Studying the effect of obesity on bone health in humans is complicated by the multifaceted factors contributing to excess adiposity; involving not only excessive fat intake, but possibly disproportionate consumption of other nutrients (e.g. protein and minerals) known to influence bone, as well as various lifestyle factors. The relationship between obesity and bone in humans

have therefore relied heavily on statistical correlation or modeling rather than controlled trials. Employment of animal models of high-fat diet (HFD) induced obesity therefore allows for singling out the effect of varying fat intake with other nutrients remaining the same when a diet is formulated on an energy basis.

The higher body weight induced by high-fat feeding of rodents was found to be detrimental to bone mineral accrual. Weaning mice (3 wk) fed a westernized diet of high sucrose, animal protein, and saturated fatty acids (C16:0 and C18:0) showed a 20% reduction in whole-body, femoral and vertebral aBMD at 30 wk of age [87]. Consistently, lower femoral BMC and aBMD (43% and 15% respectively) were also observed in mice fed an atherogenic high-fat (15.8% w/w fat and 1.25% cholesterol) diet for 4 mo [88]. From these studies, it appears that diet-induced obesity (DIO) has a generalized or systemic effect on BMC and aBMD, i.e. not site specific. However, the assessment of aBMD was based on DXA and thus bone geometry was not examined.

Trabecular bone is a metabolically active bone compartment that has a turnover rate that is 5-10 times higher than cortical bone [89]. Any changes in bone turnover related to obesity are therefore expected to first be observed in the trabecular-rich metaphyseal regions. Corn oil-fed female mice (12 mo) for 6 mo had significantly lower metaphyseal aBMD at the distal femur as well as proximal tibia [90]. Using high-resolution micro-computed tomography (μCT), growing 6-wk-old mice fed a HFD (45% kcal fat, from lard) for 14 wk had 20% lower trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), connectivity density (Conn.D), along with 15% greater trabecular separation (Tb.Sp) both at the distal femur and proximal tibia [91, 92], all of which suggested decreased bone formation and/or increased resorption with obesity.

Assessment of micro-architectural properties of cortical bone showed reductions in tibia

mid-diaphyseal cortical porosity in rats (7 mo) fed high fat (44% kcal) /high sucrose diet for up to 6 mo [93]. Contrarily, no effect on cortical porosity at the tibia mid-diaphysis or metatarsus was reported in 8 wk-old female rats fed HFD (39.5% kcal) for 10 wk [94]. Cortical geometry measurements (including total area, bone area, medullary area, bone perimeter, and cortical thickness) at the femur or tibia mid-diaphysis were also not affected in mice fed a HFD compared to controls [91, 92].

Bone strength is a function of both geometry and composition, thus changes in microarchitectural or geometrical properties do not necessarily translate into alterations in bone strength. Male rats (90 d) fed a high-energy diet composed of 'junk food' items including marshmallows, potato chips, chocolate chip cookies, popcorn and 32% sucrose for 1 mo shown significantly higher femoral maximum load, maximum deflection and failure energy; this is not surprising given stronger bones are often required to support the greater body weight (19.1%) as a result of DIO [95]. Female rats (8 wk) fed HFD (39.5% fat) for 10 wk had significantly lower tensile stress and elastic modulus at the tibia and metatarsus after correction for bone size and geometry, signifying the deterioration in material properties of cortical bone [94]. Likewise, bone calcium content was reduced with increased dietary fat content (up to 9% w/w), and cannot be restored by increasing dietary calcium in growing broiler chickens (day-old to 3 wk of age) [96]. Hence, the lower trabecular BMC could in part have contributed to the reductions in compressive loads, failure energy, as well as stress and strain of trabecular-rich regions including femoral condyles, tibial plateau and LV 6 of young rats (from 4 wk to 24 mo) and adult roosters (from 40 wk to 60 wk) fed a HFD [97, 98].

### 2.1.2 Potential mechanisms

#### *2.1.2.1 Differentiation of mesenchymal stem cells into adipocytes or osteoblasts*

Bone remodeling by resorption and formation add and reshape trabeculae and the cortex to strengthen them. The rate of bone turnover is therefore reflected in the degree of mineralization, bone collagen fiber orientation, trabecular preferential orientation (architectural anisotropy) and trabecular connectivity [99].

Together, the bone-forming osteoblasts, bone-resorbing osteoclasts, and osteocytes form the basic bone remodeling unit, and are found close to the stromal elements of the marrow. Pluripotent mesenchymal stem cells (MSC) within the bone marrow have equal ability to differentiate into osteoblasts, adipocytes and chondrocytes, among other cell types, depending on the cell-derived transcription factors present within the bone milieu (**Figure 2.1**).

Given the plasticity of MSC in becoming either adipocytes or osteoblasts, there may be decreased osteoblastogenesis with increased adipogenesis in obesity. However, increased mechanical loading with excess body weight could stimulate bone formation by decreasing apoptosis and promoting differentiation of osteoblasts and osteocytes [100]. Additionally, osteoblastogenesis is favored through the down-regulation of peroxisome proliferator-activated receptor-γ (PPARγ) [101]; or by stimulating a durable β-catenin signal that commits MSC to the osteoblast lineage [102].

Consistently, human studies have reported decreased levels of circulating bone formation markers in those with obesity compared to leaner populations. For example, serum osteocalcin concentrations were 30% lower in postmenopausal women (n=258, 55-76 y) with obesity (BMI $\geq$ 30 kg/m$^2$) than those who are of normal-weight [103]. Similarly, negative relationships between BMI and osteocalcin (r=-0.17), and procollagen type I C-peptide (r=-0.30) was

observed in healthy postmenopausal women (n=130, 46-85 y) [104]. Only one study to date has measured bone turnover markers in children with obesity (BMI Z-score 3.3 ± 0.6, 5-16 y, n=52), where serum osteoprotegerin (OPG) levels were 10% lower in children classified as obese, in addition to an inverse relationship with total body and truncal fat mass. The bone resorption marker carboxy-terminal telopeptide of type I collagen (CTx), was also approximately 30% higher in those with greater BMI [105].

Cultured bone marrow stromal/osteoblastic cells isolated from long bones of DIO (45% kcal) C57BL/6 mice showed 50-75% greater alkaline phosphatase (ALP)-positive colony forming units and calcium modules, indicative of increased bone formation presumably due to greater mechanical loading. ALP and receptor activator of nuclear factor-κB ligand (RANKL) mRNA levels were increased by 41% and 20% respectively, but not that for osteocalcin, OPG and macrophage colony stimulating-factor (M-CSF) levels [91]. Together with higher serum tartrate-resistant acid phosphatase (TRAP) and multinucleated TRAP-positive osteoclasts in bone marrow in mice fed HFD, these results suggest that osteoclast activity and bone resorption induced with high-fat feeding may blunt any positive effects of increased weight loading on bone [92]. Alternatively, marrow stromal cells from C57BL/6 mice fed a high-fat, atherogenic diet (15.8% w/w fat) for 4 mo failed to undergo osteoblastic differentiation *in vitro* [106] and led to inhibition of osteocalcin expression [88]. Feeding of a 10% w/w corn oil diet in 12 mo female C57BL/6J mice for 6 mo also significantly increased osteoclast number, superoxide production in osteoclasts, as well as expression of *Pparγ* and *ctsk,* a cytokine protease instrumental in bone matrix degradation necessary for bone resorption. This suggested high fat feeding favored lineage selection of hematopoietic cells towards osteoclast, as well as adipocyte differentiation from MSCs [90, 107]; fat infiltration of marrow space might also have replaced functional

hematopoietic and/or osteogenic cells in the cavity [108].

#### *2.1.2.2 Low-grade inflammation and production of pro-inflammatory cytokines*

Obesity is considered a state of low-grade chronic inflammation manifested with abnormal cytokine production, increased acute-phase reactants, and activation of inflammatory signaling pathways. Excess adipose tissue in obesity is infiltrated with an increased number of macrophages, which act as a major source of pro-inflammatory cytokines. Individuals with obesity have been reported to show higher circulating amounts of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and C-reactive protein [109-113].

These pro-inflammatory cytokines have been shown to mediate the process of osteoclast differentiation and bone resorption through the regulation of RANKL/RANK/OPG pathway [114, 115] (**Figure 2.2**). The expected bone loss associated with loss of active sex steroids in ovariectomized mice was prevented with the abolition of IL-1 and TNF signaling pathways [116] [117, 118]. IL-$6^{-/-}$ mice were protected against joint inflammation and bone loss, suggesting that IL-6 is essential for bone formation only in conditions of high bone turnover, possibly via enhancing osteoblast proliferation and differentiation as shown in murine embryonic fibroblasts [119].

Few studies have investigated the direct relationship between obesity-induced inflammation and its effect on bone metabolism. Adult female mice (12 mo) fed 10% corn oil diet for 6 mo had increased visceral adiposity as well as bone marrow adipogenesis [90]. LPS-stimulated IL-6 and TNF-α secretion by splenocytes and bone marrow cells was elevated [90], accompanied by 0.2-0.3 fold greater IL-6, TNF-α and IL-1β mRNA levels at the femur [107]. Lower concentrations of the anti-inflammatory cytokine IL-10 [120-122] were secreted by bone marrow-derived macrophages upon RANKL stimulation in young mice (9 wk) fed HFD (45%

energy) for 13 wk. Exogenous IL-10 administration inhibited osteoclast formation to a lesser extent in obese relative to lean mice, suggesting that obesity led to reduced IL-10 inhibiting recruitment of NF-κB onto the promoter of IL-10, and subsequently enhanced osteoclastogenesis [123].

### 2.1.3 Adipokines

Adipose tissue is known to be an active endocrine organ, secreting hormones including leptin and adiponectin that are shown increasingly to have both a direct and centrally mediated influence on the bone-fat relationship.

#### *2.1.3.1 Leptin*

Leptin is a pleiotropic polypeptide hormone produced primarily by adipocytes [124-126]. Circulating leptin enters the central nervous system (CNS) in proportion to its plasma concentration, and serves to communicate the state of body energy repletion. Through the hypothalamic peptidergic network [127-129], leptin suppresses food intake and enhances energy expenditure [130, 131]. Consequently, mouse models of leptin deficiency or modeling (*ob/ob* and *db/db* mice) demonstrates increased food intake in combination with reduced energy expenditure and a phenotype indicative of the neuroendocrine starvation response (including hypothyroidism, decreased growth, infertility, and decreased immune function) in spite of obesity [132-134].

Circulating concentrations of leptin in humans are in the range of 0.1-5 nM (1.6 – 80 ng/ml) [135] and is strongly correlated with body fat mass in both humans [136] and animals [92, 137]. Children with obesity (n=18, 8.9 ± 1.8 y) have significantly higher serum leptin concentrations (28.6 ± 17.4 vs 6.8 ± 7.1 ng/ml) than their healthy-weight age-matched counterparts [138]. Circulating leptin levels have shown a negative relationship with markers of

bone resorption (urinary CTx and N-telopeptide of type I collagen) even after adjustment for fat mass [139, 140]. Cross-sectional analyses in both healthy [141-143] and adults classified as obese [144] have suggested an overall adverse effect of leptin on BMD, after adjustment for age, body weight and fat mass. Leptin levels were also significantly lower in postmenopausal women with vertebral fractures than those without fractures [145]. However, the independent relationships between circulating leptin and BMD evaluated through cross-sectional studies are confounded by the strong association between leptin and fat mass, given the almost exclusive production of leptin by adipocytes [124].

Leptin-deficient rodents or models with impaired signaling (*ob/ob* or *db/db* mice, *fa/fa* rats) have reduced femur length, cortical thickness, Tb.N, along with lower rates of osteoblast differentiation and greater osteoclast number [146-148]. This resulted in substantial decreases in biomechanical properties in appendicular bone of adolescent mice [149], suggesting the positive effect of mechanical loading conferred by excess body weight on bone could not overcome the detrimental effects of leptin deficiency. Accordingly, leptin administration (i.p.) in *ob/ob* mice (4 wk) increased femur length, BMC and BMD despite a 40% decrease in food intake and 14% body weight loss [150]. This was mediated through promoting bone marrow adipocyte apoptosis, and increasing osteoblast function evident by greater bone forming surfaces *ob/ob* mice [151].

To the author's knowledge, the adverse effects of leptin deficiency on bone have scarcely been reported at the lumbar vertebrae. No differences in LV 4 BMC, BMD nor maximum compressive load was found between adolescent *ob/ob* mice (10 wk) and their wild-type (WT) counterparts [149]. Adult *ob/ob* mice (6-10 mo) had significantly longer vertebrae and higher trabecular BV/TV than WT mice [146, 152], in spite of the increase in osteoclast numbers and resorption parameters [152]. This was explained in part by the increase in osteoblast bone

formation rate in *ob/ob* mice [152]. In addition, the potential positive effects of leptin signaling on bone mass in long bones in *ob/ob* mice may be masked by the reduced muscle mass [146].

With regard to longitudinal growth, *ob/ob* mice (6 mo) have been observed to have shorter femora yet longer vertebral length, and this led to the speculation that the increase in vertebral body length is secondary to abnormal mechanical loading, since *ob/ob* mice begin to lose muscle mass at 3 to 4 wk of age [146, 153]. The utility of the *ob/ob* mouse as a model of normal childhood obesity is therefore questionable. Treatment of 25-day-old male ICR mice with leptin injections (i.p.) increased tibial length in spite of lower body weight, suggesting the effect of leptin on bone growth is independent of food intake [154].

The potential role of leptin in endochondral ossification has also been investigated. Administration of exogenous leptin (43 ng/day) increased overall size and thickness of the tibial epiphyseal growth plate by 11% and 4.2% respectively in young mice (37-40 d) over 4 wk [155], through stimulating growth plate chondrocytes differentiation without affecting the overall organization of the growth plate [154]. Histological analyses of the femoral growth plate of *ob/ob* mice (4 wk) revealed failure of proliferative and hypertrophic chondrocytes to form the normal columnar, and organized structure [153]. Administration of exogenous leptin at normal physiological concentrations (1-10 ng/ml) restored all abnormal phenotypes, which is indicative of the requirement of leptin in modulating events associated with terminal differentiation of chondrocytes [153].

*Direct effects of leptin on bone cells*

Bone marrow stromal cells and other cells of the osteoblastic lineage [155-157] are targets for leptin as they express both long and short forms of the leptin receptor [158]. Leptin is therefore believed to have the ability to modulate the reciprocal differentiation of stromal cells

between osteoblastic and adipocytic cells.

Leptin treatment of human marrow stromal cells (hMS2-12) resulted in up to a 2.5 fold increase in osteoblastic differentiation and mineralization of the extracellular matrix, along with a 0.2-0.6 fold reduction in leptin expression, which is indicative of inhibited adipogenesis [157]. Similarly, increased osteoblast activity evident by higher number of mineralized modules after leptin treatment has been observed in rat fetal osteoblasts [155] as well as in long-term human osteoblast cultures [156].

Incubation of human peripheral blood mononuclear cells (PBMCs) and murine spleen cells with leptin in the presence of M-CSF and soluble RANKL significantly inhibited osteoclast generation [159]. Furthermore, leptin caused a 3-fold reduction in RANKL mRNA levels but increased OPG mRNA and protein contents in PBMC and stromal cells [159, 160]. The effect of leptin on osteoclastogenesis via alterations of the RANKL/RANK/OPG system could be exerted through a dose-dependent effect. RANKL mRNA levels were initially increased when MC3T3-E1 osteoblasts were exposed to 12 ng/mL leptin, yet higher concentrations of leptin (24 and 200 ng/ml, ten-fold greater than those optimal for OPG production) were associated with inhibition of RANKL production by osteoblasts [161]. Subsequently, leptin administration prevented the initial increase in bone resorption (through increase OPG mRNA expression) and decrease in bone formation rate against disuse-induced bone loss in female rats [162]. This supported the hypothesis that leptin promotes positive OPG/RANKL balance and therefore may directly modulate bone remodeling.

*Leptin controls bone mass through a central hypothalamic relay*

Leptin controls energy homeostasis and appetite via the CNS, it is plausible that the same hypothalamic relay is used for its action on the skeleton. Intracerebroventricular (ICV) infusion

of leptin in *ob/ob* mice that lack global leptin secretion hence allows for the determination of the mode of action involves the central or peripheral pathways [163].

Leptin administration (I.C.V.) fully corrected the high bone mass phenotype at the lumbar vertebrae of *ob/ob* female mice (4 mo), and other functions including body mass and food intake, strongly arguing for the effect of leptin is mediated only via the central relay [152]. Consistently, targeted leptin overexpression at the hypothalamus of *ob/ob* mice (8-10 wk) fully restored the deficits in femoral length, total bone volume, and decreased trabecular bone volume at the distal femur and LV 3 to WT levels [164].

The downstream pathway of the antiosteogenic effects of leptin were subsequently shown to be mediated via the ventromedial hypothalamic nuclei of the hypothalamus, independent of the anorexigenic networks (arcuate nuclei) of the CNS. The peripheral mediators also appeared to be neuronal rather than through neuropeptides such as α-melanocyte stimulating hormone that is responsible for the anorexigenic function [165]. Moreover, osteoblasts from *db/db* mice showed no osteoblast defects compared to WT [152]. Transgenic mice with specific leptin overexpression in osteoblasts did not display any skeletal abnormalities [165], supporting that the effect of leptin on bone is exerted predominantly through the central relay, and specifically through the sympathetic arm. Leptin infusion in mice deficient in dopamine β-hydroxylase did not correct the high bone mass phenotype which mimics that of *ob/ob* mice [165]. Likewise, β2-adrenergic receptor-deficient mice showed high bone mass [166, 167], along with reduced bone resorption that cannot be restored to normal values with ICV leptin [168, 169].

The effects of leptin on bone metabolism likely involves multiple pathways that converge to produce the ultimate phenotype. Depending on the bone tissue, administration route, or signaling pathway, leptin may exert dual effects on the remodeling cycle. Observations in

humans showed stimulatory effects of leptin on bone size and longitudinal growth through its angiogenic and chondrogenic activities early in life [170]. Later, it may decrease bone remodeling during periods of accelerated bone loss in the mature skeleton. On the other hand, leptin controls the central sympathetic tone to correct abnormal bone mass in states of leptin deficiency by favoring bone resorption, and possibly increasing bone formation through the neuropeptide Cocaine- and Amephetamine-Regulated Transcript via an, as of yet, uncharacterized pathway [169] (**Figure 2.3**). What remains of question is whether these observations made in *in vitro* and knockout mouse models hold true in humans with obesity, especially in the face of leptin resistance where the elevated circulating leptin led to alterations in the transport of leptin across the blood-brain barrier. This is supported by a recent study in children with obesity (BMI Z-score 3.3 ± 0.6), where serum leptin levels (148 ng/mL) and free leptin index (10.6) that is beyond normal physiological range was associated with decreased OPG, resulting in up-regulation of osteoclastogenesis and failure to maximize bone mineral acquisition in children [105].

#### *2.1.3.2 Adiponectin*

Adiponectin is a protein hormone produced almost exclusively by adipocytes. In contrast to leptin, its circulating levels are reduced in obesity [171, 172]. An inverse relationship between plasma adiponectin and increases in adiposity (r=-0.37) and BMI (r=-0.44) over 5 y were reported in Pima Indian children (5-10 y) [173]. Consistently, plasma adiponectin decreased by 8.1% and 11.2% in boys and girls respectively for every unit increase in BMI Z-score in French Canadian youths (n=1632, 9-16 y) [174]. Children with obesity also had about 10% lower free adiponectin after correction for bone age and sex compared to their age-matched counterparts [105].

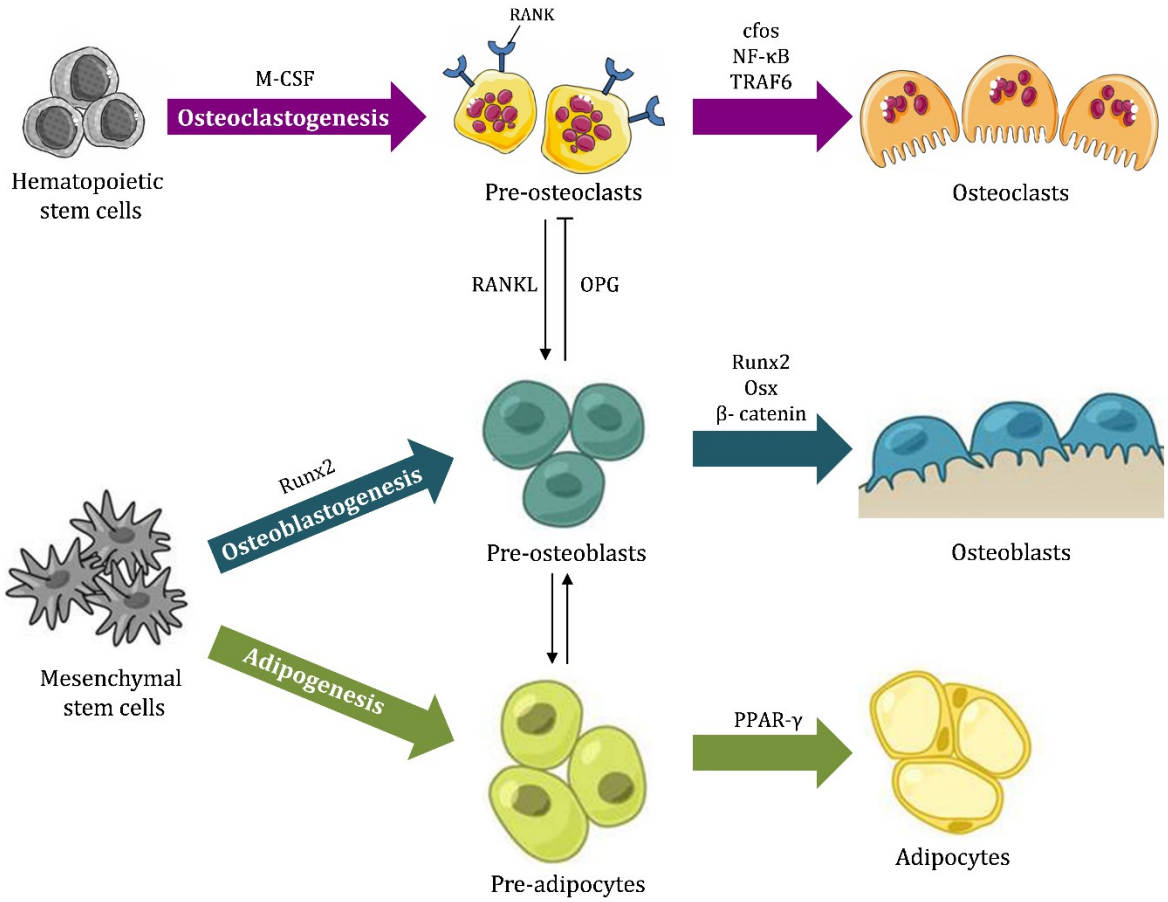
Adiponectin receptors are expressed on osteoblasts [175] and on RANKL-induced multinucleated osteoclast-like cells [176]. *In vitro,* adiponectin (0.01-1 μg/mL) stimulated the proliferation, differentiation and mineralization of osteoblast-like MC3T3-E1 cells via the AMP-activated protein kinase (AMPK) pathway [177], and is mitogenic to primary rat and human osteoblasts in a dose-dependent manner [178]. Additionally, M-CSF- and RANKL-induced osteoclast differentiation as well as bone resorption activity was reduced by adiponectin [179]. However, adiponectin also promoted RANKL expression while concomitantly reducing OPG expression on osteoblasts through stimulating the p38 MAPK pathway, indirectly resulting in increased osteoclast formation [180].

Observations from clinical and animal trials regarding the effects of adiponectin on bone to date are inconclusive. Reduced adiponectin in children that are obese (n=103, 5-16 y) was associated with higher Wnt-inhibitor Dickkopf-1, where it may favor adipogenesis over osteoblastic differentiation [105]. Contrarily, a positive association ($r^2$ = 0.79) between bone marrow adipose tissue and adiponectin concentrations in early-pubertal girls (n=59, 4-10 y) has been reported [181]. Mouse models of adiponectin deficiency had 15-56% greater trabecular and cortical bone mass at both the axial and appendicular skeletons, that resulted in almost 2-fold greater biomechanical strength [178, 182]. This was attributed to an increase in osteoblast number, bone formation rate and circulating osteocalcin [178, 182].

The mechanisms of adiponectin on bone remains uncertain. It is possible adiponectin can bind certain growth factors [183] that would oppose the direct anabolic effects on bone. Adiponectin may also signal back to osteoblasts in response to increased osteocalcin to slow osteoblast proliferation and favor apoptosis through decreasing Forkhead box O (FOXO)1 activity in a phosphoinositide 3-kinase (PI3K)-dependent pathway, altogether decreasing bone

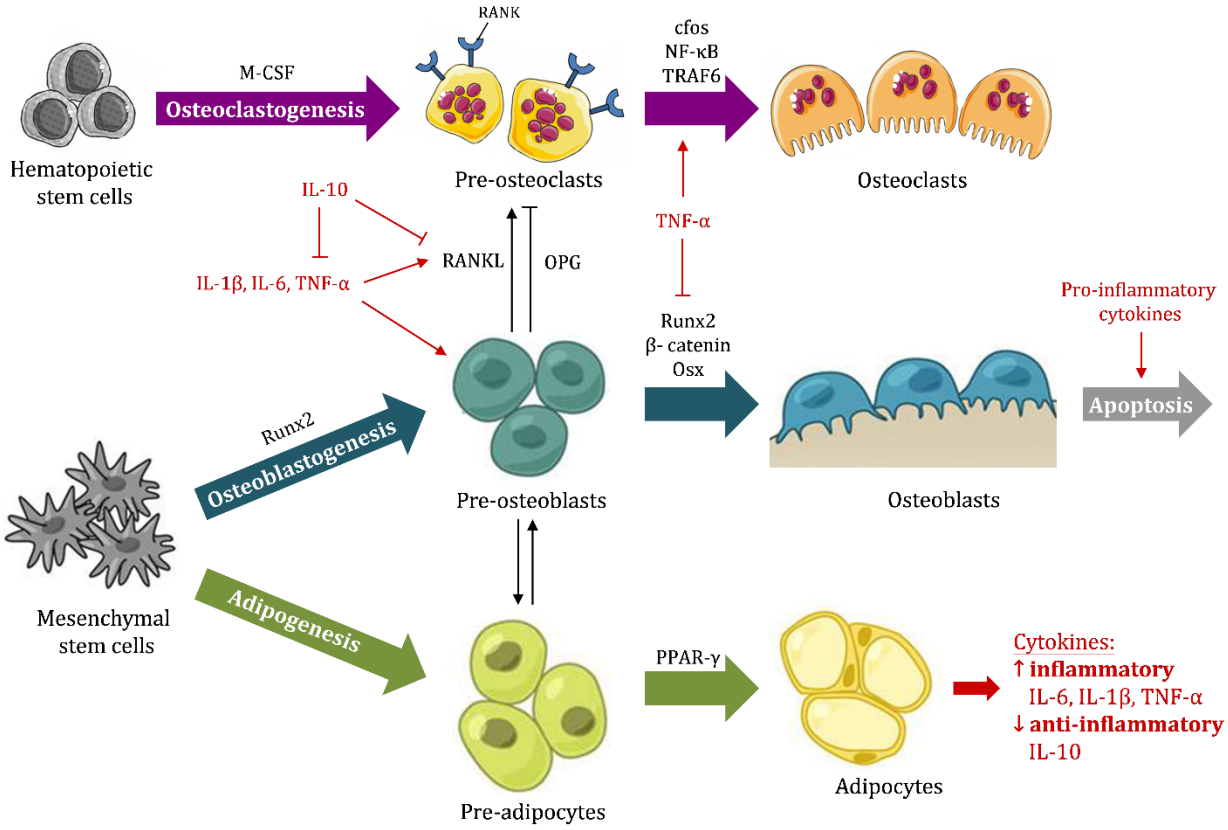
mass and circulating osteocalcin. Overtime, however, these local effects could be masked as adiponectin signals centrally to decrease sympathetic tone, partially opposing the influence of leptin on the sympathetic nervous system, thereby increasing bone mass and decreasing energy expenditure [182].

**Figure 2.1** Factors involved in differentiation of mesenchymal stem cells osteoblastogenesis and adipogenesis



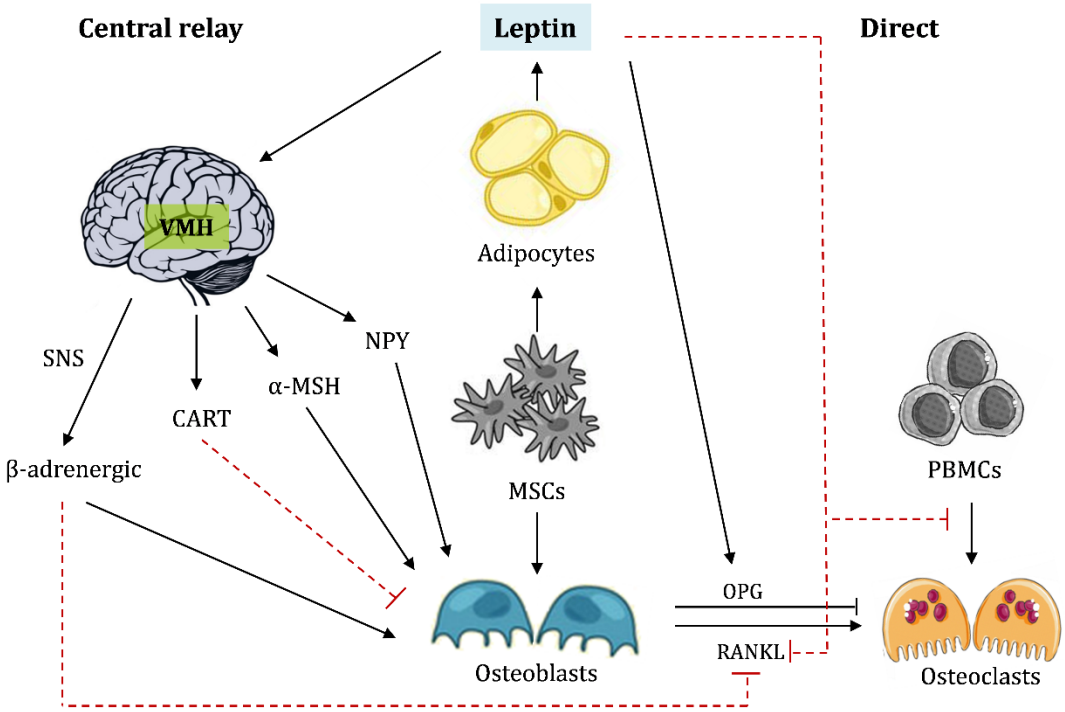

Abbreviations: M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; Osx, osterix; PPAR, peroxisome proliferator activated receptor; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; Runx, runt-related transcription factor; TRAF; tumor necrosis factor receptor associated factor

**Figure 2.2** Production of cytokines associated with obesity-mediated low-grade chronic inflammation affects processes of osteoblastogenesis and osteoclastogenesis




Abbreviations: IL, interleukin; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; Osx, osterix; PPAR, peroxisome proliferator activated receptor; RANK, receptor activator of nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; Runx, runt-related transcription factor; TNF, tumor necrosis factor; TRAF; tumor necrosis factor receptor associated factor

**Figure 2.3** Leptin-dependent regulation of bone mass through its direct action on bone cells or the central relay




Abbreviations: α-MSH, α-melanocyte stimulating hormone; CART, cocaine- and amphetamine-regulated transcript; MSC, mesenchymal stem cell; NPY, neuropeptide Y; OPG, osteoprotegerin; PBMC, peripheral bone mononuclear cells; RANKL, receptor activator of nuclear factor-κB ligand; SNS, sympathetic nervous system; VMH, ventromedial nuclear of the hypothalamus

## 2.2 Integrating relationships between childhood obesity, DHA status, and bone outcomes

Omega-3 LCPUFA have been shown repeatedly in humans [184] and animal models fed high-fat diets to protect against insulin resistance and limit adiposity accumulation [185]. The independent beneficial effects of LCPUFA on bone alone are emerging. Despite so, studies that explored the interaction among these factors are lacking.

### 2.2.1 LCPUFA status in children with obesity

Dietary intakes remains one of the most important determinant of LCPUFA status (refernce), nonetheless the intakes of LCPUFA of children in Canada are variable (**Table 2.1**). Adolescents classified with overweight condition (n=60, 12 y) had 4-9% higher saturated fatty acids (SFA) in both phospholipid (PL) and cholesterol ester (CE) fractions, but 21% lower DHA in PLs compared to their age-matched healthy weight peers [59]. Similarly, serum PL concentrations of n-3 LCPUFA, including DHA were 33% lower in adolescents with obesity (n=10, 10.4-16.4 y, BMI 26.8 – 40.4) than lean controls [186]. Phospholipid and sterol esters AA, dihomo-γ-linolenic acid (DHGLA, C20:3 n-6) and linoleic acid (LA, C18:2 n-6) concentrations were substantially higher in children with obesity (n=22, 13.7 ± 1.4 y, relative body weight 170 ± 24%), despite no differences in levels of LA were observed [60]. In younger children (6-12 y), however, concentrations of ALA, LA, DHA, total n-3 and n-6 LCPUFA in plasma total lipids and triacylglycerols were greater in children in the highest quartile of BMI Z-score, presumably due to the higher dietary intakes of n-3 LCPUFA [187].

Using the substrate:product ratio as a surrogate for fatty acid elongases and desaturases activities, ratios of AA:LA and total n-6: n-3 LCPUFA were increased in children with obesity [60, 186]. This pattern is consistent with the enhanced conversion of LA to other n-6 LCPUFA, as concentrations of eicosadienoic acid (C20:2 n-6), a direct elongation product of LA, did not

differ between the obese and non-obese groups, indicating that desaturase activity, but not chain elongation was enhanced in obesity. Only the γ-linolenic acid (GLA, C18:3 n-6) + DHGLA:LA ratio but not that of AA:DHGLA was elevated in children who are obese, suggesting Δ6 desaturase (D6D) activity is predominantly affected by obesity [60]. Obese Zucker rats had significantly lower hepatic PL AA than lean animals. It was however corrected by GLA feeding which bypasses D6D to form AA, implying an abnormality in D6D activity in obesity [188].

In addition to LCPUFA, proportions of SFA (C16:0, C18:0) and palmitoleic acid (C16:1 n-7) were up to 37% greater in children and adolescents with obesity [59, 186, 187, 189]. Unlike oleic acid, C16:1 n-7 is uncommon in the human diet, and usually reflects endogenous desaturation by the enzyme stearoyl-CoA desaturase (SCD) [190]. SCD activity (C16:1 n-7/C16:0) was elevated (30%) in children with obesity (n=59, 11.8 ± 3.8 y) compared to age-matched healthy controls. Likewise, waist-to-hip ratio and serum leptin concentrations were positive determinants of SCD activity [189]. Obesity-prone C57BL/6J mice fed HFD (40% kcal) for 8 wk showed 2-fold higher liver SCD mRNA levels versus controls [191]. Alternatively, mice with targeted SCD1 disruption had decreased expression of lipogenesis genes such as fatty acid synthase and increased expression of lipid oxidative genes including downstream targets of PPARα [192]. Interestingly, a population-based cross-sectional study of 2021 elderly people reported that plasma total, n-6, and n-3 LCPUFA were inversely associated with estimated SCD indices as well as whole-body fat mass measured by DXA. This association with body fat was markedly attenuated after adjustment for SCD indices, suggesting that high LCPUFA status may decrease SCD activity in humans [193].

### 2.2.2 Potential benefits of DHA supplementation to bone health in obesity

#### *2.2.2.1 Omega-3 PUFA as potential modulators of adiposity*

Interventions with n-3 LCPUFA in children with obesity are scarce and results are nonetheless inconclusive. Children with BMI classified as obese (≥ 95$^{th}$ percentile , n=201, 11.6 ± 0.7 y, BMI) that received 1.8 g n-3 LCPUFA for 12 wk showed almost a two-fold greater reduction in BMI than those treated with metformin (-0.55 vs -0.27 kg/m$^2$ respectively) [194]. Alternatively, supplementation of 900 mg n-3 LCPUFA (360 mg DHA + 540 mg EPA) in children with overweight condition (n=76, 9-18 y) for 1 mo reported mixed results in body weight changes, but there were significant reductions (10-24%) in serum TNF-α and leptin, and an 12% increase in adiponectin concentrations [195]. Both of these studies are promising, but of too short a duration to examine influences on bone mass.

Adults rats of healthy weight fed fish oil for 6 mo had significantly lower total body and intra-abdominal fat mass, than those that received an isocaloric diet containing lard, corn oil or medium-chain triglycerides, despite no differences in body weight or energy expenditure were observed [196]. A cross-over experiment in C57BL/6J mice showed that the hyperleptinemia and excess adiposity induced by SFA-rich HFD (58% kcal) was completely reversed by a switch to an n-3 LCPUFA diet of equal fat content, and significantly more effective than a low-fat diet (10% kcal) [197], implicating an important role of n-3 LCPUFAs in reversing body weight and fat mass gain induced by DIO [198]. Consistently, broiler chickens fed a high n-3 LCPUFA diet for 5 wk had significant reductions in abdominal fat pad masses relative to those in a high SFA diet, which was attributed to the greater portioning of energy into lean versus fat tissue in LCPUFA group [199]. Taken together, these findings provide compelling evidence that n-3 LCPUFA reduce body mass, total body fat, and/or abdominal fat in animal models of DIO,

independent of changes in energy intake and/or expenditure.

#### *2.2.2.2 Effects of DHA on obesity-mediated low-grade chronic inflammation*

The anti-inflammatory properties of n-3 LCPUFA could play a role in restoring normal adipocyte function in obesity. Feeding of *db/db* mice (7 wk) fed a moderately-HFD (30% kcal) rich in n-3 LCPUFA for 6 wk reduced macrophage infiltration induced by HFD, the changes in inflammatory gene expression, and reduced c-Jun N-terminal kinase (JNK) phosphorylation that is critically involved in the deterioration of insulin sensitivity [200]. Similarly, the extent of adipocyte hypertrophy and macrophage infiltration induced by high-fat feeding was almost halved in C57BL/6 mice fed a corn oil-based HFD (35.2% w/w) substituted with 15% n-3 LCPUFA (46% DHA, 14% EPA) [201]. This suggested that n-3 LCPUFA can ameliorate adipocyte stress, and normalize adipocyte metabolism; thereby hindering adipocyte expression of pro-inflammatory cytokines, and preventing macrophage infiltration of adipose tissue [202].

#### *2.2.2.3 Effects of DHA on leptin production*

The effects of DHA on leptin production is scarcely reported and based largely on *in vitro* models. Leptin mRNA levels in epididymal adipose tissue was almost two-fold lower in male rats fed a n-3 LCPUFA-rich diet (6.5% w/w) for 3 wk compared to controls [156]. Treatment of 3T3-L1 adipocytes and human placental throphoblast (BeWo) cells with DHA showed 22% and 49% reduction in leptin mRNA levels respectively. Leptin promoter activity was decreased by 17-18% with DHA incubation in constructs containing the human leptin promoter. While the proximal region of the promoter has the potential to bind sterol-response element binding proteins (SREBP) [203], it has no sequence similarity to any known PPAR response elements (PPRE) [204], indicating the leptin gene is not a direct target of the PPAR [156]. However, the PPARγ agonist BRL 49653 reduced human leptin promoter activity by 35-53% in a dose- and

time-dependent manner in primary adipocytes, but not undifferentiated 3T3-L1 preadipocytes lacking endogenous PPARγ. This suggests that PPARγ acts through an effector on the *ob* promoter [205]. DHA treatment of BeWo cells reduced PPARγ and SREBP-1 mRNA levels by 31% and 27% respectively. The requirement of SREBP-1 in this process was evident when the effect of DHA on leptin expression was negated when cells were starved of cholesterol [156].

Reductions in both leptin and leptin receptor mRNA levels in dorsal adipose tissue, but not plasma leptin concentrations was observed in growing pigs fed an isocaloric diet containing fish oil (5.73% w/w) compared to soybean oil for 21 days [206]. It was thus speculated that changes in leptin output in response to nutritional stimuli might be mediated post-transcriptionally by regulating both leptin secretion and synthesis. About 60% of the variation in circulating leptin could be attributed to variations in leptin secretion rate, BMI, or fat cell volume but not that of leptin mRNA levels in adipose tissue [207]. Likewise, the changes in circulating leptin did not depend on the synthesis of new protein, this suggests that leptin was released from a preformed pool within the cells where post-transcriptional regulation of leptin might occur [208].

#### *2.2.2.4 Effects of DHA on adiponectin production*

The modulating effects of DHA on adiponectin secretion have also been reported. The inclusion of 40% of total fat as n-3 LCPUFA almost completely restored the reduction (35-40%) in serum adiponectin concentrations in *db/db* mice, and could be regarded as a marker of normalized adipocyte function [200]. Likewise, partial replacement (15%) of dietary lipids with EPA/DHA in a composite HFD (35% w/w) upregulated adiponectin gene expression (*Adipoq*) and adiponectin production in epididymal white adipose tissue as well as systemic levels [209].

Studies using 3T3-L1 adipocytes suggested the up-regulation of adiponectin expression by n-3 LCPUFA could in part be mediated via PPARγ. A functional PPRE has been identified in the

human adiponectin promoter. The induction of adiponectin levels by the PPARγ agonist thiazolidiedione was completely admonished by point mutation of the PPRE [210]. Both EPA and DHA increased secreted adiponectin concentration by 44% and 102% respectively, but did not alter cellular adiponectin protein content. Incubation with the PPARγ antagonist bisphenol-A-diglycidyl ether inhibited adiponectin secretion in adipocytes induced by DHA. Furthermore, DHA enhanced both adiponectin and PPARγ mRNA levels, emphasizing that DHA acts primarily via transcriptional control that is mediated by PPARγ [211]. Contrary to observations *in vitro* however, negligible changes in adiponectin mRNA levels were observed in humans treated with pioglitazone despite a 2- to 3-fold increase in plasma adiponectin [212, 213], suggesting regulation occurs through post-transcriptional mechanisms instead. The regulation of adiponectin secretion by DHA varies among experimental models and animal species and remains to be clarified.

### 2.2.3 Influences of obesity and fatty acid status on skeletal outcomes

Only one study to date has reported bone outcomes with DHA supplementation under the constrains of childhood obesity. In adolescent boys with slight overweight conditions (n=78, BMI 23.1-25.2, 13-15 y) randomly assigned to breads enriched with fish oil (1.1g) or control for 16 wk, fish oil intervention increased red blood cell (RBC) EPA and DHA levels, and reduced LA and AA compared to controls. Despite so, examination of changes in bone mass by DXA found no significant differences in any bone outcome, nor was DHA status correlated with whole-body bone outcomes or markers of bone formation. The short duration of this study has likely limited the ability for any skeletal changes to be captured; studies of longer duration and more sensitive measures are required for determining the effects of DHA on bone mass and adiposity [214].

Long-term DHA supplementation (0.13 mg DHA/g diet) in female C57BL/6J mice from 3 mo of age for 14 mo led to 8% and 25% higher body weight and fat mass % compared to controls at the end of study. DHA significantly improved caudal vertebrae trabecular BV/TV, Tb.N and trabecular thickness (Tb.Th) by 5-30% in early adulthood (8 mo), as well as cortical bone volume (10%) of the tibia mid-diaphysis. This paralleled a continuous increase in leptin concentrations that was not accompanied by body fat mass gain observed by 8 mo [215]. Finally, in a rat model of DIO fed via total enteral nutrition varying amounts of fat (HFD, 45% kcal, medium fat 25%, or control 14%) from 27 days of age for 4 wk (i.e. to early sexual maturity to match body weight gain, those fed HFD had 3-fold lower tibia trabecular BMC and 2-fold greater retroperitoneal and gonadal fat accumulation that was independent of body weight. The expression of PPARγ and aP2 (fatty acid binding protein) in bone were significantly up-regulated, whereas Runx2, osteocalcin and β-catenin were down-regulated in the femur of rats fed HFD. Increases in serum non-esterified fatty acids (NEFA), particularly that of 16:0, 18:0, 18:1, LA and AA, possibly from dietary lipid and triglycerides hydrolysis were roughly 5-fold greater in HFD animals. ALP activity and osteoblast differentiation of mesenchymal stromal ST2 cells were suppressed when incubated serum from HFD rats or NEFA mixture based on the serum FA profile. In addition, both serum from HFD-fed rats and NEFA mixture increase PPARγ promoter activity, accompanied by greater PPARγ protein abundance in obese animals and the binding affinity of PPARγ to the aP2 enhancer. These results demonstrated that NEFA not only increased PPARγ expression at the expense of β-catenin, but also in its transcriptional and binding activity [216].

**Table 2.1** Dietary intakes of EPA, DHA and fish/shellfish in Canadian children

| Study | Participants | Assessment method | Dietary intake (mg) | | |
|---|---|---|---|---|---|
| | | | **EPA** | **DHA** | **Fish/shellfish (3 oz serving)** |
| [217] | Healthy children 2-3 y, n=20 Guelph, Ontario | Direct quantification of food samples collected over 3 days | 9.6 ± 2.9 | 19.2 ± 6.8 | |
| [218] | Healthy children 18-60 mo, n=84 Vancouver, British Columbia | FFQ | 54 ± 6 | 88 ± 10 | |
| [219] | Healthy children 4-8 y, n=41 Guelph, Ontario | Direct quantification of food samples collected over 3 days | 38.4 ± 9.3 | 54.1 ± 11.4 | |
| [220] | Healthy children 4-7 y, n=91 Edmonton, Alberta | 3-d food records and FFQ | | 16.5 (0 - 350) * | 0.03 ± 0.19 serving /mo |
| [221] | Healthy children 2-8 y, n=49 Montreal, Quebec | 3-d food records and FFQ | | | 0.14 ± 0.14 serving/d |

Mean ± SEM * median and interquartile range. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FFQ, food frequency questionnaire

## 2.3 Conclusion

Childhood growth represents a critical period for the acquisition of bone mass. However, excess adiposity at this stage may counteract the response of bone to mechanical force induced by muscle, resulting in reduced skeletal mass relative to body size. As the incidence of obesity has risen in children and adults, so has the number of studies examining the relationships between fat and bone. The considerable body of densitometry-based evidence that exists to date remains controversial as to whether fat has a detrimental effect on bone in the pediatric population, and there is a paucity of prospective trials to monitor influences and explicate mechanisms of excess adiposity on bone as children progress through adolescence into adulthood. It is important to recognize that obesity-related changes in bone metabolism have no single cause. Further complicating the bone-fat relationship in obesity is their interrelated roles in regulating energy metabolism affected by emerging genetic, molecular and hormonal cues. What remains to be determined is whether a compensatory mechanism between bone and adipose tissue exists for adapting to the dysfunctional energy homeostasis as a result of DIO.

Omega-3 LCPUFA, especially DHA is one of the potential interventions given its modulatory effects on bone acquisition as well as adiposity, although scarce reports are available concerning skeletal outcomes with DHA supplementation in childhood obesity, and even less had shed light on potential mechanisms. Inconsistencies in the sera composition used *in vitro*, fatty acid profiles analysis, source of DHA and choice of control oils in feeding trials had made comparison across studies difficult. Also, the applicability of supplementation either through food sources or synthetic oils in the pediatric population has not been determined. More data is therefore required before incorporating DHA into recommendations and guidelines for the attainment and maintenance of bone mass in children, particularly in those that are with

overweight condition or obesity.

Bone mass acquired through childhood and adolescence tracks into adulthood and may ultimately determine future osteoporotic risk. The possible detrimental effect of fat on bone mass accrual in face of an increasing incidence of obesity has significant implications for future skeletal health. Children with obesity may therefore require targeted interventions that considers changes in body composition, lifestyle and diseases, to maximize bone mass accrual during growth to achieve optimal peak bone mass and reduce the risk of osteoporosis later in life.

**Bridge statement 1**

The literature review has highlighted the potential roles of n-3 LCPUFA in dually modulating the development of bone and adiposity during growth. The proportions of LCPUFA in circulation, especially that of EPA and DHA are largely determined by their presence in the diet. Several national and international organizations have provided guidance on desirable dietary intakes of EPA and DHA. For adults, these recommendations range from approximately 250-500 mg/d and are based mainly on primary and secondary prevention of cardiovascular diseases (CVD) [222]. For infants (0-2 y), the Food and Agriculture Organization /World Health Organization recommended 20 mg DHA per kg (body weight)/d for optimal growth and development [223]. There are, however, no agreed upon recommendations for dietary intake of EPA and/or DHA for children between 2-12 y of age, due in part to the scarce information available addressing dietary intake, measures of status, and health effects in this age group. Using NHANES data from 2009 to 2012, 62% children aged 1-19 y (n=5656) reported consuming seafood in the previous month, with only half consuming it at least three times each month [224]. Unfortunately, no similar report at the national level is available for Canadian children. The CHMS cycle 2.2 reported intakes of ALA and LA, but not that of EPA, DHA or of fish intake. Of the few studies that have been conducted, a wide range of intakes was observed but consistently low relative to recommendations (**Table 2.1**); daily intakes of DHA ranged from 16.5 to 88 mg/d for children residing in inland [217] and coastal communities [218] respectively. While two of the present studies [217, 219] used direct quantification approach to assess EPA and DHA intake, this method is tedious and limited by small sample sizes. Dietary assessments methods including 24-h recalls and food frequency questionnaires (FFQ), although easily administered, are known to be limited by memory-related reporting biases and imposes a large

respondent burden. Importantly, individuals may not be aware of the species of fish they ate, particularly if consumed out of the home, and can result in substantial discrepancies in estimation of n-3 LCPUFA intakes. Hence, the quantities of individual LCPUFA in the diet are extremely difficult to estimate from reported dietary intakes; and may in part contribute to the paucity of findings on the relationship between LCPUFA intake and CVD risk factors and inflammatory markers in children [225].

The ability for dietary LCPUFA to be absorbed, incorporated into tissue and further metabolized into longer-chain derivatives vary among individuals, and affected by factors including excess adiposity. The aforementioned studies were all performed in children with normal weight, and only one [218] has quantified fatty acid composition in RBC but found no association with dietary n-3 LCPUFA intake. The following chapter describes a preliminary analysis of dietary LCPUFA intake in children with obesity, using comprehensive assessment methodologies including 3-d food records, gas chromatography quantification of fatty acid proportions in RBC, and absorptiometry-based measures of body composition in addition to anthropometry.

# CHAPTER 3

*Manuscript 1*

**Under peer review in *Pediatric Obesity***

# Increased adiposity in children with obesity is associated with low red blood cell omega-3 fatty acid status and inadequate polyunsaturated fatty acid dietary intake

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### 3.1 Abstract

The association between total dietary fat intake and measures of body fatness in children with obesity remains inconsistent. This study aimed to determine if dietary long-chain polyunsaturated fatty acids (LCPUFA) and LCPUFA status relate to body composition in children with obesity. Children (n=63, 9.0 ± 0.2 y, BMI Z-score 3.1 ± 0.2) were divided into tertiles of % body fat assessed by dual-energy X-ray absorptiometry. Diet was assessed 3-d food diaries. Fatty acid proportions in red blood cells (RBC) were measured by gas chromatography. Data stratified by sex and Tanner stages were compared with a MIXED model ANOVA. Associations between RBC fatty acid status and dietary intakes were examined with Spearman correlation. Moderate correlations were observed between RBC eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) proportions, dietary EPA and DHA ($r=0.39$, $p<0.05$) as well as fish servings ($r=0.33$, $p<0.05$). Dietary LCPUFA did not differ among tertiles. Children in tertile 3 had lower RBC α-linolenic acid (-40%) and EPA+DHA (-15%) proportions adjusted for age, Tanner stages and race compared to tertile 1. The lower omega-3 LCPUFA status in children with greater adiposity is consistent with suboptimal intakes of omega-3 LCPUFA and fish in the diet.

## 3.2 Introduction

Obesity is a key risk factor for chronic diseases. The prevalence of obesity among children and youths in Canada has increased significantly over the last 4 decades. Results from the Canadian Health Measures Survey (2014) classified approximately 23% of children between 12-17 y to be overweight or obese, respectively, compared to 14% of children considered to be overweight or obese in 1981 [226]. This is of concern as childhood obesity and its metabolic complications are known to track into adulthood [227], hence timely interventions and preventative measures are needed. While the etiology of childhood obesity is complex and multifactorial [228], it is generally agreed that it can in part be attributed to energy intake that exceeds energy expenditure. Diet quality and composition at this life stage may also have important implications in addition to quantity of food intake alone.

The roles of specific nutrients (macro- and micro-nutrients), foods or food groups contributing to energy dysregulation are unclear. Children with obesity are suggested to consume snacks and foods with added sugar more frequently than normal-weight peers [229, 230]; while others have found no association between dietary habits and the prevalence of childhood obesity in Canada (n=5890, 11-16 y) [36]. Similarly, the importance of high fat diets in the increasing rates of childhood obesity remains controversial, as obesity rates have increased even though intakes of fat have not changed since the 1970s [231]. Recent studies suggest that dietary fatty acid composition may be as important as the amount of fat consumed. For instance, long chain polyunsaturated fatty acids (LCPUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are associated with improved cardiovascular health [232], and in animal models prevent obesity and adipose-tissue specific inflammation [233, 234].

Despite the suggested benefits of LCPUFA, data collected from the 2003-2008 United

States National Health and Nutrition Examination Survey showed that median daily intake of total omega-3 LCPUFA in children aged 1-6 y (n=3354) and 7-12 y (n=2846) was 0.06 and 0.09 g/d, respectively [50]. The Canadian Community Health Survey (CCHS 2.2) reported 81.1% of Canadian children (4–8 y) met the Adequate Intake (AI) recommendation for α-linolenic acid (ALA) [235], but no data for the intakes of EPA and DHA on the national level are available to date. Of the few studies that have surveyed EPA and DHA intakes in Canadian children, a wide range of intakes was observed but consistently low; daily intakes of EPA and DHA ranged from 29 to 142 mg/d for children residing in inland [217] and coastal communities [218] respectively. The *de novo* synthesis rate of EPA and DHA from ALA is low [236], and unlikely to achieve levels to produce beneficial effects on body weight regulation and disease prevention. Pre-formed EPA and DHA are found almost exclusively in oily fish and shellfish, yet these foods do not contribute substantially to the diets of children in North America [50].

Collection of LCPUFA intakes relies heavily on dietary assessment methods (e.g., 24-h recalls, 3-d food diaries and food-frequency questionnaires), however, these methods are prone to errors, and the extent of underreporting increases with body mass, especially in children [237, 238]. In addition, food items that are infrequently consumed as part of a regular diet are difficult to capture in the recalls, leading to reporting bias. Biological samples provide an objective measurement for determining omega-3 LCPUFA status. To date, no published data exist on dietary LCPUFA and/or fish and seafood intake specifically in children with obesity, and if LCPUFA in red blood cell membranes are associated with dietary LCPUFA intakes. This study aims to describe the diet of children with obesity, and to determine if dietary intakes or LCPUFA status relates to body composition outcomes. Fatty acid desaturation and elongation is adversely impacted by excess adiposity in children [56], potentially resulting in lower endogenous

synthesis and availability of LCPUFA. Hence, we hypothesize that children with obesity will have suboptimal LCPUFA intake, and that omega-3 LCPUFA status measured in red blood cell (RBC) membranes inversely relate to adiposity, due in part to lower availability in the diet.

## 3.3 Methods

### 3.3.1 Study population

Data and biological samples for this study were obtained from the baseline visit of a trial, as previously described [239]. Eligible participants were otherwise-healthy overweight or obese children (6-13 y) living in or near Montreal, Quebec. Participants were recruited through public and private elementary schools, primary healthcare organizations including physician referrals, word of mouth and local advertisements (newspaper, internet, radio commercials and postal mailings). The weight-for-height body mass index (BMI) cut-off for overweight or obese was based on the World Health Organization criteria of BMI Z-score $\geq 1$ or $\geq 2$ respectively. Only children in Tanner stages 1-3 were included in the study to minimize the confounding effects of maturation on body composition and LCPUFA status. Assessments were performed at the McGill University Mary Emily Clinical Nutrition Research Unit. In addition to parental consent, participants provided written assent at the grade 1 reading level. Socio-demographic information about parents and the child was collected from a parent-completed questionnaire developed based on the CCHS, and data on parental education level, total household income and ethnicity were collected.

### 3.3.2 Anthropometric and blood biochemistry

Anthropometry was measured using standard protocols. Height was measured to the nearest 0.1 cm using a stadiometer (Seca 214, Hamburg, Germany), and weight was measured to the nearest 0.2 kg using a balance-beam scale (Detecto, Missouri, USA). BMI Z-score was

derived using the World Health Organization's ANTHRO software (version 3.2.2, Switzerland, Europe). Waist circumference was measured to the nearest 0.1 cm. Maturation status was assessed by parental survey of physical development using the Tanner criteria [240, 241]. Body composition was obtained from a whole-body scan using dual-energy X-ray absorptiometry (DXA, Hologic QDR-4500A Discovery Series, Bedford, MA). DXA provides useful information on relative fat and lean masses in a single scan in an individual, and has been found to be more accurate than density-based water displacement for estimating total body fat [242]. Gynoid and android fat distribution was automatically-segmented by the analysis software (APEX version 13.3:3). Fat-mass index (FMI; kg/height$^2$) and fat-free mass index (FFMI) that estimates fat mass and fat-free mass relative to height respectively was calculated. Quality control was performed using Hologic lumbar spine phantom (#14774). The coefficient of variation (CV%) for bone mineral content measurements of the phantom was 0.5%.

### 3.3.3 Dietary assessment

Dietary intake was assessed using 3-d food diaries that reflected 2 weekdays and 1 weekend day that reflected the week after the baseline visit. Parents were instructed by a registered dietitian to complete the food records, and once completed the records were mailed back to the study unit. Food intakes were reported by a parent or caregiver. Recalls were analyzed using Nutritionist Pro software (Axxya System, Stafford, TX) and the Canadian Nutrient File 2010. Macronutrients (carbohydrates, protein and fat), as well as micronutrient (vitamins and minerals) contents were averaged from the 3-d diaries. Individual food items were categorized into one of four main food groups (i.e. Vegetables and Fruits (V&F), Grain Products, Milk and Alternatives (Milk & Alt), and Meat and Alternatives (Meat & Alt)). Examples of recommended serving sizes for common foods in each food group with reference to the Canada's

Food Guide (CFG, 2007) and MyPlate servings is provided in **Supp Table S3.1.** Remaining food items such as high-fat and/or high-sugar foods (e.g. candies, sugar-sweetened beverages, cookies, syrups and sauces) were classified as "sometimes" foods.

### 3.3.4 Fatty acid profiling

Blood was drawn from the child in the 12-h fasted state. RBC were separated by centrifugation from samples collected in EDTA-coated tubes with 1:1 saline, flushed with $N_2$, stored at -80ºC ≤ 2 y and were not thawed until analyses. Fatty acids were measured in RBC to reflect habitual dietary consumption as the half-life of RBC measures approximately 120 days [243], and the fatty acid profile resembles that of tissue cell membrane as RBC are composed predominately of phospholipids [244]. RBC lipids were extracted using the modified Lepage-Roy direct methylation method [245]. Fatty acid methyl esters (FAME) were separated using a 60-m CP Sil-88 capillary column (Varian-Chrompack CP 7489) installed in a gas chromatograph (Varian CP-3800, Walnut Creek, CA) with a flame-ionization detector. An aliquot of a single pooled blood sample was analyzed in triplicate during each extraction for quality control. Intra- and inter-assay CV was <5%, the inter-run CV was <8%. Recovery was determined against methylated C17:0 standard with known concentration and found to be > 90%. Chromatogram peaks were identified against standard Supleco™ 37 Component FAME mix (catalogue #47885-U; Sigma-Aldrich Co, St Louis, MO). In total, 24 fatty acids from C:14 to C24:0 were identified [246] and summed to calculate the total fatty acid content of the RBC membranes. Fatty acids were expressed as the percentage of total as it offers the practical advantage of lower variability and is distributed normally. The sum of RBC EPA and DHA as a proportion of total fatty acids was calculated. While by definition the HS-Omega-3 Index® (O3I) is the proportion of total EPA and DHA in RBC, we have refrained from using the term as integral to the definition of O3I was

a specific and standardized analytical procedure used only in laboratories that are also mandated to conform to regular proficiency testing [247].

### 3.3.5 Statistical analysis

All data were tested for normality and homogeneity of variances with the Shapiro-Wilk test and Levene's test prior to analysis. Variables that were not normally distributed (i.e., dietary EPA and DHA) were either log-transformed or a non-parametric test was performed. For nutrients where the Dietary Reference Intake (DRI) values set by the Institute of Medicine are available, dietary intakes were compared against the Estimated Average Requirement, Recommended Dietary Allowance and AI. Spearman correlation coefficients were calculated to estimate the association between RBC fatty acids and dietary fatty acid intakes from 3-d food diaries. Multiple regression models were used to estimate the relationship between LCPUFA status and dietary intake after adjusting for demographic factors (age, sex, Tanner stage, race and DXA-measured body fat %). Participants were stratified by sex and Tanner stages, then divided into tertiles according to body fat % measured by DXA, and subsequently pooled to obtain tertiles. While increased abdominal or visceral adipose tissue during adulthood is known to be associated with dyslipidemia and other symptoms of the metabolic syndrome [248], we have not divided children by android fat %, as the skeletal landmarks (e.g. acromion, iliac crests, greater trochanter) required for proper recognition of android fat are not fully developed in children. This would lead to a misplacement of the region of interest of analysis, generating data of greater variability that are less reproducible. The Chi-squared test was used to detect differences in proportions among groups for categorical variables. Differences in anthropometry, biochemistry and dietary intakes among groups were tested using a MIXED model ANOVA with *post-hoc* Tukey's HSD. Tertile classification and sex were included as fixed effects, while time (year and

month) of visit was included as a random variable. Covariates explored included age, race and parental socio-demographic characteristics. Significant interactions across most outcomes were observed hence results were presented stratified by sex. All tests were 2-sided with a $p<0.05$ used to estimate the probability of differences. Analyses were performed with SAS 9.4 (Cary, NC).

### 3.3.6 Ethics approval

Ethics approval for the study was obtained from McGill University Faculty of Medicine Institutional Review Board and English Montreal School Board.

## 3.4 Results

### 3.4.1 Participant characteristics

This analysis includes 63 children with parent-reported 3-d food diaries returned and who provided adequate blood for the RBC LCPUFA analyses. Only children in Tanner stages 1-3 were included in the study to minimize the confounding effects of maturation on body composition and LCPUFA status. Overall, 48% of the study group was female, the majority (89%) were classified as obese (mean BMI Z-score: 3.3 ± 0.2), and 68% were classified as pre-pubertal (Tanner stage 1), 8% and 24% were classified as stage 2 and 3, respectively.

Children were subdivided into tertile groups based on body fat % measured by DXA (**Table 3.1**). The proportion of mothers classified as overweight or obese (BMI > 25 kg/m$^2$) was significantly greater in tertiles 2 and 3 compared to tertile 1. No differences in age, Tanner stages, or height and its age- and sex- Z-score were observed among groups or between sexes. Body weight and waist circumference were significantly different across tertiles and was 20% and 10% greater in tertile 3 compared to tertile 1. An interaction between tertile and sex was observed for BMI Z-score; an increase in BMI Z-score was observed across tertiles in boys, but not in girls. DXA-measured gynoid fat% increased across groups in both sexes. Android fat%

was 8% greater in girls compared to boys, yet the increase across tertiles was apparent in boys only (1st tertile: 34.2 ± 1.6% vs 3rd tertile: 46.5 ± 1.6%).

### 3.4.2 Dietary intakes

No differences were observed in average daily energy intake nor any nutrients in children among tertiles of % body fat (**Supp Table S3.2**). The average distribution of macronutrients in the diet were all within the acceptable macronutrient distribution range (AMDR). Intakes of total sugars averaged 20.7 ± 5.5% of total energy, which is within the recommended Canadian limit of 25% of total energy. There were no differences in the proportion of children meeting the recommendation for all nutrients among tertiles (**Table 3.2**)**.** Differences in sex was observed for the intakes of monounsaturated fatty acids and PUFA as a percentage of total energy, where boys had 15% and 30% lower average intake compared to girls respectively. Intakes of LCPUFA were low; overall the median intake of ALA was 0.79 g/d (IQR 0.54 – 1.16), with up to 63% children not meeting the AI for ALA of 0.9 g/d. Expressed as a proportion of energy intake, 89% of children had ALA intakes below the lower limit of the AMDR (i.e. 0.6% kcal). Similarly, 84% of children did not meet the AI for LA. Consistently, despite the wide ranges observed for combined EPA and DHA intakes, the median (32.3 mg/d; IQR 12.0 - 86.7) was below the recommended 250-500 mg/d by various expert committees [249].

The number of food servings from the V&F group was lower in the third tertile compared to the first (tertile 1: 6.4 ± 0.8 vs tertile 3: 5.0 ± 0.7 servings; p=0.02 (**Supp Figure S3.1**). The difference was mainly attributed to the consumption of fewer fruits (tertile 1: 4.4 ± 0.7 vs tertile 3: 2.3 ± 0.5 servings; p=0.008). Boys consumed more Milk & Alt servings, especially as cow's milk (M: 1.2 ± 0.3 vs F: 0.8 ± 0.3; p=0.006) and yogurt (M: 0.5 ± 0.1 vs F: 0.3 ± 0.1; p=0.018) compared to girls. Intakes of whole grain products averaged to 0.8 ± 0.3 servings in the tertile 3

and was lower compared to children in tertile 2 (1.8 ± 0.4 servings; p=0.004).

### 3.4.3 RBC fatty acid status and correlation with dietary fatty acids

The proportion of ALA in RBC membranes was greater in tertile 1 compared to tertiles 2 and 3 (tertile 1: 0.095 ± 0.01; 2: 0.067 ± 0.01; 3: 0.062 ± 0.01 %). Likewise, children in tertile 1 had higher proportions of EPA+DHA than tertile 3 (2.24 ± 0.86% vs 1.86 ± 0.35%) (**Figure 3.1**). A sex difference was observed for EPA where boys had 14% greater proportions than girls. Spearman correlation coefficients were calculated to compare dietary intakes of fatty acids with their respective proportions in RBC membranes. Dietary intakes of EPA (r=0.33, p=0.008), DHA (r=0.37, p=-.003) as well as combined EPA+DHA intakes (r=0.39, p=0.002) were significantly correlated with their proportions in RBC membranes. No association between dietary total SFA, LCPUFA, oleic acid, LA or ALA and RBC status was observed. The RBC EPA+DHA proportion was positively related to total fish and seafood servings (average intake 0.2 ± 0.04 servings per day) (**Figure 3.2**). The relationship was weaker but remained significant when the outlier (fish intake > 2SD, 1.5 servings) was excluded from the analysis (r=0.29, p=0.02). In a multiple regression model (**Table 3.3**) containing demographic factors (sex, age, Tanner stage and race), body fat % and reported LCPUFA or fish consumption, only the latter was significantly associated with their respective proportions in RBC. There was an estimated 0.5% and 1.8% increase in RBC EPA+DHA proportions, respectively for each additional 1 g of dietary EPA and/or DHA consumption; and a 1.1% increase for each additional fish serving. The relative abundance of fatty acids in RBC membranes by % body fat tertiles are described in **Supp Table S3.3**.

**Table 3.1** Characteristics of male and female children with obesity by tertiles of % body fat

| | Females | | | | | | Males | | | | | | Overall | | | | | | Main effect | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st tertile, n=10 (29.0-35.9%) | | 2nd tertile, n=10 (36.0-40.9%) | | 3rd tertile, n=10 (41.0-47.2%) | | 1st tertile, n=11 (25.7-32.2%) | | 2nd tertile, n=11 (32.3-38.7%) | | 3rd tertile, n=11 (38.8-48.7%) | | 1st tertile, n=21 (25.7 -37.1%) | | 2nd tertile, n=21 (32.9-40.8%) | | 3rd tertile, n=21 (35.0-48.7%) | | $P_{tertile}$ | $P_{sex}$ | $P_{tertile*sex}$ |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | | | |
| Age (y) | 9.3 | 0.4 | 10.1 | 0.5 | 10.2 | 0.4 | 10.2 | 0.4 | 10.3 | 0.6 | 10.2 | 0.4 | 9.75 | 0.34 | 10.22 | 0.47 | 10.19 | 0.34 | | NS | |
| Height (cm) | 140.5 | 2.6 | 143.3 | 2.8 | 141.3 | 2.8 | 143.4 | 2.8 | 143.0 | 3.8 | 143.3 | 2.8 | 142.0 | 2.24 | 145.6 | 2.87 | 142.3 | 2.27 | | NS | |
| Age-and-sex Z-score | 1.17 | 0.38 | 1.38 | 0.42 | 0.58 | 0.58 | 0.60 | 0.41 | 0.89 | 0.56 | 0.65 | 0.41 | 0.89 | 0.32 | 1.14 | 0.43 | 0.62 | 0.32 | | NS | |
| Weight (kg) | 48.8 | 3.3 | 51.6 | 3.7 | 53.3 | 3.6 | 49.8 | 3.6 | 56.6 | 4.9 | 61.6 | 3.6 | 49.3 | 2.9 | 59.1 | 3.7 | 57.4 | 2.9 | **0.03** | 0.55 | 0.52 |
| BMI (kg/m$^2$) | 24.0 | 1.2 | 27.5 | 1.3 | 26.2 | 1.2 | 23.8 | 1.2 | 27.3 | 1.7 | 30.2 | 1.2 | 23.9 | 1.0 | 27.4 | 1.3 | 28.2 | 1.0 | **0.005** | 0.17 | 0.10 |
| Age-and-sex Z-score | 2.32 [a] | 0.36 | 2.56 [a] | 0.41 | 2.78 [a] | 0.39 | 2.48 [a] | 0.39 | 3.25 [ab] | 0.54 | 4.32 [b] | 0.39 | 2.40 | 0.30 | 3.00 | 0.41 | 3.35 | 0.31 | **0.08** | **0.003** | **0.03** |
| Waist circumference (cm) | 82.4 | 2.8 | 93.4 | 3.1 | 86.4 | 3.1 | 83.1 | 3.0 | 91.4 | 4.1 | 95.8 | 3.1 | 82.8 | 2.4 | 92.4 | 3.1 | 91.1 | 2.4 | **0.01** | 0.20 | 0.11 |
| **DXA** | | | | | | | | | | | | | | | | | | | | | |
| Whole body fat (%) | 34.3 | 1.0 | 38.3 | 1.2 | 42.6 | 1.1 | 32.0 | 1.1 | 35.9 | 1.6 | 41.3 | 1.1 | 33.1 | 0.9 | 37.1 | 1.2 | 42.0 | 0.9 | **<.0001** | **0.01** | 0.83 |
| Whole body lean mass (%) | 62.9 | 1.9 | 59.1 | 1.1 | 55.1 | 1.1 | 65.3 | 1.1 | 61.6 | 1.5 | 55.9 | 1.1 | 64.1 | 0.8 | 60.4 | 1.1 | 55.5 | 0.9 | **<.0001** | **0.01** | 0.64 |
| Android fat (%) | 40.5 [a] | 1.5 | 44.8 [ab] | 1.7 | 45.2 [a] | 1.6 | 34.2 [b] | 1.6 | 41.3 [ab] | 2.2 | 46.5 [a] | 1.6 | 37.3 | 1.3 | 43.0 | 1.7 | 45.8 | 1.3 | **<.0001** | **0.01** | 0.03 |
| Gynoid fat (%) | 39.3 | 1.0 | 42.0 | 1.1 | 46.7 | 1.1 | 35.9 | 1.1 | 39.6 | 1.5 | 43.4 | 1.1 | 37.6 | 0.9 | 40.8 | 1.1 | 45.0 | 09 | **<.0001** | **.0002** | 0.84 |
| FMI (kg/m$^2$) | 8.2 | 0.7 | 10.6 | 0.7 | 11.2 | 0.7 | 7.7 | 0.7 | 9.9 | 1.0 | 12.7 | 0.7 | 8.0 | 0.6 | 10.2 | 0.7 | 12.0 | 0.6 | **<.0001** | 0.84 | 0.18 |
| FFMI (kg/m$^2$) | 15.7 | 0.57 | 16.9 | 0.6 | 15.2 | 0.6 | 16.0 | 0.6 | 17.6 | 0.8 | 17.5 | 0.6 | 15.9 | 0.5 | 17.3 | 0.6 | 16.3 | 0.5 | | NS | |
| | n | % | n | % | n | % | n | % | n | % | n | % | n | % | n | % | n | % | | | |
| **Tanner staging** | | | | | | | | | | | | | | | | | | | NS | NS | |
| Pre-pubertal / Stage 1 | 6 | 60 | 6 | 60 | 7 | 70 | 8 | 73 | 8 | 73 | 8 | 73 | 14 | 67 | 14 | 67 | 15 | 71 | | | |
| Stage 2 | 1 | 10 | 1 | 10 | 1 | 10 | 1 | 9 | 0 | 0 | 1 | 9 | 2 | 10 | 1 | 5 | 2 | 10 | | | |
| Stage 3 | 3 | 30 | 3 | 30 | 2 | 20 | 2 | 18 | 3 | 27 | 2 | 18 | 5 | 24 | 6 | 29 | 4 | 19 | | | |
| Race, white | 6 | 60 | 10 | 100 | 9 | 90 | 9 | 82 | 7 | 64 | 8 | 73 | 15 | 71 | 17 | 81 | 17 | 81 | | | |
| Household income (>$75,000 CAD/y) | 3 | 30 | 9 | 90 | 5 | 50 | 9 | 82 | 8 | 73 | 6 | 55 | 12 | 57 | 17 | 81 | 11 | 52 | | | |
| **Mother's BMI category** | | | | | | | | | | | | | | | | | | | 0.01 | NS | |
| Normal (18-24.9 kg/m$^2$) | 5 | 56 | 1 | 11 | 2 | 25 | 7 | 64 | 3 | 27 | 0 | 0 | 12 | 60 | 4 | 20 | 2 | 12 | | | |
| Overweight (25-29.9 kg/m$^2$) | 2 | 22 | 1 | 11 | 4 | 50 | 2 | 18 | 6 | 55 | 4 | 44 | 4 | 20 | 7 | 35 | 8 | 47 | | | |
| Obese (> 30 kg/m$^2$) | 2 | 22 | 7 | 78 | 2 | 25 | 2 | 18 | 2 | 18 | 5 | 56 | 4 | 20 | 9 | 45 | 7 | 41 | | | |
| **Father's BMI category** | | | | | | | | | | | | | | | | | | | NS | NS | |
| Normal (18-24.9 kg/m$^2$) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 22 | 1 | 13 | 0 | 0 | 2 | 11 | 1 | 8 | | | |
| Overweight (25-29.9 kg/m$^2$) | 3 | 60 | 3 | 33 | 1 | 20 | 4 | 44 | 4 | 44 | 1 | 13 | 7 | 50 | 7 | 39 | 2 | 15 | | | |
| Obese (> 30 kg/m$^2$) | 2 | 40 | 6 | 67 | 4 | 80 | 5 | 56 | 3 | 33 | 6 | 75 | 7 | 50 | 9 | 50 | 10 | 77 | | | |

BMI, body mass index; DXA, dual-energy X-ray absorptiometry; FMI, fat mass index; FFMI, fat-free mass index. n=63. Within rows, different superscripts denote a probability of differences among tertiles groups

**Table 3.2** Proportion of children meeting the DRI recommendations defined by the Institute of Medicine

| | % children meeting recommendation | | | | | | | | | DRI / CFG 2007 recommendation |
|---|---|---|---|---|---|---|---|---|---|---|
| | Females | | | Males | | | Overall | | | |
| | 1st tertile | 2nd tertile | 3rd tertile | 1st tertile | 2nd tertile | 3rd tertile | 1st tertile | 2nd tertile | 3rd tertile | |
| **Total sugar** | 70 | 90 | 70 | 73 | 45 | 82 | 71 | 86 | 76 | < 25% kcal |
| **Dietary fiber** | | | | | | | | | | |
| AI | 30 | 30 | 0 | 9 | 18 | 0 | 19 | 24 | 0 | 4-8 y: 25 g; 9-13 y: M: 31 g; F: 26 g |
| **ALA** | | | | | | | | | | |
| AI | 40 | 40 | 40 | 18 | 18 | 36 | 29 | 29 | 38 | 4-8 y: 0.9 g; 9-13 y: M: 1.2 g; F: 1.0 g |
| AMDR | 0 | 0 | 30 | 9 | 9 | 9 | 5 | 5 | 19 | 0.6 – 1.2% |
| **LA** | | | | | | | | | | |
| AI | 40 | 20 | 30 | 9 | 0 | 18 | 24 | 10 | 24 | 4-8 y: 10 g; 9-13 y: M: 12 g; F: 10 g |
| AMDR | 30 | 10 | 40 | 9 | 0 | 9 | 19 | 5 | 24 | 5 – 10 % |
| **Calcium** | | | | | | | | | | |
| EAR | 70 | 20 | 40 | 73 | 73 | 82 | 72 | 48 | 62 | 4-8 y: 800 mg; 9-13 y: 1100 mg |
| RDA | 40 | 10 | 0 | 55 | 27 | 64 | 48 | 19 | 33 | 4-8 y: 1000 mg; 9-13 y: 1300 mg |
| **Vitamin D** | | | | | | | | | | |
| EAR | 0 | 10 | 0 | 0 | 9 | 9 | 0 | 10 | 5 | 400 IU |
| RDA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 600 IU |
| **Potassium** | | | | | | | | | | |
| AI | 50 | 60 | 40 | 36 | 45 | 45 | 43 | 52 | 43 | 4-8 y: 2300 mg; 9-13 y: M: 2500 mg; F: 2300 mg |
| **Sodium** | | | | | | | | | | |
| AI | 90 | 100 | 100 | 100 | 100 | 100 | 95 | 100 | 100 | 4-8 y: 1000 mg; 9-13 y: 1200 mg |
| > limit | 90 | 90 | 90 | 100 | 100 | 100 | 95 | 95 | 95 | 4-8 y: > 1200 mg; 9-13 y: >1800 mg |
| **CFG food groups** | | | | | | | | | | |
| V&F | 70 | 60 | 50 | 55 | 55 | 36 | 62 | 57 | 43 | 4-8 y: 5 servings; 9-13 y: 6 servings |
| Grain Products | 80 | 80 | 70 | 91 | 91 | 100 | 86 | 86 | 86 | 4-8y: 4 servings; 9-13 y: 6 servings |
| Meat & Alt | 90 | 100 | 100 | 91 | 100 | 100 | 90 | 100 | 100 | 4-8 y: 1 serving; 9-13 y: 1-2 servings |
| Milk & Alt | 60 | 30 | 30 | 64 | 64 | 82 | 62 | 48 | 57 | 4-8y: 2 servings; 9-13 y: 3-4 servings |

n=63. AI, adequate intake; AMDR, acceptable macronutrient distribution range; CFG, Canada's Food Guide; EAR, estimated average requirement; RDA, recommended dietary allowance; V&F, Vegetables and Fruits

**Table 3.3** Correlates of n-3 LCPUFA status with dietary LCPUFA, fish intake and demographic characteristics in children with obesity

| | Beta | SE | p | Model p | Model $r^2$ |
|---|---|---|---|---|---|
| **RBC EPA (% total)** | | | | **0.016** | **0.25** |
| Intercept | 0.34 | 0.11 | 0.0048 | | |
| Dietary EPA (g) | 0.54 | 0.18 | 0.0037 | | |
| Sex, male | 0.029 | 0.023 | 0.21 | | |
| Age (y) | -0.0048 | 0.010 | 0.64 | | |
| Body fat (%) | -0.0021 | 0.0023 | 0.39 | | |
| Race, white | -0.053 | 0.028 | 0.06 | | |
| Tanner stage | 0.0095 | 0.021 | 0.65 | | |
| **RBC DHA (% total)** | | | | **0.03** | **0.23** |
| Intercept | 2.45 | 0.84 | 0.005 | | |
| Dietary DHA (g) | 1.78 | 0.58 | 0.0034 | | |
| Sex, male | 0.16 | 0.17 | 0.35 | | |
| Age (y) | -0.031 | 0.075 | 0.68 | | |
| Body fat (%) | -0.013 | 0.017 | 0.47 | | |
| Race, white | -0.32 | 0.20 | 0.12 | | |
| Tanner stage | 0.096 | 0.15 | 0.53 | | |
| **RBC EPA+DHA (% total)** | | | | **0.013** | **0.26** |
| Intercept | 2.76 | 0.89 | 0.0032 | | |
| Dietary EPA+DHA (g) | 1.44 | 0.43 | 0.0016 | | |
| Sex, male | 0.19 | 0.18 | 0.29 | | |
| Age (y) | -0.035 | 0.080 | 0.66 | | |
| Body fat (%) | -0.014 | 0.018 | 0.44 | | |
| Race, white | -0.38 | 0.22 | 0.09 | | |
| Tanner stage | 0.10 | 0.16 | 0.54 | | |
| **RBC EPA+DHA (% total)** | | | | **0.0059** | **0.29** |
| Intercept | 2.88 | 0.87 | 0.018 | | |
| Fish (75 g serving/day) | 1.06 | 0.29 | 0.0005 | | |
| Sex, male | 0.12 | 0.018 | 0.50 | | |
| Age (y) | -0.051 | 0.079 | 0.52 | | |
| Body fat (%) | -0.012 | 0.18 | 0.49 | | |
| Race, white | -0.41 | 0.16 | 0.36 | | |
| Tanner stage | 0.15 | 0.21 | 0.06 | | |

n=63. Values in this table are beta coefficients and are interpreted as the percentage point change in RBC fatty acid for a one-unit change in the characteristic after adjusting for other characteristics. Characteristics were entered based on previous report [250] in adults on factors hypothesized to affect RBC fatty acid status

**Figure 3.1** Proportions of α-linolenic acid (C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and EPA + docosahexaenoic acid (DHA, C22:6 n-3) in red blood cells membrane by tertiles of % body fat. Data are mean ± SEM. n=63. Different superscripts indicate significant differences ($p<0.05$) among tertiles

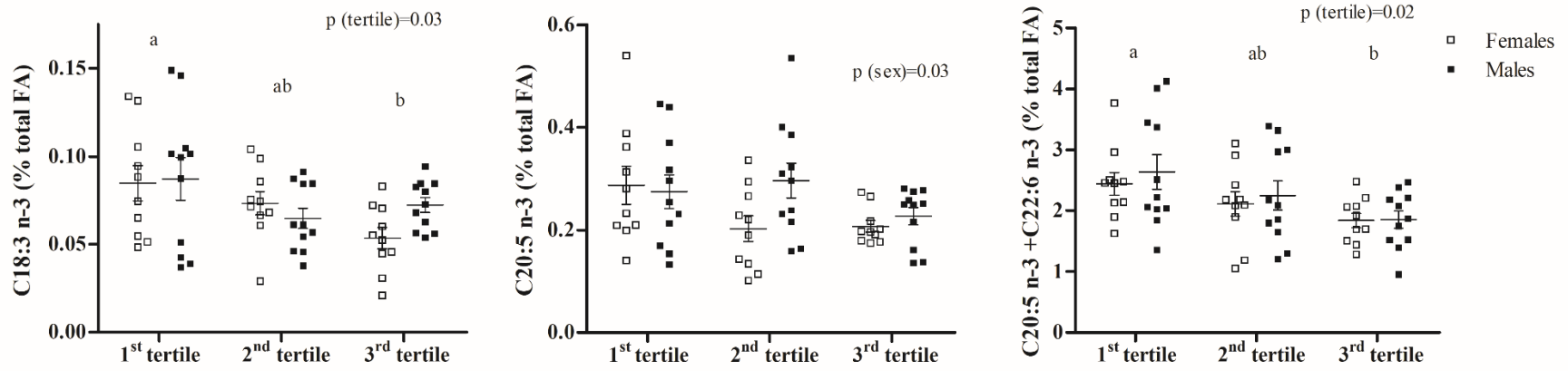
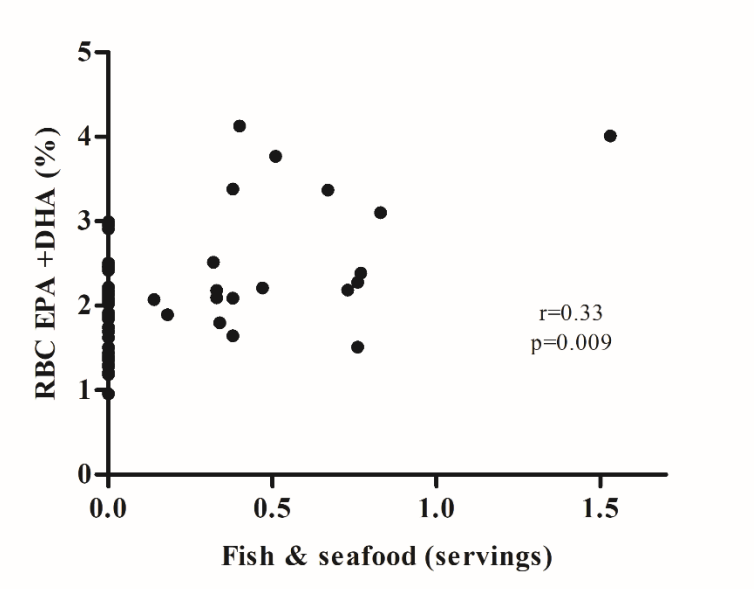


**Figure 3.2** Spearman correlation between total fish & seafood intake and eicosapentaenoic acid (EPA)+ docosahexaenoic acid (DHA) proportions in red blood cells (RBC). n=63. Dietary intakes are presented as the daily average of 3-d food diaries



### 3.5 Discussion

This study presents results from a cross-sectional analysis of school age children with obesity. Children in the highest tertile of % body fat had lower consumption of vegetables and fruits compared to the first tertile. Both quantity and quality of fat intake did not differ among tertiles. EPA and DHA status were associated with dietary EPA, DHA and fish intakes irrespective of degree of adiposity. However, higher % body fat was related to lower ALA and EPA+DHA status in RBC, possibly due in part to low n-3 LCPUFA from fish and/or seafood intakes in the diet.

The role of excess dietary fat consumption in the cause of childhood obesity remains questionable. This is supported by our results where only 11% children exceeded the AMDR for fat of 35%. The fatty acid composition of ingested fat may have equal importance on the increased obesity prevalence [186, 251]. In our study, median intakes of neither ALA or LA met the recommended AI values. This is unexpected, and contrary to the CCHS (cycle 2.2) results that showed a low prevalence of inadequate ALA intake in children 4-8 y (median intake 1.23 g/d) [235]. It is possible that there is a general avoidance of high-fat foods, irrespective of fat quality, amongst children with obesity and/or their parents. We also cannot rule out the likelihood of selective under-reporting of foods perceived as high in dietary fat in our participants, as ALA is found predominantly in canola and soybean oil, as well as processed foods in Canada [252]. Added fats used in food preparation may also be missed in the reporting of dietary data.

No specific dietary recommendation to date exists for EPA and DHA, based on the premise that they can be synthesized *de novo* from dietary ALA. However, the conversion rate is limited, and influenced by factors including dietary (e.g. omega-6 fatty acids intake), hormonal (sex steroids) and metabolic changes [253]. Excess body weight due to adiposity negatively

influences fatty acid desaturation [59, 60, 186] and RBC membrane fatty acid composition [187]. The lower ALA and EPA+DHA proportions in RBC was most apparent in children in the highest tertile of % body fat in the present study. This is consistent with previous reports in children [56, 60] where inverse relationships between excess body weight or adiposity (estimated by skinfold thickness) and LCPUFA, LA and DHA status was demonstrated. This is of concern as a recent meta-analysis confirmed that a high (>8%) O3I, defined as the sum of RBC EPA+DHA proportions, was related to a 35% risk reduction of cardiovascular death in adults [232]. Children with obesity have a lower O3I than healthy weight children (4.4 ± 1.4 and 5.0 ± 1.4 respectively). A moderate inverse relationship was observed between O3I, fasting insulin levels and Homeostatic Model Assessment for Insulin Resistance scores in children (5-12 y) [254]. These studies and ours have laid ground for future intervention trials investigating the benefits of increasing EPA and DHA status on metabolic function or cardiovascular diseases risk, especially in a population with low baseline EPA and DHA status where it is more likely to produce measurable benefits.

Consumption of EPA and DHA directly from foods and/or dietary supplements is the most practical way for humans to increase levels of these fatty acids in tissues. Consistent with reports in adults [250, 255], we demonstrated that proportions of EPA+DHA in RBC is a useful biomarker to reflect dietary EPA, DHA and fish servings in children with obesity. It is noteworthy that due to possible differences in fatty acids extraction methods and analytic procedures, the range of values in this study appeared to be lower than previously reported [256]. Hence the results presented herein can provide information on trends but cannot be directly compared with other literature.

The moderate correlation between dietary LCPUFA and RBC proportions found in this

study contradicts previous research, which reported associations between dietary EPA and RBC proportions ($r=0.24$, $p<0.05$) in children ($n=93$, 5-12 y) of healthy weight, and not those with overweight status [257]. The discrepancy may be due to the overall low LCPUFA and fish intake in our participants, where the increase in fish consumption would produce more apparent effect compared to populations with higher baseline omega-3 LCPUFA status. Using multiple regression analysis, we showed that for every additional serving of fish consumed, there was a corresponding 1% increase in RBC EPA+DHA proportions. This is substantial considering the mean EPA+DHA proportion is 2.2 ± 0.1%. Given the beneficial effects that omega-3 LCPUFA has on obesity and its associated metabolic consequences, results from this study reinforces the need to encourage fish consumption as an effective mean to raise blood omega-3 levels.

We acknowledge that the CFG has since been revised in early 2019. The most recent version has eliminated the food groups and adapted an approach that focus on proportions. Nonetheless, specific servings sizes are still of help to quantify dietary patterns and were effective at the time of the data collection. The main criticism of the CFG 2007 was its focus on meeting nutrient DRI requirements [258], yet deviations from the recommendations persisted for many nutrients, including vitamin D, LA and fibre, even if the number of servings were met. In the present study, children in the highest tertiles of % body fat had less V&F servings, and only about 50% of all children met the CFG 2007 recommendation for V&F and Milk & Alt groups, reflecting a generally poor diet quality in our participants. Specifically, on the occasions where fish intake was reported, 20% was contributed by fried fish sticks/ fish burgers, which do not align with recommendations for healthy choices, both in terms of fish species and cooking methods. Children with obesity may also be more likely to exhibit fussy eating patterns and unwilling to consume unfamiliar foods, as a result parents may offer foods based on children's

tastes and preferences [259]. In addition, common barriers including cost of fish, accessibility, the lack of inspiration or cooking skills, and differences in cultural background remain to be important determinants of fish consumption [260]. Therefore, recommendations and education strategies developed for parents and children with obesity may need to address the importance of including a variety of food choices that are both healthy and enjoyable by children and parents alike.

Despite similar ranges in % body fat between sexes, BMI Z-score increased across tertiles in boys only but not in girls. The accuracy of BMI as a surrogate measure of adiposity among children vary according to the degree of fatness [261-263]. Data from the Pediatric Rosetta Project [261] showed a markedly nonlinear relation between BMI Z-score and FMI, where small increases in BMI Z-score were associated with disproportionate increases in FMI. Sex differences among older (>8 y) children were clear; at the same BMI Z-score, girls had a higher FMI and a lower FFMI than boys [261]. In girls, increases in adiposity during puberty occurs earlier and at a greater rate than lean mass accrual [264], and this change in body composition may not be captured by BMI. Given the possible earlier maturation in children with obesity, adiposity could be underestimated by examining BMI Z-score alone and represents an important limitation. In addition, clear biological differences between sexes exist in the development of fat patterning, association between adiposity and health risks, and in the effectiveness of interventions [265]. The importance of the confounding effects of sex distribution and maturation stage on body composition must be considered in accurate diagnosis of childhood obesity.

This study is not without limitations. Dietary intakes assessed using self-reported tools are prone to respondent bias; the extent of under-reporting is also greater in children as relative adiposity increases [266, 267]. While using proxy parental reports in part overcame this

limitation, the majority (90%) of the parents were overweight or obese and may underestimate food intake of the child [268]. However, total energy intake exceeded estimated needs and thus underreporting was not likely a major limitation. More importantly, 3-d food diaries have limited usefulness in capturing the "true" average intake for nutrients (e.g. EPA and DHA) that are provided in high amounts by a very small number of foods. The relatively small number of dietary recalls collected may result in a temporal disconnect with the timeframe that the biomarker reflects, contributing to the high variability in estimated intake values. This may in part explain the lack of statistically significant differences in LCPUFA intake across tertiles despite the lower status observed with greater adiposity. Inaccuracies and variations in fatty acid composition database (e.g. farmed versus wild fish species, seafood variety, cooking method), and exclusion of EPA+DHA from enriched foods further limits accuracy in estimating LCPUFA intake. The assessment of Tanner stages was performed by parents and subject to bias. Early true breast tissue is difficult to determine distinguish from fat tissue in overweight girls, similarly, the assessment of maturity status in boys is based on testicular size rather than pubertal hair growth. Measurements of sex steroids and gonadotropins would provide a more objective indication of pubertal onset. High-income households were disproportionately higher in this study; generalization of findings should therefore be cautioned, as prevalence of childhood obesity is often associated with low socioeconomic status (employment, family income and education) [269]. Finally, this study is cross-sectional and described the dietary patterns of the children at one point in time, but still does not answer the question about the etiology of obesity.

Children with obesity in this study consumed typical healthy amounts of dietary fat as part of the total diet but had suboptimal fish and EPA+DHA intakes relative to national guidelines [270] and recommendations by regulatory committees including the Food and

Agricultural Organization of the United Nations [271] and the European Food Safety Authority [272] of 200-250 mg/d. The proportions of EPA and DHA in RBC membranes reflected dietary omega-3 LCPUFA and fish intake, and can serve as a useful proxy for dietary recall methods. Excess adiposity along with poor dietary patterns in children with obesity, however, hinder the potential for reaching optimal blood omega-3 LCPUFA levels that confer benefits to body weight and metabolic regulation. This study provided unique information on fatty acid status based on biological markers in children and its association with adiposity assessed with densitometry-based methodology (DXA), representing a more sensitive measure of obesity compared to BMI. The results provide further support for increasing habitual consumption of fish and omega-3 LCPUFA as part of targeted obesity prevention efforts in children.

### 3.6 Acknowledgments

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**Supplementary Table S3.1** The Eating Well with Canada's Food Guide (CFG) 2007 and US Dietary Guidelines for Americans (DGA) 2015-2020 and MyPlate servings sizes according to the CFG food groups

| **Vegetables & Fruits** | **CFG 2007** | **US DGA 2015 / MyPlate** |
|---|---|---|
| Recommendation | 4-8 y: 5 servings; 9-13 y: 6 servings | Vegetables: 4-8 y: 1 ½ cup; 9-13 y: 2 – 2 ½ cups<br>Fruits: 4-8y: 1- ½ cups; 9-13 y: 1 ½ cups |
| Food item | Equivalent Serving | |
| Vegetables, fresh, frozen, canned | 125 mL, ½ cup | 1 cup |
| Vegetables, leafy, raw | 250 mL, 1 cup | 2 cups |
| Fruits, fresh, frozen, canned | 1 fruit, ½ cup | 1 cup |
| Juice, 100% fruit | 125 mL, ½ cup | 1 cup |

| **Grain Products** | **CFG 2007** | **US DGA 2015 / MyPlate** |
|---|---|---|
| Recommendation | 4-8 y: 4 servings; 9-13 y: 6 servings | 4-8 y: 5 oz equivalent; 9-13 y: 5-6 oz equivalent |
| Food item | Equivalent Serving | |
| Bread, slice | 35 g | 1 slice |
| Bagel | 45 g | 1" mini bagel, ¼ large |
| Rice, quinoa, cooked | 125 mL, ½ cup | ½ cup |
| Cereal, cold, ready to eat | 30 g | 1 cup, flakes or rounds |
| Pasta, cooked | 125 mL, ½ cup | ½ cup |

| **Milk & Alternatives** | **CFG 2007** | **DGA 2015 / MyPlate** |
|---|---|---|
| Recommendation | 4-8 y: 2 servings; 9-13 y: 3-4 servings | 4-8 y: 2 ½ cups; 9-13 y: 3 cups |
| Food item | Equivalent Serving | |
| Milk, skim, 1%, 2%, while | 250 mL, 1 cup | 1 cup |
| Cheese (e.g. Cheddar, Swiss) | 50 g | 1 ½ oz |
| Yogurt, plain and flavored | 175 g, ¾ cup | 8 fl. oz |
| Soy beverage, calcium-fortified | 250 mL, 1cup | 1 cup |

| **Meat & Alternatives** | **CFG 2007** | **DGA 2015 / MyPlate** |
|---|---|---|
| Recommendation | 4-8 y: 1 serving; 9-13 y: 1-2 servings | 4-8 y: 4 oz equivalent; 9-13 y: 5 oz equivalent |
| Food item | Equivalent Serving | |
| Fish, shellfish, cooked | 75 g, ½ cup | 1 oz |
| Poultry, lean meat, cooked | 75 g, ½ cup | 1 oz |
| Legumes, cooked | 175 mL, ¾ cup | ¼ cup |
| Tofu | 150 g, 175 mL, ¾ cup | 2 oz, ¼ cup |
| Eggs | 2 eggs | 1 egg |
| Peanut, or other nut butter | 30 mL, 2 tbsp | 1 tbsp |

**Supplementary Table S3.2** Dietary intakes and Canada's Food Guide food groups by tertiles of % body fat assessed by 3-d food diaries.

| | Females | | | | | | Males | | | | | | Overall | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st tertile, n=10 (29.0-35.9%) | | 2nd tertile, n=10 (36.0-40.9%) | | 3rd tertile, n=10 (41.0-47.2%) | | 1st tertile, n=11 (25.7-32.2%) | | 2nd tertile, n=11 (32.3-38.7%) | | 3rd tertile, n=11 (38.8-48.7%) | | 1st tertile, n=21 (25.7-37.1%) | | 2nd tertile, n=21 (32.9-40.8%) | | 3rd tertile, n=21 (35.0-48.7%) | |
| Intake/d | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| Energy (kcal) | 1943 | 193 | 1889 | 231 | 1683 | 218 | 1965 | 201 | 1954 | 247 | 2186 | 231 | 1954 | 165 | 1922 | 212 | 1935 | 194 |
| (kcal/kg) | 44.27 | 4.91 | 32.07 | 5.93 | 31.73 | 5.53 | 41.64 | 5.24 | 33.68 | 6.86 | 34.77 | 5.82 | 43.00 | 4.34 | 32.87 | 5.71 | 33.25 | 4.91 |
| Carbohydrates (g) | 264.99 | 25.75 | 271.62 | 28.55 | 234.77 | 27.19 | 271.31 | 26.55 | 294.63 | 33.23 | 297.47 | 27.19 | 268.15 | 20.76 | 283.12 | 26.59 | 266.12 | 21.49 |
| % total kcal | 53.9 | 1.8 | 55.7 | 2.0 | 54.7 | 2.0 | 54.1 | 1.9 | 58.8 | 2.4 | 53.6 | 1.9 | 54.0 | 1.5 | 57.3 | 1.9 | 54.1 | 1.5 |
| Dietary fiber (g) | 17.7 | 2.4 | 25.3 | 3.0 | 17.2 | 2.8 | 19.8 | 2.5 | 22.3 | 3.1 | 16.8 | 3.0 | 18.7 | 2.1 | 23.8 | 2.7 | 17.0 | 2.5 |
| g/1000 kcal | 9.3 | 1.2 | 13.7 | 1.3 | 10 | 1.3 | 10.0 | 1.2 | 10.8 | 1.5 | 7.8 | 1.3 | 9.6 | 1.0 | 12.2 | 1.2 | 9.0 | 1.0 |
| Total sugars (% kcal) | 21.0 | 2.4 | 20.3 | 3.3 | 22.2 | 2.9 | 19.6 | 2.6 | 26.1 | 3.4 | 20.9 | 3.3 | 20.3 | 2.3 | 23.2 | 3.1 | 21.6 | 2.8 |
| Protein (g) | 77.44 | 9.20 | 77.82 | 10.95 | 69.94 | 10.27 | 82.11 | 9.92 | 79.60 | 12.89 | 88.96 | 10.82 | 79.78 | 8.33 | 78.71 | 10.68 | 79.45 | 9.16 |
| g/kg | 1.71 | 0.21 | 1.35 | 0.25 | 1.34 | 0.23 | 1.71 | 0.22 | 1.48 | 0.29 | 1.44 | 0.24 | 1.71 | 0.19 | 1.41 | 0.24 | 1.39 | 0.21 |
| % total kcal | 16.2 | 1.2 | 17.3 | 1.3 | 17.0 | 1.2 | 17.7 | 1.2 | 17.1 | 1.5 | 17.4 | 1.2 | 16.9 | 0.9 | 17.2 | 1.2 | 17.1 | 1.0 |
| Fat (g) | 73.11 | 6.98 | 62.59 | 7.88 | 55.62 | 7.58 | 68.74 | 7.49 | 55.37 | 10.27 | 72.94 | 7.51 | 70.92 | 5.83 | 58.98 | 7.85 | 64.28 | 5.95 |
| % total kcal | 33.3 | 1.5 | 29.5 | 1.7 | 29.4 | 1.7 | 31.3 | 1.6 | 25.6 | 2.2 | 29.9 | 1.6 | 32.3 | 1.3 | 27.6 | 1.7 | 29.7 | 1.3 |
| SFA (g) | 21.42 | 2.62 | 20.09 | 3.17 | 17.98 | 2.97 | 22.40 | 2.74 | 19.18 | 3.36 | 25.04 | 3.18 | 21.91 | 2.24 | 19.63 | 2.90 | 21.51 | 2.66 |
| % total kcal | 9.94 | 0.81 | 989 | 0.90 | 9.44 | 0.86 | 10.09 | 0.84 | 8.98 | 1.05 | 10.43 | 0.86 | 10.02 | 0.66 | 9.43 | 0.84 | 9.94 | 0.68 |
| MUFA (g) | 19.72 | 2.77 | 19.03 | 3.36 | 16.83 | 3.16 | 17.88 | 2.89 | 14.73 | 3.55 | 21.39 | 3.37 | 18.80 | 2.37 | 16.88 | 3.07 | 19.11 | 2.82 |
| % total kcal | 9.10 | 0.66 | 9.27 | 0.73 | 9.05 | 0.69 | 8.23 | 0.68 | 6.72 | 0.85 | 8.52 | 0.69 | 8.67 | 0.53 | 8.00 | 0.68 | 8.78 | 0.55 |
| PUFA (g) | 12.80 | 1.58 | 11.50 | 1.79 | 10.00 | 1.73 | 10.59 | 1.72 | 9.47 | 2.40 | 7.80 | 1.72 | 11.69 | 1.34 | 10.49 | 1.81 | 8.90 | 1.36 |
| % total kcal | 5.71 | 0.49 | 4.59 | 0.55 | 6.24 | 0.52 | 4.79 | 0.51 | 3.56 | 0.64 | 3.77 | 0.52 | 5.25 | 0.40 | 4.07 | 0.51 | 5.01 | 0.41 |
| LA (g) | 9.74 | 1.30 | 7.59 | 1.48 | 8.93 | 1.42 | 8.39 | 1.40 | 5.83 | 1.94 | 7.77 | 1.39 | 9.06 | 1.08 | 6.71 | 1.48 | 8.35 | 1.10 |
| % total kcal | 4.38 | 0.42 | 3.46 | 0.47 | 4.89 | 0.45 | 3.79 | 0.44 | 2.62 | 0.55 | 3.10 | 0.45 | 4.08 | 0.34 | 3.04 | 0.44 | 4.00 | 0.35 |
| ALA (g) | 0.95 | 0.14 | 0.96 | 0.16 | 1.04 | 0.15 | 0.92 | 0.15 | 0.90 | 0.22 | 0.95 | 0.15 | 0.94 | 0.12 | 0.93 | 0.16 | 0.99 | 0.12 |
| % total kcal | 0.44 | 0.05 | 0.43 | 0.06 | 0.55 | 0.05 | 0.41 | 0.05 | 0.40 | 0.07 | 0.38 | 0.05 | 0.42 | 0.04 | 0.42 | 0.05 | 0.47 | 0.04 |
| EPA + DHA (mg) | (23.3, 6.3 – 43.0) | | (50.5, 34.3 -406.7) | | (29.3, 18.3 – 59.3) | | (32.3, 2.0 – 60.7) | | (54.0, 22.3 – 169.7) | | (22.3, 4.7 – 214.0) | | (28.3, 6.3-55.7) | | (54.0, 24.0-169.7) | | (24.7, 15.3-59.3) | |
| Vitamin D (IU) | 159 | 51 | 138 | 57 | 124 | 56 | 181 | 53 | 195 | 63 | 245 | 58 | 170 | 46 | 167 | 55 | 184 | 51 |
| Calcium (mg) | 950 | 158 | 841 | 182 | 801 | 175 | 1044 | 164 | 1132 | 199 | 1275 | 183 | 997 | 138 | 986 | 171 | 1038 | 156 |
| Sodium (mg) | 2755 | 279 | 2820 | 316 | 2688 | 303 | 3094 | 300 | 2827 | 415 | 3386 | 299 | 2924 | 233 | 2824 | 316 | 3037 | 237 |
| Potassium (mg) | 2454 | 380 | 2819 | 471 | 2294 | 439 | 2462 | 397 | 2992 | 487 | 2674 | 476 | 2458 | 326 | 2906 | 427 | 2484 | 399 |

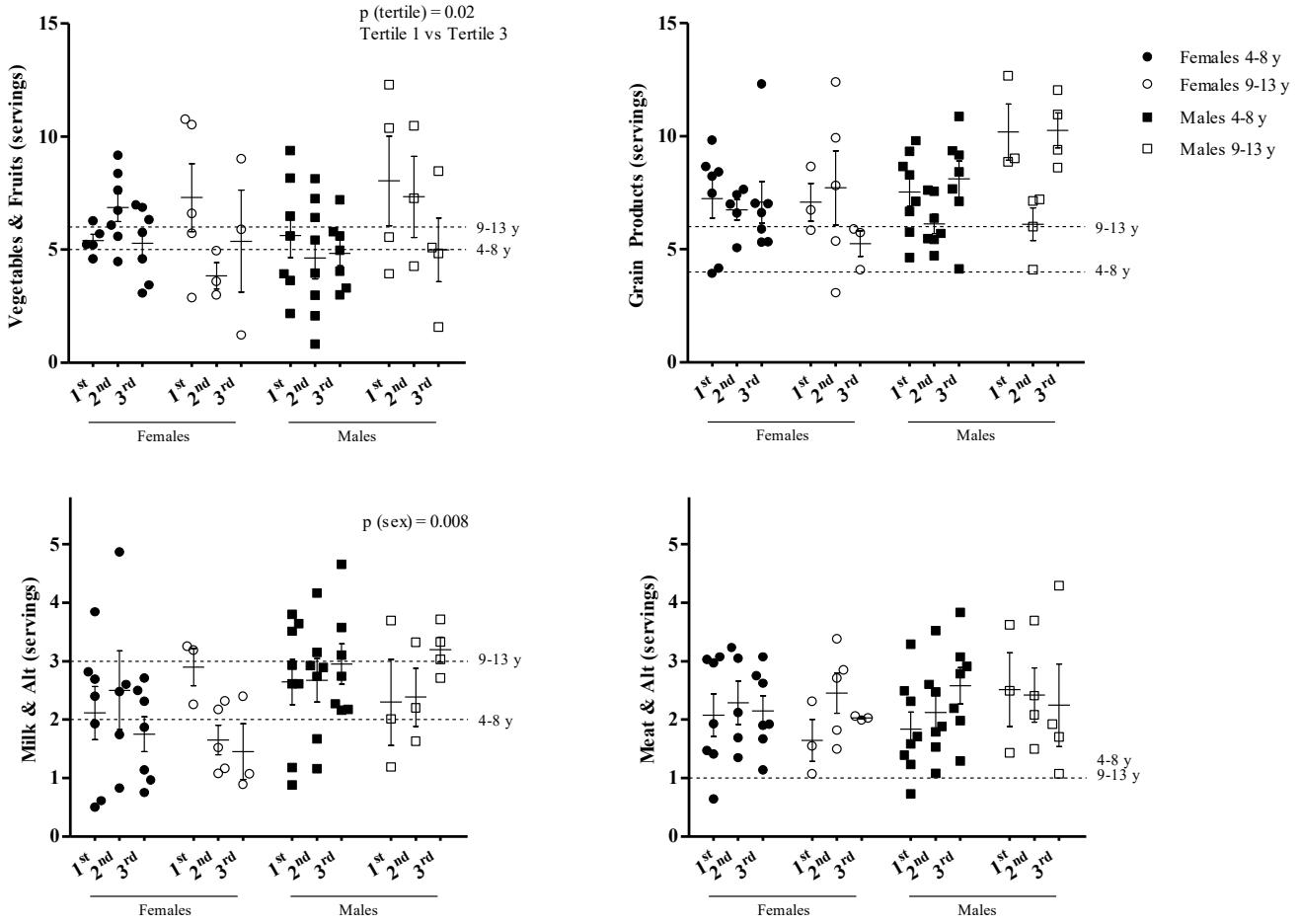
Data are presented as the average of 3-d food diaries. ALA, α-linolenic acid; DHA; docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LM, lean mass; V&F, Vegetables & Fruits. n=63. #Data presented as median, interquartile range

**Supplementary Table S3.3** Red blood cell membrane fatty acid profile of children with obesity by tertiles of % body fat

| | Females | | | | | | Males | | | | | | Overall | | | | | | Main effect | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st tertile, n=10 (29.0-35.9%) | | 2nd tertile, n=10 (36.0-40.9%) | | 3rd tertile, n=10 (41.0-47.2%) | | 1st tertile, n=11 (25.7-32.2%) | | 2nd tertile, n=11 (32.3-38.7%) | | 3rd tertile, n=11 (38.8-48.7%) | | 1st tertile, n=21 (25.7-37.1%) | | 2nd tertile, n=21 (32.9-40.8%) | | 3rd tertile, n=21 (35.0-48.7%) | | $P_{tertile}$ | $P_{sex}$ | $P_{tertile*sex}$ |
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | | | |
| C16:0 | 20.92 | 0.81 | 21.25 | 0.90 | 21.65 | 0.89 | 21.03 | 0.89 | 21.78 | 1.21 | 21.72 | 0.90 | 20.97 | 0.71 | 21.51 | 0.92 | 21.68 | 0.72 | | NS | |
| C18:0 | 18.44 | 0.80 | 19.29 | 0.90 | 19.83 | 0.88 | 18.49 | 0.88 | 18.15 | 1.20 | 19.05 | 0.89 | 18.46 | 0.70 | 18.72 | 0.91 | 19.44 | 0.71 | | NS | |
| C18:1 | 12.39 | 0.95 | 13.28 | 1.01 | 13.10 | 0.98 | 13.60 | 0.98 | 13.33 | 1.10 | 13.37 | 1.02 | 13.00 | 0.92 | 13.31 | 1.00 | 13.24 | 0.95 | | NS | |
| C18:2 n-6 | 8.74 | 0.51 | 7.91 | 0.62 | 8.36 | 0.57 | 8.43 | 0.55 | 8.04 | 0.70 | 7.62 | 0.62 | 8.59 | 0.48 | 7.97 | 0.60 | 7.99 | 0.53 | | NS | |
| C18:3 n-3 | 0.096 | 0.012 | 0.067 | 0.014 | 0.057 | 0.014 | 0.094 | 0.013 | 0.065 | 0.016 | 0.072 | 0.014 | $0.095^{a}$ | 0.011 | $0.063^{b}$ | 0.013 | $0.069^{b}$ | 0.012 | 0.03 | 0.87 | 0.82 |
| C18:3 n-6 | 0.093 | 0.009 | 0.11 | 0.011 | 0.095 | 0.10 | 0.10 | 0.01 | 0.078 | 0.013 | 0.089 | 0.01 | 0.097 | 0.008 | 0.092 | 0.010 | 0.092 | 0.008 | | NS | |
| C20:4 n-6 | 8.37 | 0.77 | 8.25 | 0.90 | 8.02 | 0.86 | 8.62 | 0.82 | 8.30 | 1.01 | 9.02 | 0.90 | 8.50 | 0.72 | 8.27 | 0.88 | 8.52 | 0.80 | | NS | |
| C20:2 n-6 | 0.27 | 0.023 | 0.26 | 0.026 | 0.28 | 0.025 | 0.29 | 0.024 | 0.25 | 0.03 | 0.25 | 0.025 | 0.28 | 0.019 | 0.235 | 0.024 | 0.27 | 0.019 | | NS | |
| C20:3 n-6 | 1.30 | 0.12 | 1.17 | 0.14 | 1.37 | 0.13 | 1.26 | 0.13 | 1.28 | 0.15 | 1.14 | 0.14 | 1.28 | 0.11 | 1.23 | 0.13 | 1.26 | 0.12 | | NS | |
| C20:5 n-3 | 0.27 | 0.051 | 0.20 | 0.054 | 0.21 | 0.054 | 0.24 | 0.052 | 0.32 | 0.059 | 0.24 | 0.055 | 0.23 | 0.043 | 0.24 | 0.048 | 0.21 | 0.046 | 0.63 | 0.03 | 0.08 |
| C22:4 n-6 | 1.81 | 0.13 | 1.89 | 0.15 | 1.69 | 0.14 | 1.91 | 0.14 | 1.77 | 0.19 | 1.92 | 0.15 | 1.86 | 0.11 | 1.83 | 0.15 | 1.81 | 0.11 | | NS | |
| C22:5 n-6 | 0.28 | 0.064 | 0.32 | 0.075 | 0.27 | 0.079 | 0.35 | 0.07 | 0.34 | 0.096 | 0.31 | 0.091 | 0.32 | 0.059 | 0.33 | 0.077 | 0.29 | 0.075 | | NS | |
| C22:5 n-3 | 1.30 | 0.15 | 1.28 | 0.17 | 1.48 | 0.16 | 1.27 | 0.15 | 1.53 | 0.19 | 1.77 | 0.16 | 1.28 | 0.12 | 1.40 | 0.15 | 1.63 | 0.12 | | NS | |
| C22:6 n-3 | 1.80 | 0.32 | 1.76 | 0.37 | 1.68 | 0.35 | 2.24 | 0.44 | 1.98 | 0.34 | 1.63 | 0.37 | 1.89 | 0.30 | 2.00 | 0.37 | 1.66 | 0.32 | | NS | |
| EPA + DHA | 2.05 | 0.35 | 1.94 | 0.40 | 1.86 | 0.39 | 2.53 | 0.48 | 2.22 | 0.37 | 1.87 | 0.40 | $2.24^{a}$ | 0.33 | $2.14^{ab}$ | 0.40 | $1.86^{b}$ | 0.35 | 0.02 | 0.56 | 0.74 |
| Total n-6 | 21.08 | 1.42 | 19.99 | 1.54 | 20.21 | 1.49 | 21.19 | 1.47 | 20.01 | 1.70 | 20.43 | 1.55 | 21.13 | 1.36 | 20.00 | 1.52 | 20.32 | 1.42 | | NS | |
| Total n-3 | 3.44 | 0.45 | 3.17 | 0.49 | 3.40 | 0.49 | 3.58 | 0.46 | 4.17 | 0.54 | 3.55 | 0.50 | 3.31 | 0.26 | 3.63 | 0.34 | 3.46 | 0.27 | | NS | |
| Total SFA | 39.35 | 1.53 | 40.54 | 1.71 | 41.48 | 1.68 | 39.51 | 1.69 | 39.94 | 2.28 | 40.78 | 1.70 | 39.43 | 1.34 | 40.24 | 1.73 | 41.13 | 1.36 | | NS | |
| Total PUFA | 24.63 | 1.80 | 23.31 | 1.93 | 23.88 | 1.88 | 24.83 | 1.85 | 24.50 | 2.12 | 24.06 | 1.93 | 24.55 | 1.68 | 23.79 | 1.88 | 23.93 | 1.75 | | NS | |
| D5D | 6.46 | 0.53 | 7.01 | 0.58 | 6.09 | 0.56 | 6.82 | 0.54 | 6.85 | 0.68 | 7.43 | 0.56 | 6.64 | 0.43 | 6.93 | 0.54 | 6.76 | 0.44 | | NS | |
| D6D | 0.012 | 0.002 | 0.013 | 0.003 | 0.013 | 0.003 | 0.014 | 0.003 | 0.013 | 0.003 | 0.014 | 0.003 | 0.013 | 0.002 | 0.013 | 0.003 | 0.013 | 0.003 | | NS | |

Total n-6 = C18:2 n-6, C18:3 n-6, C20:4 n-6, C22:4 n-6, C22:5 n-6; Total n-3 = C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3; Total SFA = C16:0, C18:0, C20:0, C22:0, C24:0; Total PUFA =Total n-6 + Total n-3. SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids. n=63

**Supplementary Figure S3.1** Canada's Food Guide food groups intakes by tertiles of % body fat assessed by 3-d food diaries. Data are mean ± SEM. n=63. The dotted lines represent recommended age-group specific serving sizes for each food group according to The Canada's Food Guide (2007). Different superscripts indicate significant differences ($p<0.05$) among tertiles



**Bridge statement 2**

The results presented in manuscript 1 highlighted the pressing issue of low n-3 LCPUFA status in children with increased adiposity, likely as a result of inadequate LCPUFA and/or fish intake. A preliminary report from the FiSK (Fish, children, health and cognition) Junior Study [260] has demonstrated that raising fish intake in children (n=199, 8-9 y) to at least 300 g per day, resulted in an absolute increase of 2.5 ± 1.5% in RBC EPA and DHA proportions over 12 wk (baseline value: 4.9 ± 1.0%). It is however, important to note that fish were provided by the researchers, hence common barriers to fish/seafood consumption including price and availability [273] have been entirely eliminated. The observations from study 1 are consistent with the literature [273-275], where tuna and processed fish (breaded or fried) are among the most popular fish/seafood choices among children with obesity. As food habits established early in life tracks into adulthood [276, 277], barriers including the lack of inspiration, cooking skills and the liking of unfamiliar foods are important considerations for devising strategies to increase fish intake in children.

As mentioned, the lack of consensus for specific dietary recommendations for n-3 LCPUFA are in part due to the scarcity of studies with functional outcomes with public health importance (i.e. CVD, cognition, behavioral and immune modulatory functions) [225]. Importantly, the potential beneficial effects of EPA and DHA intakes in children on their future risk of developing CVD have yet to been reported. A growing body of evidence are revealing a link between the common occurrence of CVD and osteoporosis with aging [278] that cannot be explained by shared common risk factors alone. It is clear that CVD starts in childhood and progresses with advancing age. Likewise, alterations in body composition during childhood has been shown to lead to a deviation from predicted bone mass tracking. An increase in % body fat

either limits the positive influence of lean mass, or results in a negative deviation from normal bone mass tracking in both sexes [279, 280]. Hence, benefits derived from early interventions that both optimize bone and cardiovascular health may extend into adulthood.

Traditional approaches (i.e. DXA) for skeletal assessments in growing children are fast, readily available, and a wealth of reference data exists for comparison. However, densitometry-based methods have neither the sensitivity nor specificity for optimal fracture risk assessment as they are unable to assess aspects of bone's complex design [281]. Evidence demonstrates that modeling adaptations occur during growth lead to important structural changes independent of bone mass that may not only determine peak bone strength (thus resistance to fracture), but also track throughout life [282]. Thus, there has been a shift toward measuring biomechanically relevant determinants (i.e. bone geometry) of bone strength in children.

The following chapter describes a cross-sectional analyses of fatty acid status in children with obesity and bone health outcomes, using a combination of DXA with pQCT, which allows for assessment of bone density and geometry in the cross-sectional plane, providing surrogate measurements of bone strength.

# CHAPTER 4

*Manuscript 2*

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# Arachidonic acid status negatively associates with forearm bone outcomes and glucose homeostasis in children with overweight condition or obesity

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## 4.1 Abstract

Long-chain polyunsaturated fatty acids are implicated in musculoskeletal health in adults. This study examined whether fatty acid status relates to bone health outcomes in children with overweigh condition or obesity (BMI Z-score 3.1 ± 0.1, 9.0 ± 0.2 y, n=108). Non-dominant forearm bone density (distal 1/3rd), geometry (4% site) and soft tissue composition (66%) were assessed using dual-energy X-ray absorptiometry and peripheral quantitative computed tomography. Red blood cell (RBC) fatty acid profile and indices of glucose homeostasis were measured. Differences in outcomes among RBC arachidonic acid (AA, C20:4 n-6) tertiles were tested using mixed model ANOVA. Ultra-distal, mid- and total- distal forearm bone mineral content, adjusted for sex, age percentage body fat, race and forearm length were 10 to 13% greater in children in the first AA tertile relative to the third. Children in the second tertile had the highest bone cross-sectional area and estimated strength at the 66% radius. Muscle cross-sectional area was 15% lower in the third tertile compared to the first, along with higher fasting insulin concentrations (27%) and HOMA-IR (31%). Higher RBC AA status aligns with deficits in forearm bone mass, geometry, and muscle mass in children with excess adiposity and early signs of insulin resistance.

## 4.2 Introduction

Childhood represents the most critical stage for developing optimal bone strength as approximately 90% of bone mineral acquisition occurs before 18 y of age [2]. While excess body weight and obesity are associated with elevated areal bone mineral density (aBMD), children with obesity (3-19 y, BMI ≥ 95$^{th}$ percentile) are presented with lower whole body bone mineral content (-9.1%, BMC) and bone area (-7.6%) relative to body weight [78], suggesting bone quality may be compromised. However, measures using peripheral quantitative computed tomography (pQCT) have shown that overweight children present with greater radial cortical cross-sectional area, but have disproportionate bone strength for the estimated load from greater body weight [85]. Hence, the increase in growth velocity in children with excess adiposity may occur at the expense of optimal mineral deposition, compromising bone quality and elevate fracture risk.

Dietary intake of long-chain polyunsaturated fatty acids (LCPUFA, ≥18C, ≥ 2 double bonds) from fish or supplemental oils has been implicated in bone health in humans and animals [283]. Data from the National Health and Nutrition Examination Survey (NHANES, 2005-2008) showed significant positive associations between omega-3 (n-3) LCPUFA supplement use and lumbar spine BMD in adults ≥ 60 y [284]. Conversely, serum linoleic acid (LA, C18:2 n-6), and total n-6: n-3 ratio negatively associated with whole body and lumbar spine BMD in healthy children (8.2 ± 0.3 y) [285]. Arachidonic acid (AA, C20:4 n-6), when consumed or present in high or disproportionate amounts, can stimulate bone resorption as a result of elevated metabolites such as prostaglandin $E_2$ ($PGE_2$) [286, 287]. These data suggest that dietary LCPUFA of the n-3 or n-6 series can differentially influence bone health and that the n-6 series might be implicated in adverse bone health outcomes.LCPUFA and their metabolites have shown

beneficial effects in the etiopathology of obesity in adults [184]. Obesity can be associated with imbalances between n-6 and n-3 LCPUFA status [60, 186], therefore having a dual impact on bone health of children. Youths who are overweight have lower docosahexaenoic acid (DHA, C22:6 n-3) but increased n-6 LCPUFA status [59, 60]. Further, adipose tissue AA proportions explained the variance in BMI (38.2%) more strongly than any other LCPUFA in pre-pubertal children (n=88, 10-12 y), with higher AA proportions in children with greater adiposity [288].

The n-6 LCPUFA are also involved in regulating adipose tissue function and the development of insulin resistance. Positive correlation between hyperinsulinemia and insulin-resistance was found with adipose AA content in otherwise healthy children (n=83, 6.2 ± 3.6 y) [289]. Obesity-related insulin resistance in children may contribute to the alterations in bone. In a recent study, higher levels of insulin resistance negatively relate to spine BMC and total body aBMD in adolescents (7-15 y) [290]. Negative associations between adiposity and trabecular bone microarchitecture were attenuated after adjusting for insulin resistance, suggesting its intermediary role between adiposity and bone [291]. Despite current knowledge of the linkages between LCPUFA, obesity and related health outcomes, few studies have examined associations between LCPUFA and the bone-fat relationship in childhood obesity. This study aimed to examine the relationships between LCPUFA status, bone mass and geometry in children with overweight condition or obesity. Based on previous reports on altered fatty acid metabolism in individuals with obesity, we hypothesized that the higher omega-6 LCPUFA status in children with overweight condition or obesity is negatively related to bone health outcomes, particularly at sites where fractures are common.

## 4.3 Materials and methods

### 4.3.1 Study population and protocol

This is a cross-sectional study using baseline data from two randomized controlled trials (Trial registration: ClinicalTrials.gov: NCT01290016). Details of the trial protocol have been previously described [239]. Healthy children with obesity (n=138, 6-12 y) living in or near Montreal, Quebec (Canada) were recruited through public and private elementary schools, primary healthcare organizations including physician referrals, word of mouth and local advertisements (newspaper, internet, radio commercials and postal mailings).

Assessments were performed at the McGill University Mary Emily Clinical Nutrition Research Unit (Ste-Anne-de-Bellevue, QC, Canada). Participants provided written informed assent by reading and signing an assent form written at the grade 1 level; parental consent was also obtained. Height was measured to the nearest 0.1 cm using a stadiometer (Seca 214, Hamburg, Germany), and weight was measured to the nearest 0.2 kg using a balance-beam scale (Detecto, Missouri, USA) to calculate BMI. BMI Z-score was derived using the World Health Organization's ANTHRO software (version 3.2.2, Switzerland, Europe). The cut-off for overweight or obese status was based on the WHO criteria of BMI for-age-and-sex Z-scores $\geq 1$ or $\geq 2$, respectively (World Health Organization, 2012). Demographics information and sun exposure were collected through a questionnaire that contains questions adapted from the Canadian Community Health Survey [292]. Physical activity data was collected using the Physical Activity Questionnaire for Children [293] by parents/guardians. The questionnaire reflected the child's physical activity level the week prior to study visit and included questions on total screen time (sedentary behavior) (time spent on television, video and computer games).

### 4.3.2 Body composition and bone assessment

Body composition and bone was examined using whole-body and non-dominant distal 1/3$^{rd}$ forearm (radius and ulna), and lumbar spine scans using DXA (Hologic QDR-4500A Discovery Series, Bedford, MA) with APEX software (version 13.3:3), for whole body lean and fat mass, as well as measurements of BMC, aBMD and bone area. The forearm was chosen as the assessment site as it represents the most common site of fractures among children [21]. The proximal femur / hip scan was not included given the skeletal landmarks for proper positioning and ROI placement are not well-developed in children [294]. BMD Z-scores were calculated using the Hologic age- and sex-specific database for children that are based on reference data from NHANES III. Quality control was performed using Hologic lumbar spine phantom (#14774) and yielded a coefficient of variation (CV%) for aBMD and BMC of <0.5%.

Peripheral quantitative computed tomography (pQCT, XCT-2000; Stratec, Pforzheim, Germany) was used to assess volumetric BMD (vBMD) and cross-sectional area (CSA) of bone, muscle and fat of the non-dominant radius. A single 2.5-mm thick slice with in-plane voxel size of 0.5 mm$^3$ was obtained at 4% and 66% proximal from the distal epiphysis of the radius. Total BMC was calculated as the product of total CSA and total vBMD [295]. The strength-strain index (SSI), a surrogate index for bone strength as a function of mass and geometry, as well as estimated load were measured at the same sites. Age- and sex-specific Z-scores were calculated for total BMC, vBMD, CSA and trabecular vBMD at the 4% distal radius, using reference data (n=371, aged 6 to 20 years, 185 males, 186 females) as described [295]. Quality assurance scans were performed prior to each visit using a hydroxyapatite cone phantom of known density and mineral content supplied by the manufacturer, accuracy was >99%.

### 4.3.3 Dietary intake

Dietary intake was assessed using 3-d food diaries completed by caregivers, as instructed by a registered dietitian. The diaries reflected 2 non-consecutive weekdays and 1 weekend day. Dietary nutrient intake was analyzed using Nutritionist Pro software (Axxya System, Stafford, TX) that uses the Canadian Nutrient File 2010b. Diets were assessed for total energy intakes (kcal/d), macronutrients and micronutrients of interest (e.g., calcium), and presented as mean of 3 days. Children were sub-divided into two groups based on life stage (4-8 and 9-13 y), and when appropriate, dietary intakes were compared with age- and sex-specific Dietary Reference Intake recommendations [296].

### 4.3.4 Fatty acid measurements

Blood was sampled (12-h fasted state) by a registered nurse and separated into plasma and red blood cells fractions, then stored immediately in 1:1 saline at -80ºC until analyses. Fatty acids were measured in RBC membranes to reflect longer term dietary consumption, as well as, tissue incorporation of fatty acids [297]. The methodology for lipid extraction, analysis and reporting of data conformed to the recent guidelines recommended by the International Society for the Study of Fatty Acids and Lipids [298]. In brief, RBC membrane fatty acids were extracted using a modified direct methylation method [245]. RBC were combined with an internal standard (heptadecanoic acid, C17:0) in toluene, fatty acid methyl esters (FAME) were prepared by acidification by acetyl chloride and precipitated with $K_2CO_3$. FAME (C14:0 to C22:6 n-3) were separated using a 60-m CP Sil-88 capillary column (Varian-Chrompack CP 7489) installed in a GC (Varian CP-3800, Walnut Creek, CA, USA) with a flame-ionization detector. Hydrogen was used as the carrier gas and nitrogen as the make-up gas. A single pooled blood sample was analyzed in triplicate at each extraction for quality control. Intra-batch extraction CV were <5%,

the inter-run CV was <8%. Recovery was determined against C17:0 standard with known concentration and found to be > 90%. Peaks were identified against standard Supleco$^{TM}$ 37 Component FAME mix (catalogue #47885-U; Sigma-Aldrich Co, St Louis, MO). Fatty acids were expressed as the weight percentage of total RBC fatty acids analyzed (C12:0 to C24:0). Individual fatty acids were summed to calculate major lipids classes of SFA (C16:0, C18:0), total n-3 (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3), total n-6 (C18:2 n-6, C18:3 n-6, C20:3 n-6, C20:4 n-6, C22:2 n-6, C22:4 n-6) and total LCPUFA.

### 4.3.5 Blood biochemistry

Fasting blood glucose, lipids (low-density lipoprotein, high-density lipoprotein, total cholesterol and triacylglycerols) and sex hormones (luteinizing hormone and estradiol) were measured in plasma, and fasting insulin in serum were measured using autoanalyzers (Beckman Access and Meckman DxC600 CA, USA) at the Montreal Children's Hospital (certified by the provincial quality assurance program, the Laboratoire de santé publique du Québec). The homeostasis model of assessment estimate of insulin resistance (HOMA-IR) was calculated as fasting glucose (mg/dL) x fasting insulin (μU/mL)/405 [299]. Plasma 25-hydroxyvitamin D (25(OH)D), parathyroid hormone, bone specific alkaline phosphatase, and osteocalcin concentrations were examined using a chemiluminescence autoanalyzer (Liaison, DiaSorin, Ontario, Canada). The laboratory participates in the Vitamin D External Quality Assessment Scheme and follows the National Institute of Standards and Technology (NIST) guidelines. The inter-assay CV% for 25(OH)D was 2%; and inter-kit CV% with NIST standards was 2.6% and 4.0% for high and low controls respectively. ELISA were used to measure levels of C-terminal telopeptide of type 1 collagen (Immnunodiagnostic Systems Inc, MD, USA) and receptor activator of NF-κB ligand (EMD Millipore, MA, USA). Adipokines (leptin and adiponectin) and

osteoprotegerin were measured in plasma using multiplex assays per the manufacturer's instructions (EMD Millipore, MA, USA). The inter-assay CV% for all assays were <10%.

Maturation status was assessed by parent's identification of physical development as per the Tanner criteria [240, 241]. Serum luteinizing hormone and estradiol concentrations were used to confirm Tanner staging.

### 4.3.6 Statistical analysis

This study uses secondary data from a randomized controlled trial [239] where the primary objective was to determine effectiveness of a 1-year family-centered lifestyle intervention on changes in BMI for-age-and-sex Z-scores in children with obesity. This paper includes 108 children; thirty children were excluded due to inadequate blood sampling for RBC membrane LCPUFA assessments. No differences in baseline characteristics were observed between children included and excluded in the analyses. Based on previous work in healthy pre-pubertal children [285], the sample size of n=108 has sufficient power to detect a correlation of at least r=0.27 between serum FA concentrations and whole body aBMD.

All data were tested for normality and homogeneity of variances with the Shapiro-Wilk test and Levene's test respectively, prior to analyses. Differences between sexes were assessed using Student's *t* tests for continuous variables; the Chi-squared test or Fisher's exact test was used to detect differences in proportions among groups for categorical variables. To determine whether individual LCPUFA were related to bone outcomes, children were first stratified by Tanner stages (stage 0-1, 2 and 3) and divided into tertiles based on RBC LCPUFA status to minimize confounding effects of maturation on bone mass.

Differences in anthropometry, bone outcomes and blood biochemistry among tertiles of FA were determined using a MIXED model ANOVA with *post-hoc* Tukey-Kramer comparison, to

ensure family-wide error rate of less than 5%. Tertile classification and Tanner stage were included as fixed effects, and time of visit (year and month) as a random effect. Covariates explored included sex, age, BMI, calcium and vitamin D intake, % body fat, race, and forearm length. This paper reports on AA tertiles as significant differences were not observed among other LCPUFA tertile categories. All tests were 2-sided with $p<0.05$. Analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

#### 4.3.7 Ethics approval

Ethics approval was obtained from the McGill University Faculty of Medicine Institutional Review Board, the Lester B. Pearson School Board and the English Montreal School Boards. McGill University Faculty of Medicine Institutional Review Board approval was granted for this secondary use of samples and data.

### 4.4 Results

#### 4.4.1 Participant characteristics

The majority (90.7%) of participants were classified as obese with BMI-for-age-and-sex Z-scores of ≥2. When children were divided by RBC AA tertiles (**Table 4.1**), no differences in age, anthropometric and body composition measurements by DXA, or demographics were observed. Inherent to the study design, the proportion of children in each Tanner stage were balanced across tertiles. There were no differences in luteinizing hormone and estradiol concentrations, nor was the proportion of each sex different across tertile groups.

Average physical activity level estimated by the physical activity questionnaire did not differ significantly among tertiles ($1^{st}$: 80.4 ± 13.3; $2^{nd}$: 82.4 ± 11.8; $3^{rd}$: 78.9 ± 11.3 minutes / day; p=0.34). Similarly, total screen time was not different among groups ($1^{st}$: 6.5 ± 0.6; $2^{nd}$: 6.5 ± 0.6; $3^{rd}$: 5.9 ± 0.7 hours/week; p=0.21).

### 4.4.2 Bone outcomes by AA tertiles

No differences were observed in whole body or lumbar spine (**Table 4.1**) bone area, BMC, aBMD or aBMD Z-score among AA tertiles. After adjusting for forearm length, race, age and sex, 1/3 ultra-distal forearm BMC and total area in children in the lowest (first) RBC AA tertile had a 5% greater projected bone area compared to the highest (third) tertile (p=0.04) (**Figure 4.1**). Similarly, BMC in the ultra-distal, mid- and total forearm were also 10-13% greater in the first tertile relative to the third. Total bone area was greatest in the second tertile (p=0.03). However, aBMD did not differ among the groups. Observations from the radius and ulna followed the same pattern hence these results are not reported in detail.

Based on pQCT assessments at the 4% radius (**Figure 4.2**), children in the first tertile had significantly greater total BMC (7%, p=0.04) and bone CSA (6%, p=0.03) than the third tertile. Total bone CSA was also lower in the third relative to the second tertile. Total BMC Z-score was approximately 2-fold greater in tertile 1 (0.08 ± 0.10) compared to tertile 3 (-0.11 ± 0.12) (p=0.01) but there were no differences in CSA Z-score. Total vBMD, trabecular vBMD or their respective Z-scores did not differ among groups at this site. At the 66% radius (**Figure 4.3**), children in the second tertile had approximately 30% greater total area (p=0.02) than those in the third tertile. Cortical bone area and area ratio were not different among groups. $SSI_{polar}$, as well as estimated fracture load, were significantly higher (p=0.03) in the second tertile relative to the third.

Evaluation of muscle and fat at the 66% radius by pQCT showed lower forearm muscle CSA in the third tertile compared to the first (**Table 4.1**). Fat CSA was also lower in the second tertile compared to both other groups (p=0.02). There were no differences in muscle density, fat-muscle density, bone-to-muscle and fat-to-muscle CSA, or forearm length among tertiles.

### 4.4.3 Serum biochemistry and RBC fatty acid status

Mean plasma 25(OH)D concentration of all children was 63.8 ± 1.8 nmol/L. No differences in sun exposure (data not shown) or vitamin D status were observed among tertiles (**Table 4.2**). Seventeen (14.3%) children had plasma 25(OH)D concentrations of ≤50 nmol/L, which is below the Institute of Medicine cutoff for sufficient vitamin D status [300], whereas n=47 (39.5%) children had 25(OH)D between 50 and 75 nmol/L. The proportion of children with vitamin D status below the healthy threshold (25(OH)D ≤50 nmol/L) were not different among AA tertiles. Fasting insulin concentrations and HOMA-IR index increased with AA tertiles, although no differences in fasting glucose concentrations were observed. Bone alkaline phosphatase concentration was approximately 15% lower in the third tertile compared to other groups. There were no significant differences in concentrations of other biomarkers of bone metabolism (bone alkaline phosphatase, receptor activator of NF-κB ligand, osteoprotegerin, C-terminal telopeptide and osteocalcin).

Fatty acid status, when expressed as a proportion of total FA in RBC, also differed across AA tertiles (**Supp Table S4.1**). Oleic acid (C18:1) was lowest in the third AA tertile, while the AA:DHA ratio decreased with increasing AA status. Total LCPUFA, total n-6 and the AA:LA ratio increased across AA tertiles. Proportions of LA, ALA and total n-3 were also greater in the second and third AA tertiles relative to the first.

### 4.4.4 Dietary intake by AA tertiles

There were no significant differences in energy or nutrient intakes amongst RBC AA tertiles based on the 3-d food diaries (n=63) (**Table 4.3**). The distribution of dietary carbohydrates, protein and fat were all within the respective Acceptable Macronutrient Distribution Ranges.

When examining the dietary data by age groups (4-8 y) and (9-13 y), the median intake of ALA was 0.73 g/d (IQR 0.51 – 0.85) and 0.74 g/d (IQR 0.49 – 0.96) respectively, which classified 78% children from both age-groups as not meeting the Adequate Intake (AI) 0.9 g/d of ALA. Similarly, intakes of LA were low across AA tertiles, with 84% of all children not meeting the AI of 10-12 g/d. Median combined intakes of EPA and DHA (25.667 mg/d, IQR 12.000 – 60.667) were also notably lower than the 250-500 mg EPA+DHA/d recommended by various expert committees [249]. Mean dietary calcium intake (948 ± 132 mg/d) was close to the EAR of 800-1100 mg/d, where 34% and 64% of children between 4-8 y and 9-13 y respectively had intakes below the EAR. Similarly, mean intakes of dietary vitamin D was low (155 ± 31 IU/d), and none of the children met the EAR of 400 IU/d.

**Table 4.1** Anthropometry and demographics by tertiles of arachidonic acid in red blood cells

| | **Tertile 1** (4.67 - 7.72%, n=36) | | **Tertile 2** (7.73 - 9.26%, n=36) | | **Tertile 3** (9.27 - 14.06%, n=36) | |
|---|---|---|---|---|---|---|
| | N | % | N | % | N | % |
| **Demographics** | | | | | | |
| Sex, male | 16 | 44.4 | 15 | 41.7 | 19 | 52.8 |
| Ethnicity, white † | 30 | 83.3 | 29 | 80.6 | 25 | 69.4 |
| Family income >$75,000 CAD/y | 21 | 58.3 | 22 | 61.1 | 20 | 55.6 |
| Parental self-repoted BMI | | | | | | |
| Mother, BMI >25 kg/m$^2$ | 22 | 61.1 | 22 | 61.1 | 18 | 50 |
| Father, BMI >25 kg/m$^2$ | 17 | 47.2 | | | | 63.9 |
| Highest household education | | | | | | |
| Elementary school | 0 | 0 | 0 | 0 | 0 | 0 |
| Secondary school | 1 | 2.8 | 2 | 5.6 | 1 | 2.8 |
| Vocational eduation, apprentice | 4 | 11.1 | 2 | 5.6 | 3 | 8.3 |
| College | 13 | 36.1 | 11 | 30.6 | 12 | 33.3 |
| University | 16 | 44.4 | 20 | 55.6 | 18 | 50.5 |
| | Mean | SEM | Mean | SEM | Mean | SEM |
| **Anthropometry** | | | | | | |
| Age (y) | 9.1 | 0.3 | 9.1 | 0.3 | 8.9 | 0.3 |
| Height (cm) | 143.3 | 1.7 | 144.0 | 1.7 | 139.9 | 1.8 |
| Age-and-sex Z-score | 0.9 | 0.1 | 1.1 | 0.1 | 0.9 | 0.1 |
| Weight (kg) | 56.2 | 2.2 | 55.4 | 2.2 | 53.5 | 2.4 |
| Age-and-sex Z-score if < 10 y* | 3.4 | 0.7 | 3.4 | 0.5 | 3.1 | 0.5 |
| BMI (kg/m$^2$) | 27.0 | 0.80 | 26.2 | 0.80 | 26.3 | 0.9 |
| Age-and-sex Z-score | 3.1 | 0.2 | 2.8 | 0.2 | 3.2 | 0.3 |
| Waist circumference (cm) | 90.5 | 2.6 | 88.4 | 2.7 | 87.0 | 2.5 |
| **DXA whole-body** | | | | | | |
| Percentage fat (%) | 38.7 | 1.3 | 37.0 | 1.4 | 39.1 | 1.3 |
| Fat mass (kg) | 21.9 | 1.3 | 20.4 | 1.3 | 21.0 | 1.2 |
| Percentage lean mass (%) | 58.5 | 1.1 | 59.9 | 1.0 | 58.9 | 1.3 |
| Lean mass (kg) | 32.7 | 1.1 | 33.3 | 1.1 | 31.1 | 1.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bone area ($cm^2$) | 1430.51 | 40.59 | 1446.60 | 39.66 | 1356.70 | 42.41 |
| BMC (kg) | 1.31 | 0.64 | 1.34 | 0.63 | 1.24 | 0.67 |
| aBMD ($g/cm^2$) | 0.904 | 0.028 | 0.910 | 0.027 | 0.919 | 0.029 |
| Age-and-sex Z-score | 1.8 | 0.3 | 2.0 | 0.3 | 1.8 | 0.3 |
| **DXA lumbar spine** | | | | | | |
| Bone area ($cm^2$) | 42.98 | 0.90 | 42.42 | 0.90 | 41.44 | 0.97 |
| BMC (g) | 29.60 | 1.10 | 30.25 | 1.09 | 27.81 | 1.18 |
| aBMD ($g/cm^2$) | 0.686 | 0.019 | 0.710 | 0.019 | 0.665 | 0.021 |
| Age-and-sex Z-score | 0.9 | 0.2 | 1.0 | 0.2 | 0.9 | 0.3 |
| **pQCT (66% radius)** | | | | | | |
| Muscle density ($mg/cm^3$) | 68.88 | 5.28 | 67.24 | 5.53 | 66.40 | 5.91 |
| Muscle CSA ($mm^2$) | $2070.20^{a}$ | 247.51 | $1991.70^{ab}$ | 261.19 | $1767.99^{b}$ | 281.30 |
| Fat CSA ($mm^2$) | $2407.88^{a}$ | 232.94 | $1954.83^{b}$ | 249.40 | $2124.72^{ab}$ | 290.15 |
| Fat-muscle density ($mg/cm^3$) | 34.2 | 3.98 | 36.48 | 4.30 | 34.33 | 4.43 |
| Bone-muscle CSA ($mm^2$) | 5.31 | 1.69 | 3.82 | 1.83 | 4.78 | 2.03 |
| Fat-to-muscle CSA ratio | 102.99 | 15.35 | 90.51 | 16.62 | 95.75 | 18.46 |
| Forearm length (cm) | 22.64 | 0.75 | 22.65 | 0.74 | 22.35 | 0.77 |

aBMD, areal bone mineral density; BMI, body mass index; BMC, bone mineral content; DXA, dual-energy X-ray absorptiometry; CSA, cross-sectional area; pQCT, peripheral quantitative computed tomography. Within row, mean values with different superscripts indicate significant differences across AA tertiles ($p<0.05$). *weight Z-score are calculated for children <10y only; n=24 for tertile 1, n=24 for tertile 2 and n=25 for tertile 3. [†]non-white races include Black, African American, Hispanic, Latino, First Nations, Asian, Native Hawaiian / Pacific Islanders and others

**Table 4.2** Blood biochemistry by tertiles of arachidonic acid in red blood cells

| | **Tertile 1** (4.67 – 7.58%, n=36) | | **Tertile 2** (7.59 – 8.85%, n=36) | | **Tertile 3** (8.86 – 14.06%, n=36) | |
|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM |
| **Lipids** | | | | | | |
| Total cholesterol (mmol/L) | 4.17 | 0.28 | 4.07 | 0.25 | 4.41 | 0.26 |
| High-density lipoprotein (mmol/L) | 1.16 | 0.09 | 1.20 | 0.08 | 1.25 | 0.08 |
| Low-density lipoprotein (mmol/L) | 2.42 | 0.28 | 2.46 | 0.23 | 2.77 | 0.24 |
| Triacylglycerols (mmol/L) | 1.23 | 0.22 | 0.89 | 0.20 | 0.87 | 0.20 |
| **Glucose metabolism** | | | | | | |
| Glucose (mmol/L) | 4.9 | 0.1 | 5.0 | 0.1 | 4.9 | 0.1 |
| Insulin (pmol/L) | 7.7[a] | 1.1 | 9.1[ab] | 0.9 | 10.2[b] | 1.0 |
| HOMA-IR | 1.0[a] | 0.1 | 1.1[b] | 0.1 | 1.4[c] | 0.1 |
| **Adipokines** | | | | | | |
| Leptin (μg/L) | 20.1 | 2.9 | 16.1 | 2.5 | 17.3 | 2.7 |
| Adiponectin (μg/L ) | 10.6 | 4.0 | 9.3 | 4.1 | 14.8 | 4.7 |
| **Bone metabolism** | | | | | | |
| 25-hydroxy vitamin D (nmol/L) | 64.1 | 5.7 | 58.3 | 5.7 | 56.8 | 5.4 |
| Parathyoid hormone (pmol/L) | 2.0 | 0.13 | 1.77 | 0.14 | 1.80 | 0.15 |
| Bone alkaline phosphatase (μg/L) | 93.6[a] | 6.3 | 89.3[a] | 6.6 | 76.6[b] | 5.9 |
| RANKL (pg/mL) | 101.9 | 30.2 | 78.0 | 53.0 | 104.1 | 38.2 |
| Osteoprotegerin (pg/mL) | 234.8 | 25.6 | 214.1 | 45.8 | 200.3 | 33.09 |
| CTx (ng/L) | 1532.7 | 86.4 | 1781.1 | 108.5 | 1486.7 | 79.9 |
| Osteocalcin (μg/L) | 55.9 | 7.6 | 60.0 | 7.3 | 50.9 | 7.5 |

CTx, C-terminal telopeptide of type 1 collagen; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; RANKL, receptor activator of NFκB ligand. Fasting glucose and CTx were measured in serum, while all other biomarkesr were measured in plasma. Within row mean values with different superscripts indicate significant differences across AA tertiles ($p<0.05$)

**Table 4.3** Dietary intake assessed by 3-day food diaries by tertiles of arachidonic acid in red blood cells

| | **Tertile 1** (4.67 – 7.72%, n=23) | | **Tertile 2** (7.73-9.26%, n=20) | | **Tertile 3** (9.27 – 14.06%, n=20) | | **DRI recommendation** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Energy (kcal) | 1559 | 139 | 1599 | 113 | 1590 | 150 | |
| Protein (g) | 65.59 | 5.4 | 62.93 | 4.61 | 63.28 | 5.87 | RDA: 19 g (4-8 y); 34 g (9-13 y) |
| g/kg | 1.39 | 0.18 | 1.41 | 0.15 | 1.37 | 0.20 | |
| % total kcal | 17.31 | 1.30 | 17.54 | 1.05 | 16.72 | 1.40 | AMDR: 10-30% |
| Carbohydrate (g) | 217.36 | 21.95 | 227.92 | 17.80 | 221.80 | 23.56 | RDA: 130 g |
| % total kcal | 55.74 | 2.22 | 53.56 | 1.85 | 54.45 | 2.35 | AMDR: 45-65% |
| Total fiber (g) | 13.0 | 1.7 | 16.4 | 1.4 | 18.1 | 1.9 | AI: 25 g (4-8 y); 31 g (9-13y) |
| Fat (g) | 49.68 | 6.64 | 60.74 | 5.52 | 55.89 | 7.19 | |
| % total kcal | 28.33 | 2.28 | 32.01 | 1.94 | 31.84 | 2.55 | AMDR: 25-35% |
| SFA (% kcal) | 9.29 | 1.02 | 9.64 | 0.85 | 9.82 | 1.14 | |
| MUFA (% kcal) | 8.50 | 0.85 | 8.76 | 0.70 | 8.99 | 0.92 | |
| LCPUFA (% kcal) | 3.40 | 0.73 | 4.59 | 0.57 | 5.06 | 0.76 | |
| ALA (g) | 0.752 | 0.117 | 0.816 | 0.092 | 0.914 | 0.129 | AI: 0.9 g (4-8 y); 1.0 -1.2 g (9-13 y) |
| % total kcal | 0.41 | 0.06 | 0.42 | 0.05 | 0.50 | 0.06 | AMDR: 0.6 – 1.2% |
| LA (g) | 6.363 | 1.407 | 6.887 | 1.171 | 6.818 | 1.563 | AI: 10 g (4-8 y); 10-12 g (9-13 y) |
| % total kcal | 3.06 | 0.59 | 3.61 | 0.50 | 3.93 | 0.65 | AMDR: 5 – 10% |
| EPA + DHA (mg)[#] | 18.3, 6.3 – 39.0 | | 34.5, 13.3 – 103.3 | | 25.2, 15.5 – 72.5 | | |
| Calcium (mg) | 948 | 132 | 897 | 104 | 803 | 138 | EAR: 800 mg (4-8 y); 1100 mg (9-13y) |
| Vitamin D (IU) | 155 | 31 | 147 | 25 | 120 | 33 | EAR: 400 IU |

AI, Adequate Intake; AMDR, Acceptable Macronutrient Distribution Range; DHA, docosahexaenoic acid; DRI, Dietary Reference Intakes; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; RDA, Recommended Dietary Allowances; SFA, saturated fatty acids. [#] Data presented as mean, interquartile range

**Figure 4.1** Differences in DXA measurements of non-dominant forearm bone area, bone mineral content (BMC) and areal bone mineral density (aBMD) among tertiles of arachidonic acid in red blood cells. Mean ± SEM. n=108. Different superscripts indicate significant differences ($p<0.05$) among tertiles after adjustments for forearm length and race. UD, ultra-distal; Mid, mid-forearm; 1/3 Dist, distal 1/3rd epiphysis

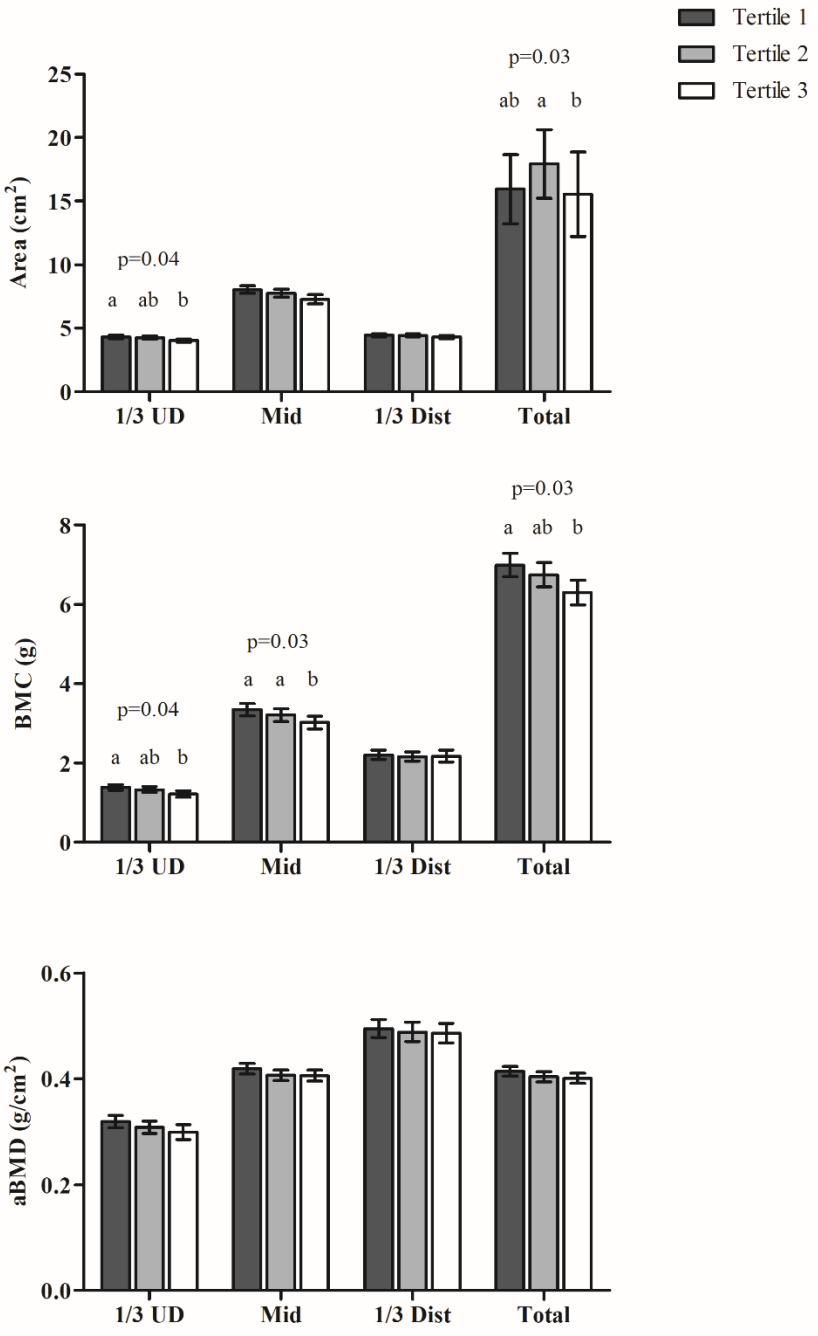


**Figure 4.2** pQCT measurements of total bone mass and geometry at the non-dominant 4% radius (metaphysis) among tertiles of arachidonic acid in red blood cells. Mean ± SEM. n=108. Different superscripts indicate significant differences ($p<0.05$) among tertiles after adjustments for sex, age, forearm length and race. BMC, bone mineral content; CSA, cross-sectional area; vBMD, volumetric bone mineral density. Z-scores were calculated using reference data collected from a population of healthy Caucasian children and adolescents

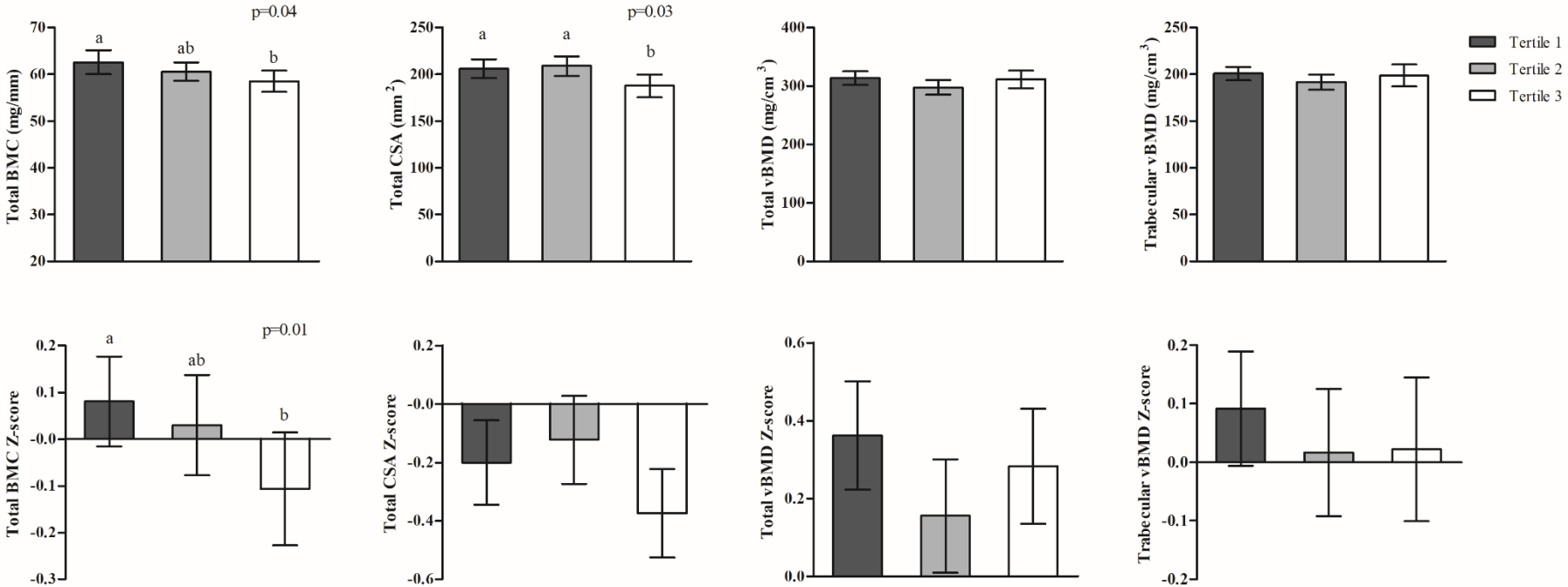
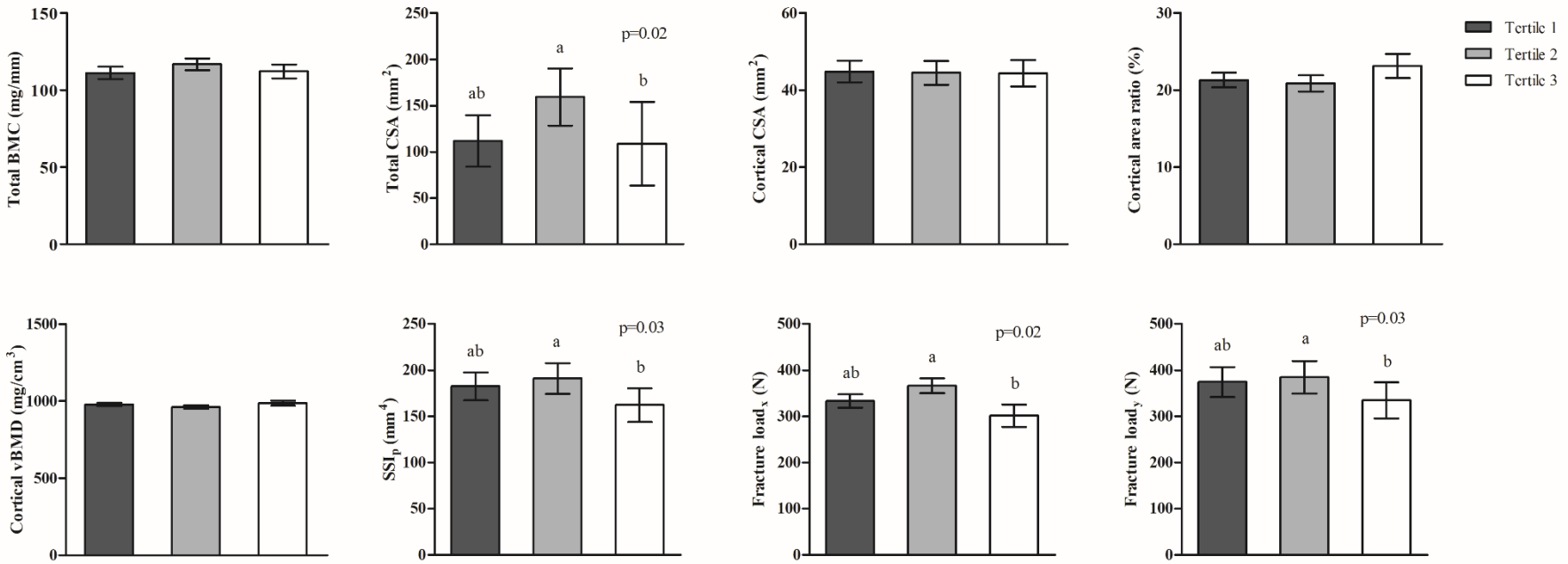


**Figure 4.3** pQCT measurements of total bone mass and geometry at the non-dominant 66% radius (diaphysis) among tertiles of arachidonic acid in red blood cells. Mean ± SEM. n=108. Different superscripts indicate significant differences ($p<0.05$) among tertiles after adjustments for sex, age, forearm length and race. BMC, bone mineral content; CSA, cross-sectional area; $SSI_p$: strength-strain index (polar); vBMD, volumetric bone mineral density



## 4.5 Discussion

Obesity is now recognized to negatively impact LCPUFA status and the bone-fat interaction [59, 186]. This study showed that children with the highest RBC AA status had deficits in forearm bone mass, geometry and muscle mass, despite no difference in whole body fat mass or aBMD among tertiles. In addition, AA status positively associated with biomarkers of insulin resistance. The observed bone outcomes may be attributed to direct effects of LCPUFA on bone growth, the etiopathology of obesity, and obesity-related metabolic consequences including impaired glucose homeostasis.

Few studies have examined the relationships between LCPUFA intakes or status and bone mass in children and the existing literature remain inconclusive. This study showed that while moderate AA levels in RBC are related to greater bone mineral content and CSA, higher amounts of AA may not confer additional benefits. In a cross-sectional study in children (8.2 ± 0.3 y) of healthy weights, serum phospholipid AA proportion positively correlated with whole body BMC (r=0.23), aBMD (r=0.33) and BMD Z-score (r=0.32) [285]. Positive associations between the AA:LA ratio and whole body BMD Z-score (r=0.35), as well as the AA: dihomo-γ-linoleic acid ratio with whole body BMC, aBMD, BMD Z-score and femur aBMD were also reported [285]. Although higher RBC AA did not associate with greater whole body assessments of bone health in the present report, it did align with compromised outcomes at the forearm including $SSI_p$. In addition, while the magnitude of differences in forearm BMC observed in this study were modest (10-13%), others have only observed a 4-6% deficit in ultra-distal radius aBMD in normal-weight children who presented with forearm fractures compared to controls [279]. Retrospective pQCT assessment of children (5-16 y) with recent (1 month) forearm fracture showed 3.4% and 0.9% lower vBMD at the 4% and 20% sites of the radius respectively [301]. Unlike adults, most

fractures in children are a result of traumatic force from falls rather than fragility fractures. The upper limb is also the most common site of fracture in children compared to the hip or spine often seen in older individuals [22]. While we did not prospectively assessed for fracture risk, women with overweight body mass indices (n=469, 66-96 y, BMI 26.3 ± 4.2) in the second tertile of plasma phospholipid n-6 LCPUFA and AA had the highest fracture risk (HR [95% CI]: 1.43 [1.10, 1.85] and 1.42 [1.09, 1.85], respectively) compared to the first and third tertiles [302]. Hence, even a slight deficit in bone mass at the distal forearm, along with poor balance, physical inactivity and excess adiposity, may predispose children with obesity to significantly greater fracture risks.

Consistent with observations in bone health outcomes, children with higher AA status showed higher fasting insulin concentrations and HOMA-IR. Positive associations between adipose tissue AA content and glycemia (r=0.37), hyperinsulinemia (r=0.47), and HOMA-IR (r=0.47), after adjustments for age and weight have been reported in healthy weight children (n=83, 6.2 ± 3.6 y, 90% male) [289]. Since Δ6 desaturase activity is stimulated by insulin [303], the higher AA proportions in RBC could be a consequence of greater n-6 LCPUFA synthesis from LA. However, our observations may not be related to increased Δ6 desaturase activity, as RBC LA proportions also increased across AA tertiles despite similar ranges in dietary LA intake. Nonetheless, the design of the current study precludes any conclusion to be drawn with respect to causality or reverse causality between insulin concentrations and AA status.

Individuals with obesity have greater absolute muscle mass that could benefit bone strength through regional muscle contractions [304]. Children in tertiles 2 and 3 of RBC AA had significantly lower forearm muscle CSA, suggesting the deficits observed in cortical bone size and BMC could be related to lack of mechanical stimulation at that site. Further, the relationship

between AA and bone outcomes were slightly weaker when muscle mass at the distal radius was accounted for reflecting an association mediated, at least in part, through lean mass. Recent study in children (9-13 y) reported positive relationships between whole body lean mass, insulin-like growth factor-I and cortical bone outcomes, yet the beneficial effects were moderated depending on HOMA-IR ($\beta$=0.20 vs $\beta$=0.41 for high and normal HOMA-IR respectively) [305]. Overall, the positive influence of muscle on bone may be attenuated with increasing levels of insulin resistance in children in tertile 3.

The ranges of RBC AA proportion across tertiles is unlikely to be explained by differences in dietary AA intake. Although information on dietary AA intake was not available in our dietary analysis software, the contribution of AA from diet is relatively minor in children, averaging about 61.7 mg/d (0.04% energy intake) [219]. There are few foods in which AA constitutes more than 2-6% of total FA by weight, and these foods, including egg yolk, beef liver, mackerel and whale oils [306], do not contribute substantially to the diets of the average Canadian children. Interactions between dietary EPA and DHA, sex, and AA status have been reported in older adults; plasma AA positively associated with BMD in women with high EPA and DHA intakes, while a negative association with BMD was seen men with the lowest intakes of EPA+DHA [44]. However, no differences in dietary EPA+DHA or total n-3 LCPUFA were observed in the present work; and while the proportions of other FA in RBC including LA and DHA (**Supp Table S4.1**) differed across AA tertiles, similar results in forearm bone mass were not found when participants were divided into groups by other FA tertiles (**Supp Table S4.2-S4.5**), highlighting the role of AA in relation to our observations.

The inclusion of children with obesity represents one of the limitations of the present study, as it precludes us from determining how much our study population deviates from children with

normal BMI. This was mitigated in part by use of Z-scores where possible as derived from healthy populations. The relationships reported between LCPUFA and bone cannot be extrapolated to normal weight children, given children with excess adiposity exhibit different patterns in LCPUFA metabolism [56, 307]. In addition, the cross-sectional analyses cannot capture the unique attributes of pubertal maturation over time. Children with obesity can enter puberty earlier, and often presented with more advanced bone age compared to age-matched normal weight peers [308]. Transient fluctuations in insulin sensitivity occurs during puberty and does so irrespective of adiposity [309, 310]. However, the current analyses included only children from Tanner stages 1-3 and, were performed stratified by maturation status; hence the influence of puberty should be minimized. Utilization of pQCT to complement DXA lessened the potential errors in quantifying bone size and mineral content in growing bones based on projection images by DXA. It is possible that non-linear trends exist between fatty acid status and obesity-related consequences [311]. However, the relatively small sample size available in the current study limited the ability to generate a non-linear model. Furthermore, measurement of $PGE_2$ was not available in the current study. A biphasic response of bone to $PGE_2$ have been reported, showing an initial stimulatory effect at lower normal concentrations, but suppressed bone formation at higher concentrations [312]. Our observations in forearm BMC support the hypothesis that the negative relationship with AA involve $PGE_2$-related mechanisms. Whether an optimal level of n-6 LCPUFA or AA intake exist to support growth needs to be clarified. Finally, physical activity levels of children in the current analysis were collected through a parent-reported questionnaire and were most likely overestimated; 47% of children had physical activity levels that met the Canadian Physical Activities Guidelines of 60 minutes per day. This is substantially higher than the Canadian National Survey that suggested only 10% of children with

obesity met the recommendation [313]. Poor agreement was observed between a physical activity questionnaire and accelerometers in children with severe obesity [314]. The appropriateness of using questionnaires to estimate physical activity in children with obesity needs to be further validated.

This study described the relationship between RBC AA status using a well-accepted biomarker of LCPUFA status and forearm bone mass and geometry in children with excess adiposity. Those with higher AA status presented with lower bone mass and size and accordingly not vBMD. Additionally, fasting insulin concentrations and HOMA-IR were higher with increasing AA status despite no differences in fat mass. Whether these observations are mediated independently by AA through distinct pathways, or an interplay exists between LCPUFA signaling, other aspects of body composition and glucose metabolism is not clear. Given the prominent roles of LCPUFA in modulating both bone growth and obesity-related metabolic consequences, further clarification of the implication in the bone-fat relationship is warranted.

**4.6 Acknowledgments**

The authors would like to thank Dr. Tom J Hazell, PhD, Sarah-Eve Loiselle, MSc, RD and Popi Kasvis, MSc, RD for their work in conducting of the study and data collection, as well as Sherry Agellon, MSc for her technical assistance with biochemical assays.

**Supplementary Table S4.1** Red blood cells fatty acid proportions in children with obesity by tertiles of arachidonic acid (AA, C20:4 n-6) % in red blood cells

| | **Tertile 1** (4.67 – 7.51%, n=36) | | **Tertile 2** (7.52 – 8.42%, n=36) | | **Tertile 3** (8.44 – 14.06%, n=36) | | **$P_{tertile}$** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Palmitate acid (C16:0) | 18.39 | 0.82 | 18.52 | 0.88 | 19.21 | 0.76 | 0.10 |
| Stearic acid (C18:0) | 17.33 | 0.45 | 16.59 | 0.46 | 16.30 | 0.48 | 0.85 |
| Oleic acid (C18:1) | 12.23[a] | 0.37 | 11.57[ab] | 0.36 | 10.79[b] | 0.36 | 0.009 |
| Linoleic acid (C18:2 n-6) | 7.07[a] | 0.20 | 7.85[b] | 0.21 | 8.10[b] | 0.22 | 0.007 |
| α-linolenic acid (C18:3 n-3) | 0.062[a] | 0.009 | 0.078[b] | 0.009 | 0.079[b] | 0.009 | 0.01 |
| γ-linolenic acid (C18:3 n-6) | 0.16 | 0.12 | 0.12 | 0.11 | 0.08 | 0.12 | 0.92 |
| Dihomo-γ-linolenic acid (C20:3 n-6) | 1.10 | 0.11 | 1.23 | 0.11 | 1.25 | 0.12 | 0.50 |
| Arachidonic acid (C20:4 n-6) | 6.50[a] | 0.17 | 8.29[b] | 0.16 | 9.94[c] | 0.17 | <.0001 |
| Eicosapentaenoic acid (C20:5 n-3) | 0.20 | 0.03 | 0.22 | 0.03 | 0.25 | 0.03 | 0.17 |
| Docosapentaenoic acid (C22:5 n-3) | 0.98 | 0.15 | 1.14 | 0.14 | 1.22 | 0.15 | 0.85 |
| Docosahexaenoic acid (C22:6 n-3) | 1.39[a] | 0.16 | 1.79[ab] | 0.18 | 2.09[b] | 0.19 | 0.003 |
| Total saturated fatty acids | 35.61 | 0.92 | 34.86 | 0.94 | 35.63 | 0.98 | 0.55 |
| Total polyunsaturated fatty acids | 19.09[a] | 0.60 | 22.79[b] | 0.65 | 25.31[c] | 0.70 | <.0001 |
| Total omega-3 | 2.53[a] | 0.26 | 3.21[b] | 0.25 | 3.15[b] | 0.28 | 0.29 |
| Total omega-6 | 16.63[a] | 0.60 | 19.65[b] | 0.67 | 21.78[c] | 0.73 | <.0001 |

Total SFA = C16:0, C18:0, C20:0; Total PUFA = C18:2 n-6, C18:3 n-3, C18:3 n-6, C20:4 n-6, C20:3 n-6, C20:5 n-3, C22:2 n-6, C22:4 n-6, C22:5 n-3, C22:6 n-3; Total omega-3 = C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3; Total omega-6 = C18:2 n-6, C18:3 n-6, C20:3 n-6, C20:4 n-6, C22:2 n-6, C22:4 n-6. Within row, mean values with different superscripts were significiantly difference across AA tertiles ($p<0.05$)

**Supplementary Table S4.2** Anthropometry and DXA measurements of the forearm by tertiles of α-linolenic acid (ALA, C18:3 n-3) % in red blood cells

| | **Tertile 1** (0.029-0.061%, n=36) | | **Tertile 2** (0.061-0.084%, n=36) | | **Tertile 3** (0.085-0.188%, n=36) | | **$P_{tertile}$** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Age (y) | 8.9 | 0.3 | 9.0 | 0.3 | 9.1 | 0.4 | 0.92 |
| Weight (kg) | 52.7 | 2.3 | 55.2 | 2.1 | 56.8 | 2.1 | 0.41 |
| BMI (kg/m$^2$) | 26.0 | 0.8 | 26.2 | 0.8 | 27.2 | 0.8 | 0.52 |
| Age-and-sex Z-score | 3.0 | 0.3 | 3.0 | 0.2 | 3.1 | 0.2 | 0.94 |
| **DXA whole body** | | | | | | | |
| Body fat (%) | 38.1 | 1.3 | 37.9 | 1.2 | 38.6 | 1.3 | 0.89 |
| Lean mass (kg) | 31.2 | 1.2 | 32.0 | 1.1 | 33.0 | 1.1 | 0.55 |
| aBMD (g/cm$^2$) | 0.948 | 0.029 | 0.941 | 0.027 | 0.966 | 0.028 | 0.67 |
| Age-and-sex Z-score | 2.3 | 0.3 | 1.9 | 0.3 | 1.8 | 0.3 | 0.37 |
| BMC (kg) | 1.39 | 0.53 | 1.38 | 0.48 | 1.40 | 0.48 | 0.95 |
| Area (cm$^2$) | 1456.37 | 36.93 | 1463.18 | 33.36 | 1488.03 | 33.48 | 0.79 |
| **DXA distal forearm** | | | | | | | |
| UD BMC (g) | 1.30 | 0.08 | 1.35 | 0.07 | 1.27 | 0.08 | 0.72 |
| MID BMC (g) | 3.08 | 0.15 | 3.17 | 0.14 | 3.27 | 0.15 | 0.52 |
| 1/3 Dist BMC (g) | 2.02 | 0.12 | 2.13 | 0.11 | 2.19 | 0.12 | 0.90 |
| Total BMC (g) | 6.50 | 0.28 | 6.68 | 0.26 | 6.80 | 0.30 | 0.65 |
| **Biochemistry** | | | | | | | |
| Glucose (mmol/L) | 4.96 | 0.11 | 5.04 | 0.11 | 4.95 | 0.11 | 0.17 |
| Insulin (pmol/L) | 62.45 | 6.98 | 60.22 | 6.40 | 64.98 | 6.84 | 0.42 |
| HOMA-IR | 1.17 | 0.13 | 1.18 | 0.12 | 1.14 | 0.14 | 0.49 |

aBMD, areal bone mineral density; BMC, bone mineral content; BMI, body mass index; Dist, distal; DXA, dual energy X-ray absorptiometry; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; UD, ultra-distal. Data were compared with MIXED model ANOVA adjusted for age, sex, race, body fat % and time of baseline visit. DXA measurement of the forearm were adjusted also for forearm length

**Supplementary Table S4.3** Anthropometry and DXA measurements of the forearm by tertiles of linoleic acid (LA, C18:2 n-6) % in red blood cells

| | **Tertile 1** (4.37-7.25%, n=36) | | **Tertile 2** (7.26-8.13%, n=36) | | **Tertile 3** (8.15-10.46%, n=36) | | **$P_{tertile}$** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Age (y) | 9.1 | 0.3 | 9.0 | 0.3 | 9.0 | 0.3 | 0.79 |
| Weight (kg) | 57.3 | 2.4 | 53.8 | 2.1 | 54.1 | 2.2 | 0.47 |
| BMI (kg/m$^2$) | 27.5 | 0.8 | 26.2 | 0.8 | 25.8 | 0.8 | 0.29 |
| Age-and-sex Z-score | 3.1 | 0.3 | 3.1 | 0.2 | 2.8 | 0.2 | 0.53 |
| **DXA whole body** | | | | | | | |
| Body fat (%) | 38.8 | 1.1 | 38.2 | 1.0 | 37.9 | 1.0 | 0.83 |
| Lean mass (kg) | 33.5 | 1.2 | 31.4 | 1.1 | 31.7 | 1.1 | 0.37 |
| aBMD (g/cm$^2$) | 0.991 | 0.033 | 0.954 | 0.031 | 0.932 | 0.030 | 0.12 |
| Age-and-sex Z-score | 2.4 | 0.3 | 1.9 | 0.3 | 1.7 | 0.3 | 0.22 |
| BMC (kg) | 1.50 | 0.70 | 1.35 | 0.62 | 1.37 | 0.58 | 0.10 |
| Area (cm$^2$) | 1506.93 | 38.49 | 1448.28 | 33.82 | 1449.39 | 34.16 | 0.43 |
| **DXA distal forearm** | | | | | | | |
| UD BMC (g) | 1.38 | 0.08 | 1.35 | 0.07 | 1.25 | 0.07 | 0.42 |
| MID BMC (g) | 3.21 | 0.17 | 3.22 | 0.16 | 3.08 | 0.15 | 0.62 |
| 1/3 Dist BMC (g) | 2.18 | 0.12 | 2.22 | 0.12 | 2.10 | 0.11 | 0.76 |
| Total BMC (g) | 6.85 | 0.31 | 6.73 | 0.30 | 6.43 | 0.27 | 0.35 |
| **Biochemistry** | | | | | | | |
| Glucose (mmol/L) | 5.11 | 0.10 | 4.82 | 0.09 | 5.02 | 0.10 | 0.71 |
| Insulin (pmol/L) | 47.78$^{a}$ | 6.59 | 49.56$^{a}$ | 6.80 | 75.41$^{b}$ | 6.10 | **0.02** |
| HOMA-IR | 1.01$^{ab}$ | 0.12 | 0.96$^{a}$ | 0.13 | 1.39$^{b}$ | 0.12 | **0.05** |

aBMD, areal bone mineral density; BMC, bone mineral content; BMI, body mass index; Dist, distal; DXA, dual energy X-ray absorptiometry; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; UD, ultra-distal. Data were compared with MIXED model ANOVA adjusted for age, sex, race, body fat % and time of baseline visit. DXA measurement of the forearm were adjusted also for forearm length. Within row mean values with different superscripts indicate significant differences across tertiles ($p<0.05$)

**Supplementary Table S4.4** Anthropometry and DXA measurements of the forearm by tertiles of eicosapentaenoic acid (EPA, C20:5 n-3) % in red blood cells

| | **Tertile 1** (0.097-0.18%, n=36) | | **Tertile 2** (0.18-0.23%, n=36) | | **Tertile 3** (0.23-0.46%, n=36) | | **$P_{tertile}$** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Age (y) | 9.0 | 0.3 | 9.0 | 0.3 | 9.0 | 0.3 | 0.37 |
| Weight (kg) | 55.0 | 2.0 | 50.3 | 2.2 | 51.0 | 2.0 | 0.33 |
| BMI (kg/m$^2$) | 26.9 | 0.8 | 27.1 | 0.8 | 25.5 | 0.8 | 0.30 |
| Age-and-sex Z-score | 3.1 | 0.2 | 3.2 | 0.3 | 2.8 | 0.2 | 0.37 |
| **DXA whole body** | | | | | | | |
| Body fat (%) | 39.5 | 1.01 | 37.9 | 1.13 | 37.2 | 1.02 | 0.24 |
| Lean mass (kg) | 31.6 | 1.05 | 35.0 | 1.16 | 30.51 | 1.06 | 0.49 |
| aBMD (g/cm$^2$) | 0.954 | 0.027 | 0.981 | 0.025 | 0.919 | 0.026 | 0.41 |
| Age-and-sex Z-score | 2.3 | 0.3 | 2.1 | 0.3 | 1.7 | 0.3 | 0.23 |
| BMC (kg) | 1.42 | 0.46 | 1.48 | 0.51 | 1.30 | 0.46 | 0.32 |
| Area (cm$^2$) | 1474.09 | 31.75 | 1537.50 | 34.95 | 1411.48 | 31.84 | 0.18 |
| **DXA distal forearm** | | | | | | | |
| UD BMC (g) | 1.33 | 0.08 | 1.38 | 0.08 | 1.26 | 0.07 | 0.45 |
| MID BMC (g) | 3.29 | 0.15 | 3.22 | 0.13 | 2.98 | 0.14 | 0.42 |
| 1/3 Dist BMC (g) | 2.21 | 0.12 | 2.18 | 0.12 | 2.12 | 0.11 | 0.87 |
| Total BMC (g) | 6.88 | 0.29 | 6.79 | 0.26 | 6.33 | 0.26 | 0.13 |
| **Biochemistry** | | | | | | | |
| Glucose (mmol/L) | 4.97 | 0.12 | 5.12 | 0.11 | 4.87 | 0.11 | 0.17 |
| Insulin (pmol/L) | 59.97 | 6.15 | 75.00 | 7.86 | 57.58 | 5.84 | 0.19 |
| HOMA-IR | 1.08 | 0.12 | 1.40 | 0.14 | 1.10 | 0.11 | 0.18 |

aBMD, areal bone mineral density; BMC, bone mineral content; BMI, body mass index; Dist, distal; DXA, dual energy X-ray absorptiometry; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; UD, ultra-distal. Data were compared with MIXED model ANOVA adjusted for age, sex, race, body fat % and time of baseline visit. DXA measurement of the forearm were adjusted also for forearm length

**Supplementary Table S4.5** Anthropometry and DXA measurements of the forearm by tertiles of docosahexaenoic acid (DHA, C22:6 n-3) % in red blood cells

| | **Tertile 1** (0.27-1.43%, n=36) | | **Tertile 2** (1.44-1.98%, n=36) | | **Tertile 3** (2.00-3.65%, n=36) | | **$P_{tertile}$** |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| Age (y) | 9.0 | 0.3 | 9.2 | 0.3 | 9.0 | 0.3 | 0.55 |
| Weight (kg) | 54.1 | 2.1 | 57.9 | 2.3 | 53.6 | 2.1 | 0.34 |
| BMI (kg/m$^2$) | 26.6 | 0.8 | 26.7 | 0.8 | 26.1 | 0.8 | 0.86 |
| Age-and-sex Z-score | 3.1 | 0.2 | 3.1 | 0.3 | 2.9 | 0.2 | 0.74 |
| **DXA whole body** | | | | | | | |
| Body fat (%) | 38.6 | 1.4 | 37.7 | 1.4 | 37.9 | 1.2 | 0.82 |
| Lean mass (kg) | 31.4 | 1.1 | 33.6 | 1.2 | 31.7 | 1.1 | 0.34 |
| aBMD (g/cm$^2$) | 0.950 | 0.028 | 0.961 | 0.030 | 0.952 | 0.026 | 0.92 |
| Age-and-sex Z-score | 2.2 | 0.3 | 2.1 | 0.3 | 1.7 | 0.3 | 0.42 |
| BMC (kg) | 1.38 | 0.48 | 1.35 | 0.53 | 1.37 | 0.49 | 0.44 |
| Area (cm$^2$) | 1446.46 | 33.84 | 1410.67 | 37.06 | 1456.87 | 33.85 | 0.40 |
| **DXA distal forearm** | | | | | | | |
| UD BMC (g) | 1.25 | 0.08 | 1.30 | 0.08 | 1.26 | 0.07 | 0.25 |
| MID BMC (g) | 3.22 | 0.16 | 3.24 | 0.15 | 3.17 | 0.13 | 0.50 |
| 1/3 Dist BMC (g) | 2.18 | 0.12 | 2.12 | 0.12 | 0.12 | 0.11 | 0.82 |
| Total BMC (g) | 6.72 | 0.31 | 6.57 | 0.30 | 6.50 | 0.26 | 0.60 |
| **Biochemistry** | | | | | | | |
| Glucose (mmol/L) | 4.95 | 0.10 | 4.98 | 0.10 | 5.04 | 0.09 | 0.74 |
| Insulin (pmol/L) | 67.36$^{a}$ | 6.85 | 61.26$^{ab}$ | 6.69 | 59.12$^{b}$ | 6.54 | **0.03** |
| HOMA-IR | 1.21 | 0.13 | 1.14 | 0.12 | 1.15 | 0.12 | 0.67 |

aBMD, areal bone mineral density; BMC, bone mineral content; BMI, body mass index; Dist, distal; DXA, dual energy X-ray absorptiometry; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; UD, ultra-distal. Data were compared with MIXED model ANOVA adjusted for age, sex, race, body fat % and time of baseline visit. DXA measurement of the forearm were adjusted also for forearm length. Within row mean values with different superscripts indicate significant differences across tertiles ($p<0.05$)

**Bridge statement 3**

The observations from manuscript 2 suggested a possible inverse relationship may exist between the proportions of arachidonic acid (AA) in red blood cells (RBC) and forearm bone health outcomes in children with obesity. Despite so, limited conclusion can be drawn regarding the influence and mechanisms by which AA modifies bone metabolism as statistical modeling in cross-sectional datasets only provide information on trend but does not imply causality. The availability of RBC fatty acid measures at baseline only limits our ability to assess how longitudinal changes in fatty acid proportions over time may affect bone outcomes. Dietary AA information was not available in study 2, hence it is uncertain to what extent does AA intake contribute to the variation in AA status in RBC, and whether AA in circulation resembles its incorporation in biological tissues. In addition, AA is found in protein-rich animal food sources, including eggs and meat. While no association was observed between intakes of Meat & Alt servings and AA status was observed, the possible confounding effect of other nutrients and/or bioactive compounds present in these foods cannot be entirely eliminiated. Finally, the etiology of obesity in children are multi-factorial; socio-economic factors, physical inactivity, access to healthy foods and family eating behaviors all contribute to the development of the habits of the child, rendering it difficult to delineate specific nutritional factors contributing to childhood obesity.

An important observation from the children recruited in the MYLIFE study is that the diets of children are not excessively high in dietary fat content. This is in line with a recent Cochrane review that reported no consistent effects between total fat intake and measures of body fatness in generally healthy children [38]. Many animal models of diet-induced obesity have utilized high-fat diets ranging from 45-70% of total energy as fat. While these diets are effective in

inducing obesity and related metabolic complications, they are not representative of the children's diet, and may only reflect the consequences seen in the most extreme cases of obesity in humans. The development of excess adiposity in children occurs gradually; a reduction in the energy gap of as little as 110-165 kcal/d, which is equivalent to one can of sugar-sweetened beverage, was estimated to be sufficient to prevent excess weight gain in children 2-7 y [315]. Adipocytes exhibit a certain extent of metabolic flexibility and are able to buffer a certain range of energy fluctuations for maintenance of whole-body energy homeostasis. It is however uncertain how much of this buffering capacity is affected by the rate and magnitude of energy surplus. The diet used in animal models of diet-induced obesity should therefore be carefully chosen to reflect the rate of obesity development in children as much as possible, given the response of adipocytes to excess energy availability may not be linear after all. Furthermore, as rodents have relatively faster growth rate and reach sexual maturity earlier compared to that of human children, it is uncertain if the initial bone adaptations such as increased bone mass and strength to a rapid increase in adiposity observed in growing rodents can be sustained in adulthood [95, 316].

The following chapter describes an animal-trial in male Sprague-Dawley rats using a moderately high-fat diet (35% total energy) with the addition of 1% AA (w/w) to investigate the effects on body composition, bone outcomes and LCPUFA status in a model of diet-induced obesity during periods of rapid growth.

# CHAPTER 5

*Manuscript 3*

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# Arachidonic acid exacerbates diet-induced obesity and reduces bone mineral content without impacting bone strength in growing male rats

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**5.1 Abstract**

Long-chain polyunsaturated fatty acids modulate bone mass and adipocyte metabolism. Arachidonic acid (AA, C20:4 n-6) is elevated in obesity and postulated to stimulate bone resorption. This study aimed to determine the effect of AA on bone mass, quality, and adiposity in diet-induced obesity during growth. Male Sprague-Dawley rats (n=42, 4-wk) were randomized into groups fed a control diet (CTRL, AIN-93G), high-fat diet (HFD, 35% kcal fat) or HFD+AA (1% w/w diet) for 6 wk. Body composition, bone mineral density and microarchitecture were measured using dual-energy x-ray absorptiometry and micro-computed tomography. Red blood cell fatty acid profile was measured with gas chromatography. Group differences were evaluated using repeated measures two-way analysis of variance with Tukey-Kramer post-hoc testing. Total energy intake did not differ among diet groups. At wk 6, HFD+AA had significantly greater body fat % (12%), body weight (6%) and serum leptin concentrations (125%) than CTRL, whereas visceral fat (mass and %, assessed with micro-computed tomography) was increased in both HFD and HFD+AA groups. HFD+AA showed reduced whole body bone mineral content and femur mid-diaphyseal cortical bone cross-sectional area than HFD and CTRL, without impairment in bone strength. Contrarily, HFD+AA had greater femur metaphyseal trabecular vBMD (29%) and bone volume fraction (20%) compared to controls. Inclusion of AA elevated leptin concentrations in male rats. The early manifestations of diet-induced obesity on bone mass were accelerated with AA. Studies of longer duration are needed to clarify the effect of AA on peak bone mass following growth cessation.

## 5.2 Introduction

The influence of obesity on development, maturation and remodeling of the skeleton is not well studied. Body weight can be a strong determinant of bone mineral density (BMD) [72]. Individuals with obesity have higher areal BMD as a result of greater mechanical loading. However, epidemiological studies reported positive associations between obesity and risk of fragility fractures [317, 318]. Inverse relationships were described between whole body fat mass and areal BMD at multiple sites in children, despite the greater lean mass in those with obesity [79], suggesting body composition is an important determinant of BMD. In addition to suboptimal physical activity and/or diet quality in obesity, a combination of mechanisms has been proposed to explain the effect of obesity on BMD and fracture risk. These include low-grade chronic inflammatory responses that favor bone resorption [107], and imbalances in the differentiation of mesenchymal stem cells to osteoblasts, chondrocytes or adipocytes [108]. These interrelated factors that contribute to increased fracture risk despite greater bone mass in obese individuals are not well-explained and warrants further clarification.

Peroxisome proliferator activator receptors (PPAR), specifically PPARγ, have a central role in mediating the balance between adipocytes and osteoblasts during stem cell differentiation [319]. Long-chain polyunsaturated fatty acids (LCPUFA) are natural ligands for PPARs and can influence gene expression of its downstream targets [320]. Increased intakes of the omega-3 LCPUFA eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), either as supplements or from fish intake are associated with higher BMD in adults [10, 321]. Conversely, studies that report on the influence of the omega-6 arachidonic acid (AA, C20:4 n-6) on bone mass are scarce. Dietary supplementation of AA elevated whole body BMC in growing piglets with healthy body composition [322]. Of relevance to growth, AA and its metabolite

prostaglandin $E_2$ demonstrated a biphasic effect on bone, showing initial stimulatory effects but suppressed bone formation at higher concentrations [287]. *In vitro*, AA (40 μM) significantly inhibited human osteoblastic cell proliferation and the osteogenic markers expression to a greater degree than EPA or DHA [323, 324]. This was accompanied by a 4 to 5-fold induction in PPARγ mRNA levels [324], although PPARγ activation with thiazolidinedione in osteoblast lineage cells resulted in terminally differentiated adipocytes [325, 326]. Hence the effects of fatty acids on bone cells and adipocytes likely involve multiple mechanisms, producing both beneficial and potentially deleterious effects on BMD depending on the extent of exposure.

The release of adipokines including leptin and adiponectin by adipose tissue is another link between obesity, LCPUFA and regulation of bone turnover through central and peripheral signaling [182]. The response of adipokine secretion to LCPUFA is varied. Studies *in vitro* show mixed effects on triglyceride accumulation and adipokine expression in 3T3-L1 adipocytes treated with LCPUFA alone or in combination. This can be attributed to different properties of fatty acids of the n-6 or n-3 series, variation in treatment concentrations, and maturation stage of the cells. [211, 327-329]. Additionally, adolescents who are obese (10.4-16.4 y, BMI 26.8 – 40.4) have lower omega-3 LCPUFA and higher AA:DHA ratio in serum phospholipids compared to lean controls [186]. Despite the potential links among LCPUFA, adiposity and bone health, the interaction among these factors is not well characterized. This study therefore utilizes a growing rat model of diet-induced obesity to determine the effects of a moderately high-fat diet, with or without the addition of AA on bone mass and LCPUFA status during growth. We hypothesize the adverse effects of diet-induced obesity on bone mass and strength during growth may be exacerbated by the addition of AA. This effect is mediated in part through promoting adipogenesis and decreasing levels of omega-3 fatty acids in circulation and tissue.

## 5.3 Methods

### 5.3.1 Animals and protocol

Weanling male Sprague Dawley rats (n=42) from separate litters were obtained at 3 wk of age and block-randomized into 1 of 3 diet groups for 6 wk after 1 wk acclimation, during which they were provided a standard Teklad AIN-93G purified diet TD.94045 (Envigo, Madison, Wisconsin USA). Only male rats were studied in view of the higher fracture rates in obese male children compared to females [330], and males are more prone to obesity-induced metabolic complications [331]. Male rodents are also preferred as they lack the potential confound of estrous-cycle associated hormonal fluctuations. Amongst all rat strains, outbred Sprague Dawley rats are the most often used rat model of diet-induced obesity [332] as well as for bone growth [333]. The Sprague-Dawley rat also offer practical advantages over mouse models as they allow for greater volumes of sample collection, and for bone imaging to be performed at relatively lower resolution hence minimizing exposure to anesthesia [334].

Rats were housed in pairs under controlled lighting (0600 to 1800 h), humidity (50%) and temperature (21-22°C). Body weight was recorded weekly. Food was provided daily to minimize LCPUFA oxidation, and food consumption determined by disappearance between feedings. Fasted blood samples (≤ 5% blood volume) were taken from the saphenous vein at baseline, midpoint and end of study. Whole blood was collected into heparinized capillary tubes for immediate measurement of ionized calcium. Plasma and serum were collected for immunoassays. Red blood cells (RBC) were stored with 1:1 normal saline and flushed with $N_2$ for fatty acid analysis. All samples were stored at -80°C immediately until analysis.

Rats were euthanized with an overdose of isoflurane and exsanguinated using cardiac puncture at necropsy. Soft tissues including the liver, quadriceps, subcutaneous (inguinal) and

visceral (epididymal) adipose tissue were weighed and flash-frozen in liquid $N_2$ for fatty acid extraction. The right femur was excised, carefully cleaned of soft tissue, and stored in saline-moistened gauze at -20°C for micro-computed tomography (μCT) scanning and biomechanical tests.

### 5.3.2 Diets

Rats were randomized into one of three diet groups: (1) standard AIN-93G growth purified diet (CTRL, Harlan Teklad Global 19% Protein Rodent Diet, Harlan Laboratories Inc., Madison, WI); (2) a moderately high-fat diet (HFD) with 35% energy from fat (lard was added as additional source of saturated fat); and (3) the same HFD with 1% w/w AA (HFD+AA) in the form of ARASCO (Martek Biosciences Corporation, MA, USA; **Table 5.1**). ARASCO contains approximately 40% AA derived from the fermentation of *Mortierella alpine*, and may contain trace amounts of C22:5 n-3 [335]. The amount of AA supplementation is based on a previous study in 6 wk old rats to double tissue enrichment of AA without causing neurotoxicity over 90 days [336]. All diets met the calcium requirement (2.5 g/kg) necessary to support normal growth in Sprague-Dawley rats [337]. The energy supplied by fat in the HFD (35%) mimics the average % fat intake of North American children and adolescents [338]. It is also within the accepted macronutrient distribution range defined in the 2015-2020 Dietary Guidelines for America recommendations for children and adolescents [339].

### 5.3.3 *In vivo* dual-energy X-ray absorptiometry

Rats were scanned under anesthesia with a fan-beam QDR-4500A densitometer (Hologic Inc., Bedford, MA) in a prone position with limbs extended at baseline, midpoint, and end of study. Prior to scanning, nose-to-rump and nose-to-tail lengths were measured. Whole body and regional (scans of the left and right femurs and tibias, and lumbar vertebrae (LV) 1 to 4) scans

were performed and analyzed using small animal software (QDR 4500A version 12.5). Quality control was performed each day of scanning using a spine phantom provided by the manufacturer and yielded a coefficient variation (CV%) of <0.5% for areal BMD (aBMD). Triplicate scans of each region of interest were done prior to necropsy and CV% for whole body and regional scans were below 5 and 7% respectively for all measurements in 10-wk-old rats.

### 5.3.4 *In vivo* micro-computed tomography

Whole body and regional bone and abdominal fat were assessed with a small laboratory animal CT system (LaTheta LCT-200; Aloka, Tokyo, Japan). Scans were performed at baseline and wk 6 only, to minimize X-ray exposure in growing rats. Whole-body and regional scans of LV2-5 were performed as previously reported [340]. Thresholds for segmenting bone from soft tissue and trabecular from cortical bone were set at 160 and 500 mg/cm$^3$ respectively; the datasets were automatically segmented by the LaTheta software (version 1.3). Similarly, distinction between lean and fat body mass was based on differences in X-ray attenuation. Visceral and subcutaneous abdominal fat depots were discriminated based on detection of the abdominal muscle wall [341] and manually corrected slice-by-slice by a single operator (I.M.). Quality control was performed each day of scanning using the manufacturer's hydroxyapatite phantom with known density. Triplicate scans of the LV2-5 were performed in 10-wk old rats prior to necropsy, and CV% for all measurements were < 2%. In addition, a 1% variability was noted between the *in vivo* and *ex vivo* (Skyscan, Belgium, Germany) μCT when cross-calibrated with the 0.75 mg/cm$^3$ phantom provided by the Skyscan manufacturer.

### 5.3.5 *Ex vivo* micro-computed tomography

*Ex vivo* microarchitecture of the right femur distal metaphysis, mid-diaphysis and LV3 were assessed using a Skyscan 1174 scanner (Skyscan, Belgium, Germany) as described [342].

An isotropic voxel size of 11.2 μm and 30.6 μm was used for trabecular and cortical regions respectively. Images were reconstructed with the manufacturer's software (NRecon v1.6.4.1; Skyscan) and segmented into bone and marrow compartments based on adaptive thresholding for trabecular bone, and fixed global threshold (120-255 gray level value) for cortical bone (CT Analyzer v1.11.8.0; Skyscan). The threshold is optimized by visually comparing the binarized images with original gray-scale images. All segmenting was performed by the same operator (I.M.).

The trabecular region at the distal femur was selected by manually contouring the region of interest (ROI) away from the endosteal surface on a slice-by-slice basis as recommended [343]. The trabecular region of interest (ROI) at the distal femur was set at a longitudinal distance of 0.8 mm proximal to the epiphysis, contained 90 slices with the same thickness (11.2 μm), reaching a total volume of interest (VOI) of approximately 1 $mm^3$. Cortical bone at the femur mid-diaphysis was analyzed on 100 slices (50 slices above and below the mid-point) and included a total VOI of 3 $mm^3$. A set of 2 hydroxyapatite phantoms (0.25 and 0.75 $mg/cm^3$) was used to calibrate and compute vBMD.

### 5.3.6 Bone strength testing

*Ex vivo* bone strength of the femur was assessed by three-point bending test with a servohydraulic material testing system (Instron version 5544; Canton, MA) as described [342]. The femur was centered between the fulcrums with a constant span length of 17 mm. Load was applied at the mid-diaphysis in the antero-posterior direction at a constant rate of 0.1 N/s until failure (defined as 35% decrease in detected load and/or until bone fractured).

### 5.3.7 Biochemistry

Biomarkers were measured in fasted blood samples. Plasma 25-hydroxyvitamin D

(25(OH)D) was assessed using a chemiluminescence autoanalyzer (Liaison, DiaSorin, Stillwater, MN). Ionized calcium was measured in heparinized capillary tubes (ABL800 FLEX analyzer, Radiometer, Copenhagen, Denmark). Serum receptor activator of nuclear factor-κB ligand (RANKL) and osteocalcin concentrations were measured using singleplex immunoassays (Milliplex, Millipore, MA, USA). Leptin and OPG were quantified using multiplex magnetic rat bone panel 1 (Milliplex, Millipore). Adiponectin (Milliplex, Millipore) insulin (Alpco, Salem, NH), bone turnover markers C-telopeptide (CTx, IDS, Bolden, UK) and procollagen Type-I N-terminal propeptide (P1NP, IDS, Bolden, UK) were measured in serum with standard ELISA methods per kit manufacturer's instructions. Adiponectin and leptin production in media by 3T3-L1 adipocytes after 48 h fatty acids treatment were measured using mouse-specific singleplex immunoassays (Milliplex, Millipore). Pooled rat serum (n=9) and culture media (n=4) were analyzed in triplicate, the CV% for all parameters (inter- and intra-assay) was less than 10% for all assays.

### 5.3.8 Fatty acid measurements in blood, tissue and 3T3-L1 adipocytes

RBC were used to measure fatty acid composition as they reflect fatty acid composition of the habitual diet [243]. A modified Lepage-Roy RBC direct methylation method [245] was used to prepare fatty acid methyl esters (FAME) for gas chromatography (GC) separation. RBC (100 µl) were combined with toluene containing 20 µg/ml C17:0 internal standard. The mixture was methylated with acetyl chloride, followed by precipitation with 6% $K_2CO_3$ and extracted with hexane for GC analysis. Fatty acids in soft tissues (subcutaneous and visceral adipose tissue, liver, quadriceps) and rodent feed pellets were extracted using the O'Fallon method [344]. Samples were hydrolyzed in KOH in methanol containing C17:0, and free fatty acids were methylated by concentrated sulfuric acid. The mixture was vortex-mixed with hexane and

collected for analyses.

Following 48 h of fatty acid treatment, 3T3-L1 adipocytes were trypsinized, washed twice in cold phosphate-buffered saline (PBS), and frozen in saline until analysis. Adipocytes were combined with freshly-prepared 1:10 acetyl-chloride:methanol with internal standards C19:1 (40 μg/ml) and C21:0 (20 μg/ml). The reaction was precipitated with NaCl, and then a second extraction was performed with hexane, and the upper hexane layer was collected [345].

FAME were separated using a 60-m CP-Sil-88 capillary column (Varian-Chrompack, CP7489), installed in a Varian CP-3800 Gas Chromatograph (Varian, Inc., Walnut Creek, CA) with a flame-ionization detector. FAME peaks on the chromatogram were identified against standard Supelco$^{TM}$ 37 Component FAME mix (Sigma-Aldrich Co, St. Louis, MO), and reference standards of known concentration were used to calculate recovery (>85%). Quality control was performed using a single pooled sample analyzed in triplicate with each extraction. The fatty acids were expressed as the weight percentage of the total fatty acids analyzed from C10:0 to DHA, the sum of fatty acids reported herein represented (93 ± 3%) of total fatty acids. Individual fatty acids were summed to calculate major lipid classes of total n-3 (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3) and total n-6 (C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C22:4 n-6, C22:5 n-6).

**5.3.9 Adipocyte differentiation and fatty acid treatment**

3T3-L1 pre-adipocytes were passaged every 2-3 days for approximately 7 days in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Sigma-Aldrich, MO, USA) in a 5% $CO_2$ humidified environment at 37°C until about 80% confluent. Pre-adipocytes were seeded on 6-well plates with a seeding density of 0.8 x $10^5$ cells/well and kept in the same growth medium until confluent. Two days

post-confluence, differentiation was induced (day 0) by DMEM/F12 supplemented with: 10% FBS, 1 μM dexamethasone (Sigma), 1.5 μg/ml insulin (Sigma), 500 μM isobutylmethylxanthine (Sigma), and 1 μM rosiglitazone (Sigma) for 3 days. After differentiation, medium was replaced with DMEM/F12 with 10% FBS, antibiotics, and 1.5 μg/ml insulin and changed every 2 days. Cells were maintained in this medium over an 8-d period, with day 8 representing mature adipocytes with lipid droplet accumulation.

Stock solutions of AA, EPA, docosapentaenoic acid (DPA, C22:5 n-3) and DHA (Nu-Chek Prep Inc., Elysian, MN) were dissolved in 100% ethanol to a final concentration of 0.1 M. Fatty acids were complexed to 1% fatty-acid free bovine serum albumin (BSA) (Sigma) in serum-free DMEM at 37°C, corresponding to a free fatty acid: BSA ratio of approximately 0.16-0.66:1. Fatty acid treatment was preceded by overnight starvation in serum-free DMEM supplemented with 1% BSA and antibiotics. Differentiated adipocytes (day 8) were then cultured for 48 h in the presence of 10, 25, 50 or 100 μM of AA, EPA, DPA and DHA, while controls were treated with BSA and 0.1% ethanol. The concentrations of fatty acids and BSA used were based on previous studies [52, 211] in 3T3-L1 adipocytes to influence adipokine secretion without causing cell toxicity. In addition, normal physiological ranges of plasma FFA levels have been reported to be around 0.5 – 2.3 mmol/L in rats [346] and 0.25-0.73 mmol/L in humans [347]. The selected concentration of fatty acids is therefore within the physiological range for free fatty acids for rodents and humans.

### 5.3.10 Nile Red staining

Nile Red staining was used to confirm adipocyte differentiation and lipid accumulation in 3T3-L1 adipocytes. Adipocytes were washed with PBS and fixed with 4% paraformaldehyde. Cells were subsequently washed twice in PBS and stained in Nile Red working solution (10

μg/mL) for 15 min, and then washed and stained again with DAPI for 5 min. Images of stained cells were taken using the EVOS FL Cell Imaging System (Invitrogen) and processed with ImageJ (NIH, Maryland, USA).

### 5.3.11 Total RNA isolation and quantitative RT-PCR measurements

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity and concentration were quantified spectrophotometrically after extraction. Total RNA was treated with DNase (New England BioLabs), cDNA was synthesized from 1 μg of total RNA using MultiScribe reverse transcriptase with RNase inhibitor (Applied Biosystems) and RT random primers. Relative gene expressions of leptin, adiponectin and PPARγ were determined with quantitative RT-PCR using SYBR Advanced qPCR Master Mix (Winsent Inc., St-Bruno, QC) with the 7500 RT-PCR System. (Applied Biosystems). HPRT was amplified in parallel as the house keeping gene. The following primers as previously described [329, 348] were used: 5'-GACCATTGTCACCAGGATCA-3' (forward) and 5'-TGAAGCCCAGGAATGAAGTC-3' (reverse) for leptin, 5'-GCACTGGCAAGTTCTACTGCAA-3' (forward) and 5'-GTAGGTGAAGAGAACGGCCTTGT-3' (reverse) for adiponectin, 5'-CTCCTGTTGACCCAGAGCAT-3' (forward) and 5'-TCTTCCATCACGGAGAGGTC-3' (reverse) for PPARγ, and 5'-GTTGAGAGATCATCTCCACC-3' (forward) and 5'-AGCGATGATGAACCAGGTTA-3' (reverse) for HPRT. All primers were validated by PCR to ensure the absence of multiple amplification products. Cycling conditions started with an initial denaturation step at 50°C for 2 min and 95°C for 3 min, amplification was performed for 40 cycles of 95°C for 10 s and 60°C for 45 s. Relative expressions of target genes were calculated using $2^{-\Delta\Delta Ct}$ method [349]. All cell treatments and PCR reactions were performed in triplicate.

### 5.3.12 Statistical analyses

Based on a previous study in 12-wk old male rats [350], a sample size of n=9 per group was estimated to detect a 10% difference for distal femur metaphysis vBMD in a two-sided t-test with $\alpha$=0.05 and power of 0.80. Using a total sample size of 42 (14 per group) provided coverage in the event of loss of an animal or smaller effect size. One rat in the HFD+AA group died during the 1-wk acclimation period, thus a total of 41 rats (control: n=14; HFD: n=14; HFD+AA: n=13) were included in this analysis. Analysis with and without the single-housed rat led to same interpretation of the results.

Data were analyzed using SAS statistical package (version 9.4, SAS Institute Inc., Cary, NC). Summary statistics were computed for all baseline characteristics to ensure treatment groups were comparable. Differences among diet groups were examined using repeated-measures MIXED model ANOVA with diet and time as fixed effects, and study block as a random effect nested within groups. Covariates explored included body weight, body composition, food intake and bone length for bone measurements (*ex vivo* μCT femur scans, biomechanics). A post-hoc Tukey-Kramer multiple comparison test was used to ensure family-wide error rate of 5%. All data was tested for normality and homogeneity of variances prior to analysis.

### 5.3.13 Ethics approval

All procedures were reviewed and approved by The Macdonald Campus Facility Animal Care Committee, which follows the guidelines established by the Canadian Council on Animal Care and the McGill University Animal Care Committee.

**Table 5.1** Diet composition

| | CTRL (AIN-93G) | HFD | HFD+AA |
|---|---|---|---|
| Energy (kcal/g) | 3.9 | 4.3 | 4.3 |
| Protein (% kcal) | 18.7 | 18.7 | 18.7 |
| Carbohydrate (% kcal) | 65.1 | 46.1 | 46.1 |
| Fat (% kcal) | 16.4 | 35.5 | 35.3 |
| **Formula (g/kg)** | | | |
| Casein | 200 | 225 | 225 |
| L-Cystine | 3 | 3.4 | 3.4 |
| Corn Starch | 397 | 277 | 277 |
| Maltodextrin | 132 | 132 | 132 |
| Sucrose | 100 | 100 | 100 |
| Soybean oil | 70 | 70 | 70 |
| Lard | | 95 | 70 |
| ARASCO[1] | | | 25 |
| Cellulose | 50 | 50 | 50 |
| Mineral Mix, AIN-93G-MX | 35 | 35 | 35 |
| Vitamin Mix, AIN-93G-VX | 10 | 10 | 10 |
| Choline Bitartrate | 2.5 | 2.5 | 2.5 |
| TBHQ (antioxidant)[2] | 0.014 | 0.014 | 0.014 |
| **Fatty acid composition (% total FA by weight)** [3] | | | |
| C12:0 | 0.019 ± 0.001 | 0.054 ± 0.002 | 0.041 ± 0.003 |
| C14:0 | 0.12 ± 0.008 | 0.84 ± 0.014 | 0.66 ± 0.004 |
| C16:0 | 11.26 ± 0.22 | 18.2 ± 0.14 | 16.37 ± 0.10 |
| C16:1 | --- | 1.13 ± 0.007 | 0.85 ± 0.004 |
| C18:0 | 4.18 ± 0.10 | 9.97 ± 0.07 | 9.02 ± 0.03 |
| C18:1 | 20.80 ± 0.12 | 31.09 ± 0.087 | 28.80 ± 0.06 |
| C18:2 n-6 | 54.99 ± 0.41 | 32.51 ± 0.055 | 31.15 ± 0.04 |
| C18:3 n-3 | 7.32 ± 0.09 | 3.47 ± 0.012 | 3.38 ± 0.003 |
| C18:3 n-6 | --- | --- | --- |
| C20:0 | --- | --- | 0.37 ± 0.004 |
| C20:1 | 0.16 ± 0.01 | 0.45 ± 0.02 | 0.39 ± 0.007 |
| C20:4 n-6 | --- | --- | 6.56 ± 0.03 |
| C22:0 | --- | --- | 0.36 ± 0.0001 |
| C22:5 n-3 | --- | --- | 0.023 ± 0.0003 |
| C24:0 | --- | --- | 0.21 ± 0.007 |

Purified diets were provided in pellet form and made by Harlan (Indianapolis, IN, USA), ARASCO provided *in-kind* by DSM (Columbia, MD, USA)

[1] ARASCO contained about 40.6% AA

[2] TBHQ = tert-Butylhydroquinone

[3] Mean ± SD. Average of samples analyzed in triplicate by gas chromatography

## 5.4 Results

### 5.4.1 Body weight, body composition and food intake

There were no differences in body weight or body composition among diet groups at baseline. Beginning at wk 4, HFD+AA showed greater rates of weight gain, resulting in a 6% higher body weight compared to CTRL at wk 6 (**Figure 5.1A**). Body weight of rats fed the HFD did not differ significantly from controls or HFD+AA. Similarly, HFD+AA had significantly higher total body fat % (assessed by DXA) compared to controls and but not HFD (**Figure 5.1B)**. Both HFD and HFD+AA had 15% greater abdominal fat mass compared to controls, predominantly in the form of visceral adipose tissue (**Figure 5.1C,D**). Addition of AA did not result in greater gains in abdominal fat mass than the HFD alone. Epididymal fat pads were 25% and 16% greater in HFD and HFD+AA rats compared to controls respectively; whereas only HFD+AA group showed greater (30%) inguinal fat pad weight compared to controls or the HFD group. Body length (nose-to-rump and nose-to-tail) and total lean mass were similar among groups throughout the study (data not shown).

Daily food intake was similar among the three dietary groups until wk 2 of study; the control group consumed a greater absolute quantity (in grams) of food than the HFD groups from wk 3 until end of study (data not shown). However, feed efficiency (food consumed / change in body weight (**Figure 5.1E**), total energy intake, food intake (grams) adjusted for lean mass, total protein intake (grams), and protein intake adjusted for body weight did not differ among groups (data not shown).

### 5.4.2 Blood and tissue fatty acids

The proportion of RBC and soft tissue (visceral and subcutaneous adipose, liver, quadriceps) AA was significantly elevated in the HFD+AA group at wk 6 compared to both

control and HFD (**Figure 5.2, Supp Tables S5.1-2**). This was accompanied by lower DHA proportions in RBC membranes, liver and quadriceps in the HFD+AA compared to both control and HFD, however both HFD and HFD+AA had lower DHA in adipose tissues. High-fat feeding led to lower EPA in RBC membranes and liver, with greater reductions in RBC EPA when AA was added to the diet; whereas EPA was not observed in detectable amounts in adipose or quadriceps tissue. Detailed FA composition of tissues is provided in **Supplementary Tables S5.1 and S5.2**.

### 5.4.3 Blood biochemistry

Serum RANKL concentrations were lower in the HFD group beginning wk 3 of study (**Table 5.2**), with no difference between control and HFD+AA groups. A significant time x diet interaction was observed in the RANKL/OPG ratio, with an initial decrease in the ratio in the HFD groups at wk 3, but the HFD+AA group showing the greatest reduction compared to baseline at the end of study. A similar interaction was found for osteocalcin; the HFD group had the highest concentrations at wk 3, yet the levels decreased significantly by the end of study, with HFD+AA showing intermediate levels at wk 6. There were no differences in CTx or P1NP.

Serum leptin concentrations were significantly elevated in HFD+AA beginning at wk 3 (**Figure 5.3A**), leptin continued to increase where HFD+AA had 1 and 2.5-fold greater concentrations than HFD and controls respectively at the end of study. There was an initial increase in adiponectin concentrations in the control and HFD+AA groups only, followed by a reduction in all groups with HFD having 15% lower values than controls at wk 6. No differences in circulating 25(OH)D (range: 37.8 – 79.6 nmol/L) or ionized calcium (range: 1.18 – 1.41 mmol/L) were observed at wk 6.

### 5.4.4 DXA and *in vivo* μCT measurements of bone mass and density

Longitudinal assessment by *in vivo* μCT (**Table 5.3**) showed that the HFD+AA group had significantly lower whole body BMC at wk 6. Total bone volume did not differ among groups, yet vBMD was higher in both the HFD and HFD+AA groups compared to controls. This difference in BMC and vBMD was contributed predominantly by the trabecular compartment, where the HFD+AA group had the lowest trabecular bone volume and BMC. In LV2-5, total and trabecular BMC as well as bone volume at wk 6 were reduced in the HFD+AA group but not in the HFD group. There were no differences in cortical bone measurements in neither whole body nor vertebrae scans among all groups.

Results from the DXA imaging **(Supp Table S5.3**) showed no differences in whole body or LV1-4 aBMD, BMC or area among groups throughout the study. At wk 6, the HFD and HFD+AA groups had smaller projected area in the left femur than the control groups. No differences in femur BMC were observed, resulting in slightly greater aBMD in the HFD (10%) and HFD+AA (5%) groups relative to the control group.

### 5.4.5 *Ex vivo* bone microarchitecture

Distal femur metaphysis vBMD was increased by 29% in the HFD and HFD+AA groups relative to controls (**Figure 5.4**). The HFD+AA group also showed improved microarchitectural properties (increased BV/TV, BS/TV, Tb.N, Conn.D and lower SMI) compared to controls. However, no differences in trabecular vBMD were observed at LV3 (**Supp Figure S5.1**).

At the femur mid-diaphysis (**Table 5.4**), total cross-sectional and medullary area (area enclosed by the periosteal and endosteal envelope, respectively) were lower in the HFD+AA group. However, there were no differences in cortical bone area, resulting in a slightly (5%) greater cortical area/ total area in the HFD+AA group. Mid-diaphyseal diameter measured at

anterior-posterior direction was also reduced in the HFD+AA group compared to controls. There were no significant differences in cortical vBMD or intra-cortical porosity among groups.

### 5.4.6 Bone biomechanics

The HFD group had significantly lower energy absorbed at break at the femur mid-diaphysis compared to controls (**Table 5.4**). No significant differences in other measurements of bone strength or bone length (data not shown) were observed.

### 5.4.7 Fatty acid treatment of 3T3-L1 adipocytes

The effects of varying concentrations of LCPUFA on adipose development and adipokine secretion were explored *in vitro* using 3T3-L1 pre-adipocytes. Analysis of lipid accumulation by Nile Red staining showed multiple small lipid droplets in cells treated with 25 μM of all lipids, comparable to that of vehicle-treated control culture (**Figure 5.5A**). Accumulation of lipid droplets increased in a dose-dependent manner. The effect was most pronounced in DPA- and DHA-treated cells, although there were no significant differences in average droplet size among treatments at 100 μM (**Figure 5.5B**). The relative proportion of adipocytes with small/ multiloculus-like lipid droplets (<20 $\mu m^2$) was greater in AA-treated cells; whereas for cells treated with EPA, DPA and DHA, a higher proportion of cells that contained uniloculus-like lipid droplets was observed (**Figure 5.5C**). Except for DHA, the enrichment of respective LCPUFA (AA, EPA and DPA) was notable, showing linear increases from 10-55% of total fatty acids weight as LCPUFA treatment concentration increased (**Supp Table S5.4**). The proportion of DHA in adipocytes plateaued at 30-35% at treatment concentrations above 50 μM. EPA treatment increased accumulation of its elongation product n-3 DPA and *vice versa*, suggesting these fatty acids are interconvertible in 3T3-L1 cells. AA treatment also resulted in 1.2- to 1.5-fold greater leptin secretion relative to controls at all concentrations (**Figure 5.3B**), with the

greatest effect observed at 25 μM. Contrarily, all n-3 LCPUFA decreased leptin production. All LCPUFA treatments lowered adiponectin secretion. The effect of suppression was greatest with DHA (20-35%) and least with AA (10-15%).

The effects on leptin, adiponectin and PPARγ mRNA levels were explored with AA and DHA treatment at 25, 50 and 100 μM (**Figure 5.3C**), as these fatty acids produced the most prominent effects on adipokine secretion. Changes in leptin mRNA levels closely resembled the secretion pattern in media. AA at 25 μM significantly increased leptin mRNA compared to vehicle-treated cells. Conversely, mRNA levels of leptin, adiponectin and PPARγ were lowered by DHA treatment at 50 and 100 μM.

**Table 5.2** Assessment of bone biomarkers concentrations in rats fed a control, HFD, or HFD+AA diet for 6 wk

| | Time (wk) | CTRL | HFD | HFD+AA | Main effect | | |
|---|---|---|---|---|---|---|---|
| | | | | | $P_{diet}$ | $P_{time}$ | $P_{diet*time}$ |
| RANKL (pg/mL) | 0 | 63.37 ± 10.72 | 48.63 ± 8.61 | 55.07 ± 8.14 | <.0001 | <.0001 | 0.86 |
| | 3 | 52.26 ± 14.47[a] | 37.35 ± 12.55[b] | 49.63 ± 12.92[a] | | | |
| | 6 | 45.49 ± 16.02[a] | 35.28 ± 9.65[b] | 43.71 ± 18.54[a] | | | |
| OPG (pg/mL) | 0 | 874.79 ± 245.52 | 842.20 ± 187.22 | 882.03 ± 260.87 | 0.11 | 0.76 | 0.56 |
| | 3 | 1057.74 ± 288.39 | 778.90 ± 281.74 | 1006.17 ± 360.50 | | | |
| | 6 | 925.85 ± 220.63 | 707.52 ± 200.26 | 1211.59 ± 161.41 | | | |
| RANKL/OPG | 0 | 0.078 ± 0.02 | 0.060 ± 0.01 | 0.068 ± 0.02 | 0.23 | 0.002 | 0.038 |
| | 3 | 0.057 ± 0.02[a] | 0.052 ± 0.02[b] | 0.058 ± 0.03[b] | | | |
| | 6 | 0.055 ± 0.02[ab] | 0.054 ± 0.02[ab] | 0.046 ± 0.02[a] | | | |
| Osteocalcin (mg/mL) | 0 | 292.72 ± 112.10 | 300.33 ± 78.54 | 252.19 ± 104.31 | 0.15 | <.0001 | 0.022 |
| | 3 | 334.95 ± 65.03[ab] | 358.95 ± 44.26[a] | 315.48 ± 37.02[ab] | | | |
| | 6 | 265.46 ± 47.50[ab] | 235.18 ± 26.93[b] | 245.74 ± 31.65[b] | | | |
| CTx (pg/mL) | 0 | 140.00 ± 10.24 | 134.29 ± 12.72 | 138.61 ± 11.66 | 0.72 | <.0001 | 0.73 |
| | 3 | 121.83 ± 16.52 | 120.41 ± 9.10 | 122.32 ± 11.53 | | | |
| | 6 | 110.71 ± 17.69 | 114.59 ± 11.46 | 115.38 ± 11.67 | | | |
| P1NP (ng/mL) | 0 | 59.22 ± 0.94 | 59.27 ± 1.21 | 58.64 ± 1.60 | 0.64 | <.0001 | 0.26 |
| | 3 | 55.84 ± 1.19 | 56.12 ± 0.93 | 56.85 ± 1.44 | | | |
| | 6 | 50.87 ± 2.00 | 49.88 ± 2.44 | 50.71 ± 0.82 | | | |
| Insulin (ng/mL) | 6 | 0.15 ± 0.08 | 0.14 ± 0.04 | 0.16 ± 0.08 | 0.68 | | |

Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Different superscript indicates significant differences among diet groups over time ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. CTx, C-terminal polypeptide; OPG, osteoprotegerin; P1NP, procollagen type-I N-terminal propeptide; RANKL, receptor activator of nuclear factor-κB ligand

**Table 5.3** Bone mass in whole body and lumbar vertebrae 2-5 assessed by *in vivo* μCT in rats fed a control, HFD, or HFD+AA diet at baseline and wk 6

| | | Time (wk) | CTRL | HFD | HFD+AA | Main effect | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | $P_{diet}$ | $P_{time}$ | $P_{diet*time}$ |
| **Whole body** | | | | | | | | |
| Total | Volume ($cm^3$) | 0 | 3.2 ± 0.3 | 3.2 ± 0.3 | 3.3 ± 0.3 | 0.57 | <.0001 | 0.78 |
| | | 6 | 14.4 ± 0.7 | 14.0 ± 0.9 | 13.8 ± 1.3 | | | |
| | BMC (g) | 0 | 1009.5 ± 58.1 | 1004.3 ± 120.8 | 1009.5 ± 92.9 | 0.038 | <.0001 | 0.65 |
| | | 6 | 6828.9 ± 387.6[a] | 6819.4 ± 488.7[a] | 6671.7 ± 492.2[b] | | | |
| | vBMD ($g/cm^3$) | 0 | 313.4 ± 7.3 | 314.1 ± 11.2 | 307.9 ± 8.8 | 0.042 | <.0001 | 0.34 |
| | | 6 | 475.0 ± 15.8[a] | 486.0 ± 12.9[b] | 483.6 ± 19.3[b] | | | |
| Trabecular | Volume ($cm^3$) | 0 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.01 | 0.036 | <.0001 | 0.71 |
| | | 6 | 6.2 ± 0.4[a] | 6.0 ± 0.5[ab] | 5.9 ± 0.7[b] | | | |
| | BMC (g) | 0 | 173.3 ± 15.2 | 171.9 ± 29.9 | 174.3 ± 18.2 | 0.033 | <.0001 | 0.56 |
| | | 6 | 2151.8 ± 185.2[ab] | 2201.2 ± 266.7[a] | 2118.5 ± 205.5[b] | | | |
| | vBMD ($g/cm^3$) | 0 | 195.3 ± 5.0 | 197.0 ± 14.3 | 193.1 ± 5.8 | 0.041 | <.0001 | 0.28 |
| | | 6 | 346.6 ± 15.9[a] | 364.0 ± 22.2[b] | 359.9 ± 20.7[ab] | | | |
| **Lumbar Vertebrae 2-5** | | | | | | | | |
| Total | Volume ($cm^3$) | 0 | 0.1 ± 0.01 | 0.1 ± 0.01 | 0.2 ± 0.00 | 0.039 | <.0001 | 0.46 |
| | | 6 | 0.82 ± 0.05[a] | 0.84 ± 0.02[ab] | 0.80 ± 0.02[b] | | | |
| | BMC (g) | 0 | 41.5 ± 2.7 | 40.5 ± 5.2 | 41.04 ± 3.9 | 0.027 | <.0001 | 0.37 |
| | | 6 | 331.3 ± 24.3[ab] | 330.0 ± 27.6[a] | 309.7 ± 19.3[b] | | | |
| | vBMD ($g/cm^3$) | 0 | 294.7 ± 9.7 | 286.8 ± 11.3 | 281.8 ± 9.5 | 0.25 | <.0001 | 0.92 |
| | | 6 | 405.2 ± 18.6 | 404.8 ± 16.1 | 406.3 ± 21.0 | | | |
| Trabecular | Volume ($cm^3$) | 0 | 0.06 ± 0.001 | 0.06 ± 0.001 | 0.06 ± 0.002 | 0.034 | <.0001 | 0.88 |
| | | 6 | 0.40 ± 0.01[a] | 0.41 ± 0.01[ab] | 0.35 ± 0.01[b] | | | |
| | BMC (g) | 0 | 11.3 ± 1.4 | 11.3 ± 2.0 | 11.7 ± 1.5 | 0.028 | <.0001 | 0.41 |
| | | 6 | 138.7 ± 13.9[ab] | 138.9 ± 18.3[a] | 126.2 ± 9.8[b] | | | |
| | vBMD ($g/cm^3$) | 0 | 201.5 ± 5.3 | 197.8 ± 11.2 | 195.7 ± 7.6 | 0.48 | <.0001 | 0.59 |
| | | 6 | 333.5 ± 13.8 | 331.3 ± 20.5 | 341.0 ± 19.0 | | | |

Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Within rows, different superscript indicates significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. BMC, bone mineral content; vBMD, volumetric bone mineral density

**Table 5.4** *Ex vivo* μCT measurements of bone mass and geometry of the femur mid-diaphysis and bone biomechanics assessed by 3-point bending

| | CTRL | HFD | HFD+AA | $P_{diet}$ |
|---|---|---|---|---|
| **Micro-CT measurements** | | | | |
| vBMD ($g/cm^3$) | 1.83 ± 0.04 | 1.83 ± 0.03 | 1.82 ± 0.04 | 0.76 |
| Tt.Ar ($mm^2$) | 13.72 ± 1.04[a] | 13.45 ± 1.11[ab] | 12.87 ± 1.40[b] | 0.038 |
| Ct.Ar ($mm^2$) | 6.71 ± 0.46 | 6.78 ± 0.34 | 6.52 ± 0.52 | 0.53 |
| Ma.Ar ($mm^2$) | 7.01 ± 0.85[a] | 6.67 ± 0.56[ab] | 6.38 ± 0.78[b] | 0.034 |
| Ct.Ar / Tt.Ar (%) | 49.01 ± 3.29[a] | 50.58 ± 2.90[ab] | 51.17 ± 3.72[b] | 0.041 |
| Mid-diaphyseal diameter (antero-posteral) (mm) | 3.60 ± 0.14[a] | 3.42 ±0.46[ab] | 3.24 ± 0.57 [b] | 0.028 |
| **Bone biomechanics** | | | | |
| Maximum load (N) | 105.05 ± 11.78 | 106.40 ± 13.76 | 108.51 ± 20.37 | 0.34 |
| Maximum extension (mm) | 1.04 ± 0.19 | 1.01 ± 0.18 | 0.94 ± 0.22 | 0.58 |
| Energy at break (mJ) | 94.51 ± 25.14[a] | 78.55 ± 28.55[b] | 90.70 ± 22.27[ab] | 0.035 |
| $Stress_{max}$ (MPa) | 72.87 ± 18.08 | 79.27 ± 30.71 | 67.06 ± 12.68 | 0.54 |
| $Strain_{max}$ (%) | 7.56 ± 1.50 | 7.14 ± 1.26 | 6.63 ± 1.79 | 0.67 |
| Extrinsic stiffness (N/mm) | 3.07 ± 0.27 | 3.22 ± 0.46 | 3.28 ± 0.57 | 0.71 |
| Young's Modulus (MPa) | 29.26 ± 5.99 | 33.77 ± 12.69 | 29.32 ± 7.23 | 0.92 |
| Moment of inertia (x-axis) ($mm^4$) | 11.15 ± 2.34 | 10.94 ± 4.06 | 12.05 ± 2.97 | 0.66 |

Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Within rows, different superscript indicates significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. Ct.Ar, cortical area; Ma.Ar, marrow area; Tt.Ar, total area; vBMD, volumetric bone mineral density

**Figure 5.1** Changes in (A) body weight (B) total body fat % (C) food intake / body weight gained per wk (D) abdominal fat % and (E) abdominal visceral fat % in growing male rats fed control, HFD or HFD+AA diet over course of study. Data are mean ± SD. Differences were determined using MIXED model ANOVA with post-hoc Tukey comparisons. *Significant differences between HFD+AA vs CTRL; † Differences are between CTRL vs HFD and HFD+AA ($p<0.05$). n=41; VAT, visceral adipose tissue

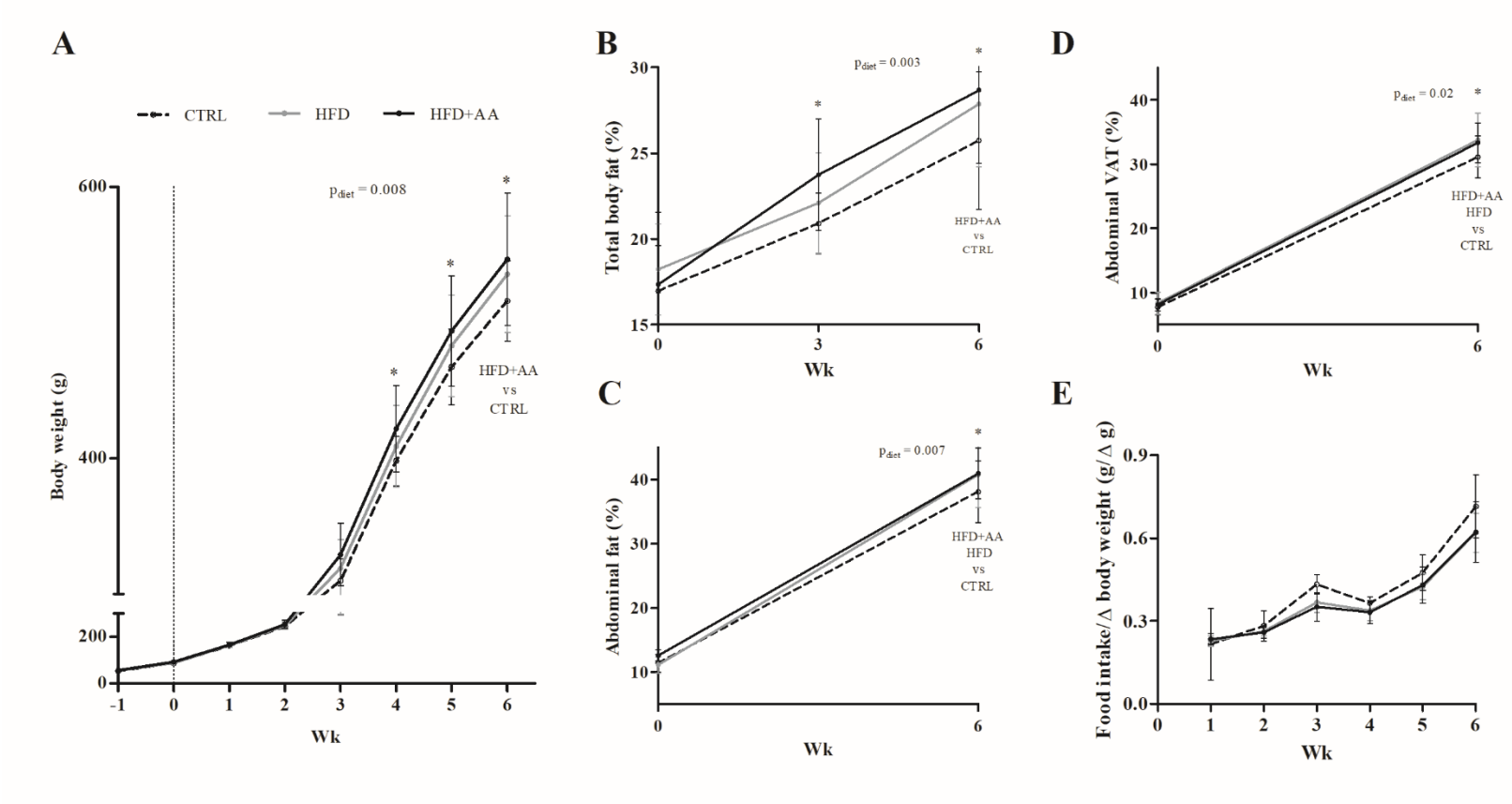


**Figure 5.2** Proportions of ALA, LA, AA, DPA and DHA in total lipids of (A) red blood cells and (B) adipose tissue in growing male rats fed control, HFD and HFD+AA diet for 6 wk. Data are mean ± SD. Differences were determined using MIXED model ANOVA with post-hoc Tukey comparisons. Different superscript indicates significant differences among diets ($p<0.05$). n=41; RBC, red blood cell; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue

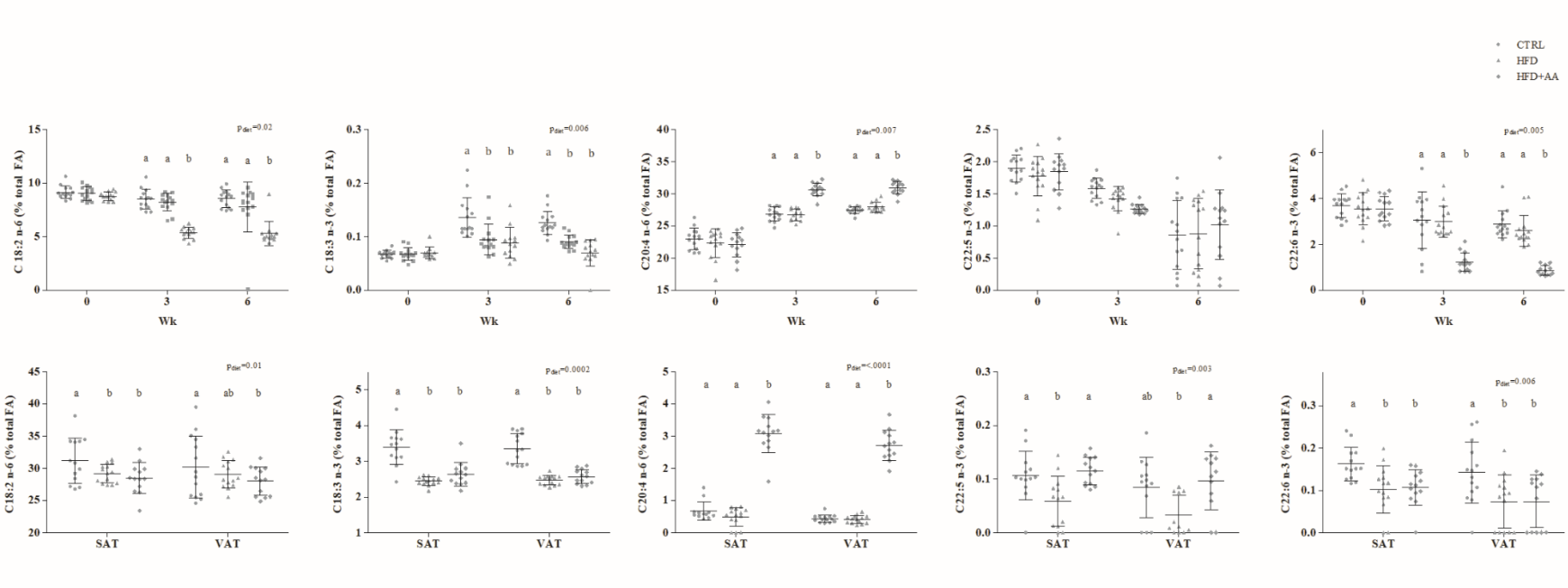


**Figure 5.3** (A) Serum leptin and adiponectin concentrations in growing male rats fed control, HFD, and HFD+AA diet for 6 wk; (B) 3T3-L1 adipocytes were treated with difference concentrations of AA, EPA, DPA and DHA (10, 25, 50 or 100 μM) for 48 h. Leptin and adiponectin concentrations were measured in culture media; and (C) mRNA levels of leptin, adiponectin and PPARγ were quantified with quantitative RT-PCR and presented as relative expression compared to vehicle-treated cells. Data are mean ± SD. Differences were determined using MIXED model ANOVA with post-hoc Tukey comparisons. Different superscripts indicate significant differences among groups ($p<0.05$).; n=14 (leptin) and n=10 (adiponectin) per group / time point; n=3 for 3T3-L1 adipocytes. * Differences are significant between treatment and vehicle-treated cells

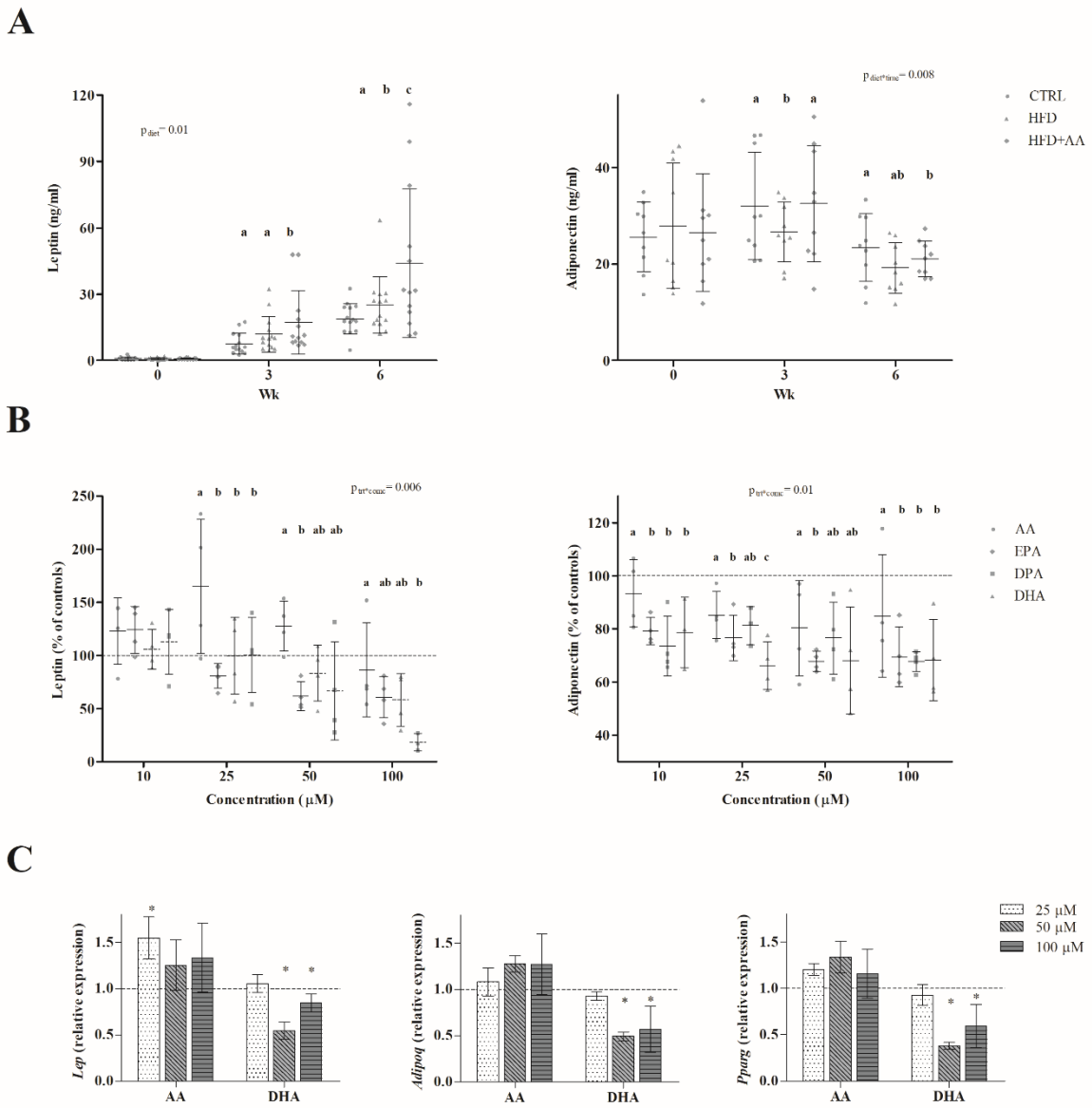


**Figure 5.4** *Ex vivo* μCT measurements of the trabecular micro-architectural properties at the (A) distal femur metaphysis (B) representative 3D reconstruction of trabecular bone. Data are mean ± SD. Differences were determined using MIXED model ANOVA with post-hoc Tukey comparisons. Different superscripts indicate significant differences among groups. n=41. BV/TV, bone volume fraction; BS/TV, bone surface density; Conn.D, connectivity density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; vBMD, volumetric bone mineral density

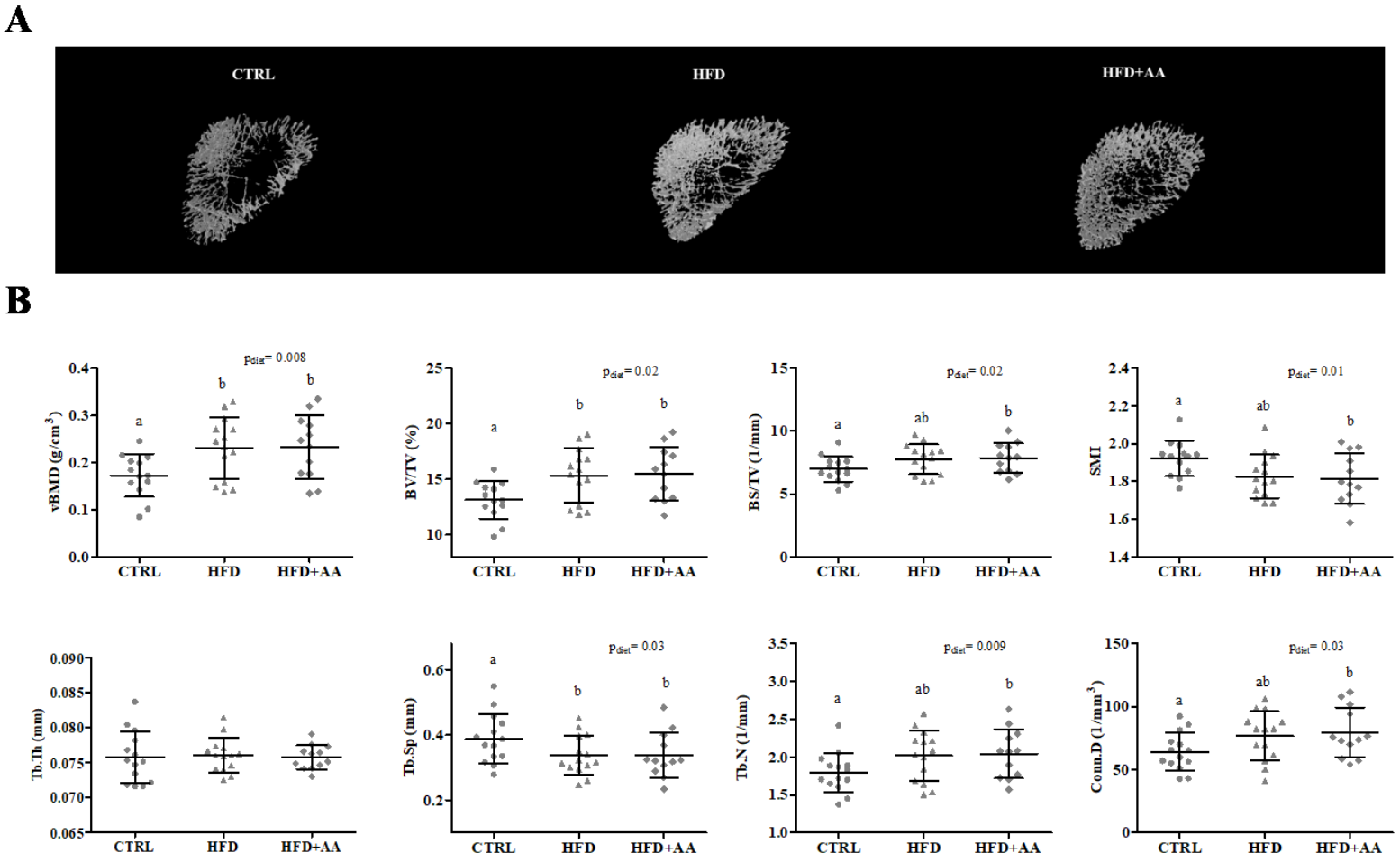
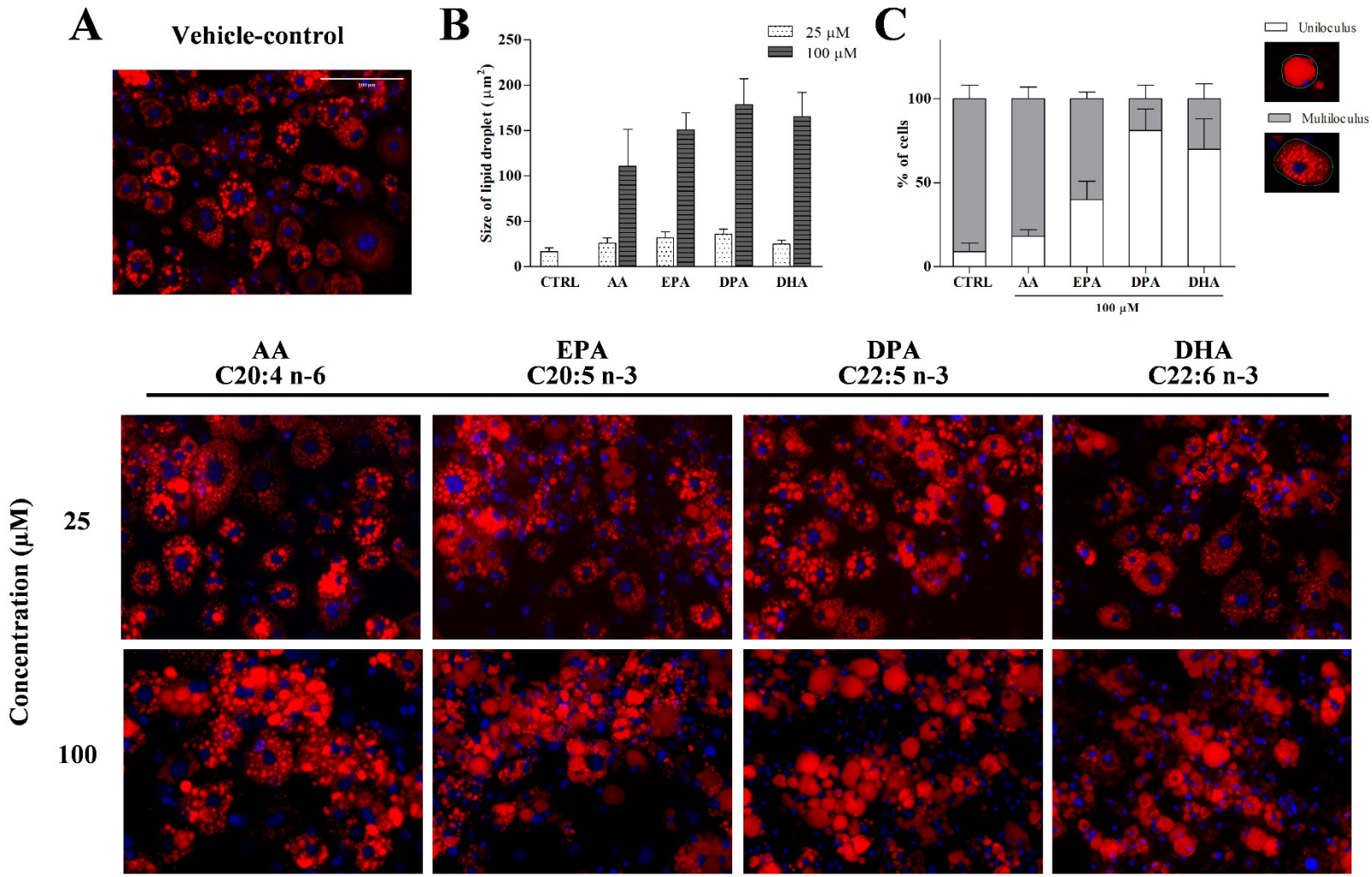


**Figure 5.5** (A) Representative images of Nile Red and DAPI-stained 3T3-L1 adipocytes (B) Measurement of lipid droplet size in cells following 48h treatment of 25 and 100 µM of AA, EPA, DPA and DHA (C) Adipocytes were categorized into containing uniloculus-like and multiloculus-like lipid droplets. Cellular distribution of each categorized cell type in adipocytes treated with 100 µM fatty acids were measured with ImageJ, the scale bar represents 100 µm. n=3 per concentration / treatment



## 5.5 Discussion

This study examined the interaction between dietary AA with bone mass and adiposity in a highly-controlled animal model of diet-induced obesity. The inclusion of AA in a HFD for 6 wk increased body weight, adiposity, and serum leptin compared to the HFD alone, suggesting that AA exacerbated diet-induced obesity. HFD+AA reduced whole body BMC and femur cortical area at the mid-diaphysis, but not bone strength. Femur metaphyseal trabecular vBMD and micro-architecture was improved in HFD+AA compared to controls. The inclusion of AA promoted adipogenesis and augmented the early consequences of obesity on bone.

Along with the present results, a moderately HFD (30-35% energy) appears sufficient to influence bone metabolism independent of weight gain, possibly through altering body composition and adipokine metabolism. HFD (25-30% energy) increased femur mid-diaphyseal cortical thickness [351] and decreased bone formation in tibia metaphysis [216]. Many studies of diet induced obesity in animals used dietary fat content ranging from 45-70% of energy [91, 216], whereas the typical rodent diet contains approximately 14% of energy as fat. While these HFD are effective in inducing weight gain and associated metabolic complications, this does not mimic the human scenario, especially in light of the rising obesity prevalence in the absence of increased dietary fat intake over the last decade [39]. It is therefore important to consider both quantity and composition of fat in experimental diets that would reflect the timing and pattern of obesity development in human as closely as possible.

It is increasingly clear that obesity is associated with lower bone vBMD or quality in both children [352] and adults [353]. Using both *in vivo* and *ex vivo* μCT, we reported decreased trabecular BMC as well as bone volume deficits in the whole body and LV2-5 in HFD+AA; whereas at the distal femur metaphysis, high-fat feeding increased trabecular vBMD and

improved microarchitectural properties in HFD+AA. This contradicted our hypothesis where both bone mass and quality are compromised in obesity. Previous studies have likewise reported increases in trabecular vBMD and microarchitecture with diet-induced obesity in growing and adult mice [316, 354]. These could be attributed to increased mechanical loading, circulating levels of anabolic hormones including insulin and leptin, and the nutritive effects of excess energy and/or fatty acid intake. The effects of obesity on bone may also manifest at non-weight bearing sites (vertebrae) in rodents, hence the lower BMC with HFD was not observed in the femur.

Leptin concentrations were notably elevated in HFD+AA. Murine models of leptin deficiency or signaling (*ob/ob, db/db* mice and *fa/fa* rats) displayed reductions in trabecular network and biomechanical properties of the femur [146, 148] but not in the lumbar vertebrae [149]. These animal models suggest the static loads conferred by excess body weight do not overcome the absence of leptin, or the potential benefits of leptin on bone may be masked by reduced muscle load at the axial skeleton. The opposing effects of AA+HFD on trabecular bone in femur and vertebrae observed in the current study are thus speculated to be mediated in part through leptin action.

Obesity is associated with imbalances of essential LCPUFA status [60]. Phospholipid and sterol esters AA, DHGLA and LA proportions were substantially higher in children who are obese [60]. Similarly, AA proportions in subcutaneous adipose tissue positively associate with the variance in BMI (38.2%), more so than any other LCPUFA in pre-pubertal children [288]. Here, HFD led to reductions in RBC and tissue proportions of n-3 ALA, EPA and DPA, while addition of AA further diminished EPA and DHA. Although studies of dietary AA on bone growth are inconsistent [322, 355, 356], the beneficial effects of EPA and DHA on bone mass

accrual are known in humans [357, 358] and animals [359]. The observed bone outcomes could therefore be attributed to the lower n-3 LCPUFA status with AA feeding and consequential to obesity.

The regulation of adipokine secretion by LCPUFA is likely nonlinear. Our elevated leptin concentrations in HFD+AA cannot be explained by adiposity alone, hence the adipocyte response to LCPUFA were tested *in vitro*. AA caused an initial increase in leptin production and mRNA levels at 25 μM followed by a gradual decrease at higher doses. Similarly, AA at 200 μM inhibited leptin expression in isolated rat adipocytes [360]. Here, we reported n-3 LCPUFA decreased leptin and adiponectin secretion. While this contradicts earlier reports where EPA and DHA (50-125 μM) upregulated leptin in differentiating and mature 3T3-L1 adipocytes [211, 327-329], higher DHA (1 mM) reduced leptin mRNA by 22-40% [156]. Thus, the responses to physiologically-relevant treatment dosages need to be characterized to guide further studies.

The involvement of PPARγ in regulating adipokine secretion is unclear. A PPAR response element was identified in the promoter region of adiponectin gene [210] but not that of leptin [204]. The changes in leptin and adiponectin mRNA levels after treatment with AA or DHA in our study strongly resembled that of PPARγ, suggesting the expression of these adipokines may relate directly to PPARγ expression and/or activity. The decreased PPARγ mRNA levels with DHA treatment is contrary to previous studies [211, 327, 329, 361]. However, DHA dose-dependently (25-200 μM) supressed lipid accumulation and droplet size in differentiating 3T3-L1 cells [52]. The decreased expression of adipokines and PPAR could therefore be a consequence of the anti-adipogenic effects of DHA. This may also explain the observation where DHA-treated adipocytes contained uniloculus-like lipid droplets that were of similar size as cells treated with AA. The potential role of LCPUFA in reducing both hyperplasic and hypertrophy of

growing adipocytes and remain to be clarified *in vivo* with longer intervention studies.

The inclusion of male rats only in this study limits the generalization of results given the known effects of estrogen on raising LCPUFA status [362], and the sex dimorphism exhibited during skeletal growth [363]. Our bone biomarker data were unable to show if the lower bone mass in HFD+AA was due to increased bone absorption or decreased formation. The use of bone biomarkers as indicators of skeletal health may be limited in our model, as the deviation from normal (healthy) states may not be large enough to be detected [364]. Further studies should include bone histomorphometry to quantify cellular activities in bone remodeling. The current study examined the fatty acid profile in total lipids; the characterization of fatty acid proportions in phospholipids or triglycerides, and/or coupled with use of fatty acid tracers would clarify the metabolic fate of fatty acids after uptake into the cells, and determine if they are preferentially stored, oxidized, or converted to other metabolites. Diet-induced obesity decreases bone mass in rodents and contrasts observed increases in some human studies [75, 76]. This could in part be related to different load-bearing patterns in rodents versus humans, and the complexities in the etiology of obesity in humans. However, our study is relevant at least in the understanding of consequences of diet-induced obesity on LCPUFA status and bone outcomes, providing insight into the possible mechanisms of bone fragility observed in children with obesity.

**5.6 Conclusion**

The addition of AA to a moderately HFD promoted obesity development in growing male rats, negatively impacting BMC and volume at the whole body and lumbar vertebra, without impairing bone strength at the femur diaphysis. Improvements in femur metaphyseal microarchitecture were also observed with AA. This study highlights the complexity of fat and bone crosstalk and points to the interaction of multiple metabolic pathways which may explain

conflicting findings within existing literature. The longer-term effects of AA on BMD and ultimately, fracture risks in children of overweight or obese weight status remains to be clarified.

### 5.7 Acknowledgments

The authors would like to thank Krystyna Wang, Julia Levy-Ndejuru and Kimberly O'Keefe for their assistance with the animal study and laboratory analyses. We would also like to thank Dr. Jingjing Li for her skillful technical assistance with cell culture experiments.

**Supplementary Table S5.1** Red blood cell fatty acid profile (expressed as % weight of total FA) at wk 0, 3 and 6 in rats fed a control, HFD, or HFD+AA for 6 wk

| | Time (wk) | CTRL | HFD | HFD+AA |
|---|---|---|---|---|
| Myristic acid (C14:0) | 0 | 0.38 ± 0.03 | 0.35 ± 0.06 | 0.43 ± 0.21 |
| | 3 | 0.22 ± 0.04 | 0.21 ± 0.08 | 0.18 ± 0.05 |
| | 6 | 0.18 ± 0.04 | 0.14 ± 0.04 | 0.16 ± 0.04 |
| Palmitic acid (C16:0) | 0 | 28.83 ± 0.92 | 29.03 ± 1.58 | 28.92 ± 0.97 |
| | 3 | 26.08 ± 0.93 | 24.28 ± 1.80 | 24.51 ± 0.74 |
| | 6 | 25.18 ± 0.71 | 23.86 ± 1.21 | 24.35 ± 0.87 |
| Stearic acid (C18:0) | 0 | 13.73 ± 0.53 | 13.77 ± 0.72 | 13.72 ± 0.66 |
| | 3 | 15.37 ± 0.57[a] | 16.19 ± 0.77[b] | 16.51 ± 0.73[b] |
| | 6 | 17.13 ± 0.83[a] | 18.45 ± 0.70[b] | 17.98 ± 0.45[b] |
| Oleic acid (C18:1) | 0 | 6.68 ± 1.95 | 6.59 ± 1.19 | 7.31 ± 0.63 |
| | 3 | 5.64 ± 1.78 | 6.92 ± 0.90 | 6.97 ± 0.75 |
| | 6 | 5.61 ± 0.43 | 6.11 ± 1.80 | 6.24 ± 0.51 |
| Linoleic acid (C18:2 n-6) | 0 | 9.12 ± 0.59 | 9.03 ± 0.64 | 8.76 ± 0.40 |
| | 3 | 8.49 ± 0.93[a] | 8.22 ± 0.82[a] | 5.38 ± 0.52[b] |
| | 6 | 8.54 ± 0.81[a] | 7.80 ± 0.91[a] | 5.30 ± 1.14[b] |
| α-linolenic acid (C18:3 n-3) | 0 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| | 3 | 0.14 ± 0.04[a] | 0.10 ± 0.03[b] | 0.09 ± 0.03[b] |
| | 6 | 0.13 ± 0.02[a] | 0.09 ± 0.01[b] | 0.07 ± 0.01[b] |
| γ-linolenic acid (C18:3 n-6) | 0 | 0.13 ± 0.05 | 0.12 ± 0.05 | 0.12 ± 0.05 |
| | 3 | 0.10 ± 0.03 | 0.11 ± 0.01 | 0.11 ± 0.01 |
| | 6 | 0.08 ± 0.03 | 0.10 ± 0.20 | 0.10 ± 0.01 |
| Eicosadienoic acid (C20:2 n-6) | 0 | 0.12 ± 0.15 | 0.16 ± 0.22 | 0.14 ± 0.17 |
| | 3 | 0.29 ± 0.23 | 0.53 ± 0.15 | 0.44 ± 0.03 |
| | 6 | 0.11 ± 0.13 | 0.22 ± 0.74 | 0.38 ± 0.05 |
| Dihomo-γ-linolenic acid (C20:3 n-6) | 0 | 0.47 ± 0.12 | 0.46 ± 0.04 | 0.49 ± 0.06 |
| | 3 | 0.45 ± 0.16 | 0.39 ± 0.04 | 0.35 ± 0.11 |
| | 6 | 0.53 ± 0.06 | 0.40 ± 0.02 | 0.37 ± 0.03 |
| Arachidonic acid (C20:4 n-6) | 0 | 23.01 ± 1.63 | 22.34 ± 2.20 | 22.13 ± 1.88 |
| | 3 | 26.84 ± 1.09[a] | 26.16 ± 2.22[a] | 30.68 ± 1.03[b] |
| | 6 | 27.47 ± 0.50[a] | 27.92 ± 0.74[a] | 30.96 ± 0.99[b] |
| Eicosapentaenoic acid (C20:5 n-3) | 0 | 0.25 ± 0.03 | 0.23 ± 0.05 | 0.23 ± 0.04 |
| | 3 | 0.26 ± 0.04[a] | 0.18 ± 0.04[b] | 0.08 ± 0.01[c] |
| | 6 | 0.24 ± 0.07[a] | 0.14 ± 0.06[b] | 0.07 ± 0.04[b] |
| Docosatetraenoic acid (C22:4, n-6) | 0 | 3.19 ± 0.30 | 3.31 ± 0.37 | 3.13 ± 0.42 |
| | 3 | 2.91 ± 0.36 | 2.79 ± 0.28 | 2.96 ± 0.35 |
| | 6 | 2.69 ± 0.32 | 2.43 ± 0.30 | 2.51 ± 0.19 |
| Docosapentaenoic acid n-6 (C22:5 n-6) | 0 | 0.042 ± 0.01 | 0.043 ± 0.02 | 0.042 ± 0.01 |
| | 3 | 0.034 ± 0.02 | 0.035 ± 0.02 | 0.029 ± 0.03 |
| | 6 | 0.029 ± 0.01 | 0.029 ± 0.01 | 0.026 ± 0.01 |

| | | | | |
|---|---|---|---|---|
| Docosapentaenoic acid n-3 (C22:5 n-3) | 0 | 1.90 ± 0.21 | 1.77 ± 0.31 | 1.84 ± 0.28 |
| | 3 | 1.59 ± 0.16 | 1.42 ± 0.19 | 1.26 ± 0.07 |
| | 6 | 0.86 ± 0.54 | 0.88 ± 0.55 | 1.02 ± 0.54 |
| Docosahexaenoic acid (C22:6 n-3) | 0 | 3.69 ± 0.52 | 3.55 ± 0.69 | 3.55 ± 0.53 |
| | 3 | 3.05 ± 1.21[a] | 2.99± 0.68[a] | 1.23 ± 0.41[b] |
| | 6 | 2.88± 0.58[a] | 2.59 ± 0.66[a] | 0.86 ± 0.21[b] |
| Total n-3 | 0 | 5.76 ± 0.78 | 5.61 ± 0.96 | 5.28 ± 1.70 |
| | 3 | 5.03 ± 1.31[a] | 4.68 ± 0.60[a] | 2.47 ± 0.81[b] |
| | 6 | 4.09 ± 0.77[a] | 3.70 ± 0.97[b] | 1.88 ± 0.84[b] |
| Total n-6 | 0 | 36.05 ± 2.30 | 35.39 ± 0.96 | 32.26 ± 9.46 |
| | 3 | 39.11 ± 1.21 | 38.22 ± 0.61 | 37.09 ± 10.76 |
| | 6 | 39.45 ± 0.78 | 39.64 ± 1.11 | 36.79 ± 10.63sv |

Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Within rows, different superscript indicates significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13

**Supplementary Table S5.2** Fatty acid profile (expressed as % weight of total FA) in subcutaneous and visceral adipose tissue, liver and quadriceps at end of study in rats fed a control, HFD, or HFD+AA for 6 wk

| | Subcutaneous adipose tissue | | | Visceral adipose tissue | | |
|---|---|---|---|---|---|---|
| | **CTRL** | **HFD** | **HFD+AA** | **CTRL** | **HFD** | **HFD+AA** |
| C14:0 | 1.78 ± 0.14[a] | 1.61 ± 0.11[b] | 1.63 ± 0.08[b] | 1.72 ± 0.12[a] | 1.57 ± 0.18[b] | 1.61 ± 0.14[ab] |
| C16:0 | 24.91± 1.57[ab] | 22.46 ± 0.92[b] | 22.37 ± 1.25[b] | 26.51 ± 2.52[a] | 23.46 ± 1.44[b] | 23.07 ± 1.48[b] |
| C18:0 | 2.53 ± 0.30[a] | 3.44 ± 0.40[b] | 3.50 ± 0.40[b] | 2.70 ± 0.53[a] | 3.40 ± 0.29[b] | 3.62 ± 0.33[b] |
| C18:1 | 26.72 ± 2.23[a] | 34.24 ± 0.48[b] | 31.51 ± 1.61[c] | 26.57 ± 1.43[a] | 34.05 ±0.70[b] | 31.97 ± 0.88[c] |
| C18:2 n-6 | 31.19 ± 3.57[a] | 29.18 ± 1.44[b] | 28.49 ± 2.41[b] | 30.19 ± 4.82[a] | 29.04 ± 2.12[ab] | 28.01 ± 2.20[b] |
| C18:3 n-3 | 3.39 ± 0.49[a] | 2.44 ± 0.12[b] | 2.63 ± 0.33[b] | 3.35 ± 0.42[a] | 2.47 ± 0.13[b] | 2.57 ± 0.20[b] |
| C18:3 n-6 | 0.12 ±0.06[a] | 0.12 ± 0.02[a] | 0.26 ± 0.09[b] | 0.10 ± 0.03[a] | 0.09 ± 0.01[a] | 0.20 ± 0.11[b] |
| C20:2 n-6 | 0.17 ± 0.05[a] | 0.26 ± 0.02[b] | 0.31 ± 0.05[b] | 0.18 ± 0.02[a] | 0.19 ± 0.05[a] | 0.29 ± 0.03[b] |
| C20:3 n-6 | 0.13 ±0.10[a] | 0.09 ± 0.29[a] | 0.35 ± 0.08[b] | 0.08 ± 0.04[a] | 0.07 ± 0.04[a] | 0.37 ± 0.05[b] |
| C20:4 n-6 | 0.77 ± 0.83[a] | 0.48 ± 0.08[a] | 3.01 ± 0.82[b] | 0.43 ± 0.12[a] | 0.41 ± 0.13[a] | 2.71 ± 0.47[b] |
| C20:5 n-3 | | ND | | | ND | |
| C22:4 n-6 | 0.09 ± 0.19[a] | 0.13 ± 0.03[a] | 0.53 ± 0.16[b] | 0.10 ± 0.04[a] | 0.15 ± 0.05[a] | 0.43 ± 0.18[b] |
| C22:5 n-3 | 0.11 ± 0.05[ab] | 0.05 ± 0.05[a] | 0.12 ± 0.03[b] | 0.08 ± 0.06[a] | 0.02 ± 0.04[b] | 0.09 ± 0.06[b] |
| C22:6 n-3 | 0.16 ± 0.04[a] | 0.10 ± 0.06[b] | 0.11 ± 0.04[b] | 0.14 ± 0.07[a] | 0.07 ± 0.06[b] | 0.07 ± 0.06[b] |
| Total n-6 | 30.23 ± 9.25 | 30.27 ± 1.39 | 32.91 ± 1.92 | 33.21 ± 4.69 | 29.59 ± 2.02 | 31.83 ± 1.97 |
| Total n-3 | 3.40 ± 1.08[a] | 2.59 ± 0.15[b] | 2.86 ± 0.32[ab] | 3.67 ± 0.39[a] | 2.49 ± 0.17[b] | 2.73 ± 0.17[ab] |

| | Liver | | | Quadriceps | | |
|---|---|---|---|---|---|---|
| | **CTRL** | **HFD** | **HFD+AA** | **CTRL** | **HFD** | **HFD+AA** |
| C14:0 | 0.86 ± 0.23[a] | 0.63 ± 0.16[b] | 0.60 ± 0.10[b] | 1.04 ± 0.42 | 0.95 ± 0.33 | 0.97 ± 0.25 |
| C16:0 | 29.71 ± 4.59[a] | 26.46 ± 4.15[b] | 22.42 ± 2.58[c] | 23.69 ± 2.03[a] | 22.53 ± 1.25[ab] | 22.04 ± 1.62[b] |
| C18:0 | 10.12 ± 2.56[a] | 9.91 ± 2.05[ab] | 7.82 ± 1.65[b] | 9.50 ± 2.98 | 10.02 ± 3.01 | 9.94 ± 2.72 |
| C18:1 | 19.43 ± 5.20[a] | 23.88 ± 4.08[b] | 19.72 ± 2.87[ab] | 16.36 ± 6.48 | 21.17 ± 8.21 | 20.11 ± 5.38 |
| C18:2 n-6 | 15.31 ± 4.46[a] | 18.44 ± 4.63[ab] | 21.09 ± 3.57[b] | 25.12 ± 2.17[a] | 23.55 ± 2.33[b] | 20.64 ± 3.05[b] |
| C18:3 n-3 | 0.67 ± 0.29[a] | 0.69 ± 0.33[a] | 1.12 ± 0.58[b] | 1.48 ± 0.54 | 1.18 ± 0.52 | 1.30 ± 0.39 |
| C18:3 n-6 | 0.21 ± 0.25 | 0.15 ± 0.10 | 0.28 ± 0.11 | 0.03 ± 0.04[a] | 0.03 ± 0.05[a] | 0.15 ± 0.07[b] |
| C20:2 n-6 | 0.31 ± 0.16[ab] | 0.17 ± 0.12[a] | 0.41 ± 0.15[b] | 0.21 ± 0.05[a] | 0.28 ± 0.03[b] | 0.32 ± 0.03[b] |
| C20:3 n-6 | 0.53 ± 0.15[a] | 0.41 ± 0.09[b] | 0.57 ± 0.12[a] | 0.33 ± 0.10[a] | 0.30 ± 0.13[a] | 0.44 ± 0.06[b] |
| C20:4 n-6 | 11.97 ± 3.71[a] | 11.43 ± 2.66[a] | 16.61 ± 2.73[b] | 8.55 ± 3.64[a] | 8.79 ± 4.77[a] | 13.99 ± 4.62[b] |
| C20:5 n-3 | 0.23 ± ??[a] | 0.09 ± ???[b] | 0.11 ± 0.??[b] | | ND | |
| C22:4 n-6 | 0.29 ± 0.09[a] | 0.49 ± 0.22[a] | 3.07 ± 0.88[b] | 0.48 ± 0.15[a] | 0.51 ± 0.18[a] | 1.35 ± 0.31[b] |
| C22:5 n-3 | 0.44 ± 0.24[ab] | 0.37 ± 0.15[a] | 0.72 ± 0.14[b] | 1.34 ± 0.54 | 1.09 ± 0.60 | 1.17 ± 0.50 |
| C22:6 n-3 | 3.64 ± 1.24[a] | 3.39 ± 0.83[a] | 1.94 ± 0.52[b] | 6.90 ± 3.33[a] | 6.18 ± 3.71[a] | 3.25 ± 1.48[b] |
| Total n-6 | 28.65 ± 8.11[a] | 31.22 ± 6.71[a] | 41.27 ± 4.88[b] | 9.52 ± 3.80[a] | 9.85 ± 4.97[a] | 16.27 ± 4.88[b] |
| Total n-3 | 5.62 ± 1.46[a] | 4.57 ± 1.12[ab] | 3.80 ± 0.59[b] | 9.72 ± 3.32[a] | 8.46 ± 3.78[a] | 5.71 ± 1.58[b] |

Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Within rows, different superscript indicates significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. Levels of EPA were below the detection limit in subcutaneous and visceral adipose tissue and the quadriceps

**Supplementary Table S5.3** Whole body, lumbar vertebrae 1-4, femur and tibia area, bone mineral content (BMC) and areal bone mineral density assessed by DXA in rats fed a control, HFD, or HFD+AA diet at baseline, wk 3 and wk 6

| | Time (wk) | CTRL | HFD | HFD+AA |
|---|---|---|---|---|
| **Whole body** | | | | |
| Area ($cm^2$) | 0 | 26.17 ± 2.09 | 27.03 ± 2.07 | 26.56 ± 3.17 |
| | 3 | 61.42 ± 3.27 | 62.49 – 4.48 | 63.74 ± 4.02 |
| | 6 | 86.01 ± 4.02 | 85.51 ± 4.37 | 87.27 ± 5.55 |
| BMC (g) | 0 | 2.32 ± 0.23 | 2.39 ± 0.21 | 2.34 ± 0.34 |
| | 3 | 7.64 ± 0.46 | 7.82 ± 0.67 | 7.89 ± 0.69 |
| | 6 | 13.05 ± 0.75 | 13.09 ± 0.84 | 13.08 ± 1.20 |
| aBMD ($g/cm^2$) | 0 | 0.088 ± 0.002 | 0.088 ± 0.003 | 0.088 ± 0.003 |
| | 3 | 0.124 ± 0.003 | 0.125 ± 0.004 | 0.123 ± 0.004 |
| | 6 | 0.152 ± 0.005 | 0.153 ± 0.004 | 0.150 ± 0.005 |
| **Lumbar vertebrae 1-4** | | | | |
| Area ($cm^2$) | 0 | 0.60 ± 0.03 | 0.62 ± 0.05 | 0.62 ± 0.06 |
| | 3 | 1.20 ± 0.10 | 1.15 ± 0.10 | 1.18 ± 0.08 |
| | 6 | 1.53 ± 0.08 | 1.49 ± 0.10 | 1.48 ± 0.12 |
| BMC (g) | 0 | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.01 |
| | 3 | 0.23 ± 0.02 | 0.22 ± 0.02 | 0.23 ± 0.02 |
| | 6 | 0.43 ± 0.02 | 0.42 ± 0.03 | 0.41 ± 0.04 |
| aBMD ($g/cm^2$) | 0 | 0.141 ± 0.009 | 0.134 ± 0.011 | 0.133 ± 0.009 |
| | 3 | 0.194 ± 0.017 | 0.195 ± 0.013 | 0.193 ± 0.011 |
| | 6 | 0.278 ± 0.015 | 0.280 ± 0.017 | 0.275 ± 0.015 |
| **Left femur** | | | | |
| Area ($cm^2$) | 0 | 0.53 ± 0.04 | 0.51 ± 0.03 | 0.52 ± 0.05 |
| | 3 | 0.93 ± 0.04 | 0.88 ± 0.06 | 0.88 ± 0.08 |
| | 6 | $1.08 \pm 0.05^a$ | $1.00 \pm 0.06^b$ | $1.02 \pm 0.07^b$ |
| BMC (g) | 0 | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.01 |
| | 3 | 0.26 ± 0.02 | 0.25 ± 0.03 | 0.25 ± 0.02 |
| | 6 | 0.44 ± 0.03 | 0.44 ± 0.04 | 0.43 ± 0.02 |
| aBMD ($g/cm^2$) | 0 | 0.148 ± 0.013 | 0.154 ± 0.017 | 0.151 ± 0.010 |
| | 3 | 0.277 ± 0.019 | 0.285 ± 0.029 | 0.284 ± 0.031 |
| | 6 | $0.404 \pm 0.027^a$ | $0.439 \pm 0.032^b$ | $0.421 \pm 0.028^b$ |
| **Left tibia** | | | | |
| Area ($cm^2$) | 0 | 0.62 ± 0.05 | 0.64 ± 0.07 | 0.66 ± 0.08 |
| | 3 | 1.14 ± 0.09 | 1.14 ± 0.10 | 1.14 ± 0.09 |
| | 6 | 1.45 ± 0.11 | 1.45 ± 0.09 | 1.44 ± 0.08 |
| BMC (g) | 0 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| | 3 | 0.22 ± 0.02 | 0.21 ± 0.02 | 0.21 ± 0.02 |
| | 6 | 0.36 ± 0.03 | 0.37 ± 0.04 | 0.35 ± 0.02 |
| aBMD ($g/cm^2$) | 0 | 0.107 ± 0.010 | 0.111 ± 0.011 | 0.108 ± 0.011 |
| | 3 | 0.189 ± 0.016 | 0.181 ± 0.018 | 0.183 ± 0.019 |
| | 6 | 0.245 ± 0.015 | 0.252 ± 0.020 | 0.242 ± 0.011 |

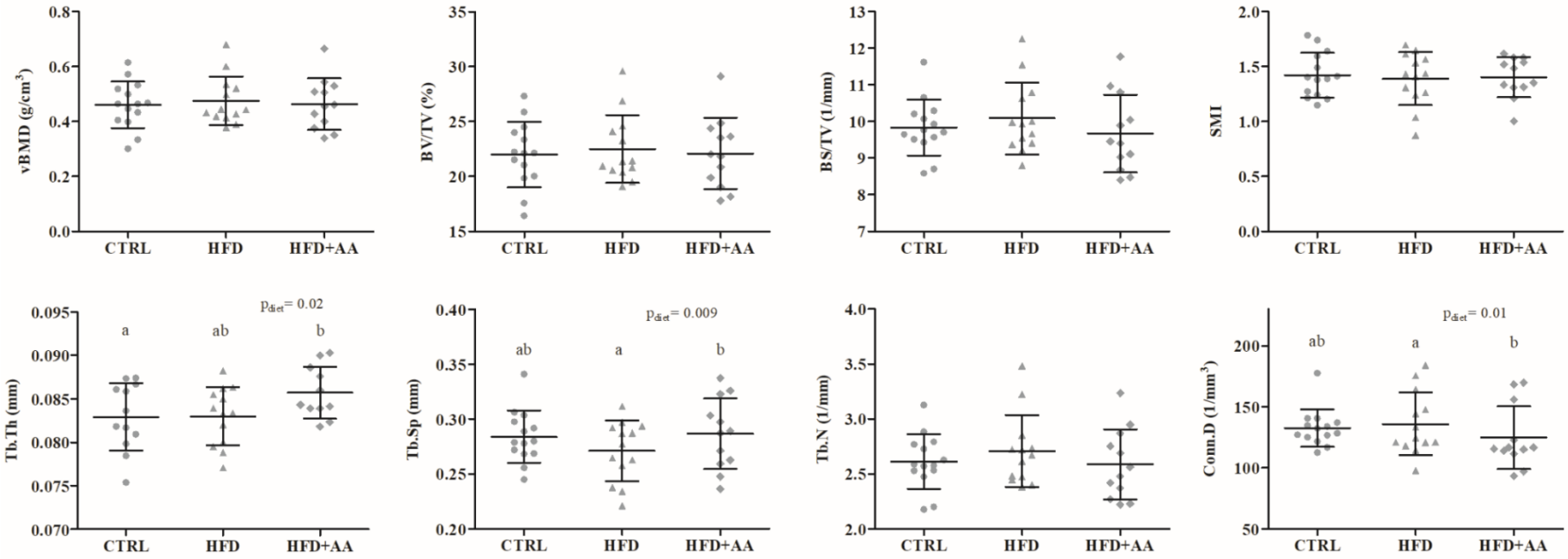
Data are mean ± SD. Significant differences determined using MIXED model ANOVA with post-hoc Tukey comparisons. Within rows, different superscript indicates significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. aBMD, areal bone mineral density; BMC, bone mineral content

**Supplementary Table S5.4** Fatty acid profile (expressed as % weight of total FA) in 3T3-L1 adipocytes following 48 h fatty acid treatment

| | Conc (µM) | AA | EPA | DPA | DHA |
|---|---|---|---|---|---|
| C16:0 | 10 | 31.41 ± 4.06$^{a}$ | 34.50 ± 0.42$^{a}$ | 32.86 ± 0.76$^{a}$ | 32.51 ± 0.32$^{a}$ |
| | 25 | 28.11 ± 3.61$^{a}$ | 27.81 ± 1.15$^{b}$ | 27.25 ± 0.62$^{b}$ | 27.08 ± 0.67$^{b}$ |
| | 50 | 24.57 ± 0.03$^{ab}$ | 23.37 ± 1.08$^{c}$ | 23.69 ± 0.29$^{c}$ | 25.22 ± 0.11$^{a}$ |
| | 100 | 18.22 ± 0.16$^{b}$ | 18.62 ± 0.21$^{d}$ | 19.58 ± 0.61$^{d}$ | 28.45 ± 0.87$^{b}$ |
| C16:1 | 10 | 16.80 ± 2.37$^{a}$ | 19.09 ± 0.46$^{a}$ | 18.11 ± 0.37$^{a}$ | 19.03 ± 0.25$^{a}$ |
| | 25 | 14.45 ± 1.90$^{ab}$ | 14.75 ± 0.71$^{b}$ | 14.08 ± 0.62$^{b}$ | 15.98 ± 0.59$^{b}$ |
| | 50 | 12.23 ± 0.15$^{b}$ | 13.85 ± 0.60$^{b}$ | 13.09 ± 0.45$^{b}$ | 15.15 ± 0.22$^{b}$ |
| | 100 | 8.55 ± 0.25$^{c}$ | 11.11 ± 0.14$^{c}$ | 10.39 ± 1.29$^{c}$ | 15.57 ± 0.62$^{b}$ |
| C18:0 | 10 | 6.91 ± 0.59$^{a}$ | 6.75 ± 0.06$^{a}$ | 7.53 ± 0.59 | 6.67 ± 0.42$^{a}$ |
| | 25 | 6.33 ± 0.60$^{a}$ | 7.46 ± 0.89$^{a}$ | 7.20 ± 1.01 | 5.72 ± 0.54$^{ab}$ |
| | 50 | 5.81 ± 0.31$^{ab}$ | 5.71 ± 0.82$^{a}$ | 5.27 ± 0.81 | 4.48 ± 0.22$^{c}$ |
| | 100 | 5.00 ± 0.48$^{b}$ | 3.88 ± 0.32$^{b}$ | 4.60 ± 2.23 | 5.97 ± 0.49$^{bc}$ |
| C18:1 | 10 | 10.67 ± 1.49$^{a}$ | 11.26 ± 0.27$^{a}$ | 12.09 ± 0.32$^{a}$ | 11.68 ± 0.14$^{a}$ |
| | 25 | 9.08 ± 1.16$^{a}$ | 9.86 ± 0.27$^{b}$ | 10.00 ± 0.11$^{b}$ | 9.67 ± 0.26$^{b}$ |
| | 50 | 8.26 ± 0.08$^{ab}$ | 8.26 ± 0.10$^{c}$ | 7.91 ± 0.09$^{c}$ | 9.31 ± 0.08$^{b}$ |
| | 100 | 6.17 ± 0.10$^{b}$ | 6.66 ± 0.09$^{d}$ | 6.37 ± 0.54$^{d}$ | 9.93 ± 0.23$^{b}$ |
| C18:2 n-6 | 10 | 1.16 ± 0.22 | 1.41 ± 0.15 | 1.74 ± 0.55 | 1.45 ± 0.42 |
| | 25 | 1.59 ± 0.12 | 1.54 ± 0.62 | 1.56 ± 0.49 | 1.56 ± 0.68 |
| | 50 | 1.21 ± 0.24 | 1.57 ± 0.73 | 1.28 ± 0.43 | 1.27 ± 0.42 |
| | 100 | 1.15 ± 0.34 | 0.70 ± 0.31 | 1.66 ± 1.56 | 1.51 ± 0.37 |
| C18:3 n-6 | 10 | 0.51 ± 0.44 | 0.21± 0.01$^{a}$ | 0.48 ± 0.18 | 0.19 ± 0.03 |
| | 25 | 0.16 ± 0.00 | 0.61 ± 0.19$^{b}$ | 0.44 ± 0.16 | 0.17 ± 0.03 |
| | 50 | 0.23 ± 0.00 | 0.36 ± 0.13$^{ab}$ | 0.35 ± 0.02 | 0.15 ± 0.02 |
| | 100 | 0.34 ± 0.04 | 0.27 ± 0.08$^{a}$ | 0.56 ± 0.56 | 0.17 ± 0.03 |
| C20:3 n-6 | 10 | 1.05 ± 0.08$^{a}$ | 0.19 ± 0.01$^{a}$ | 0.38 ± 0.26 | 0.20 ± 0.05 |
| | 25 | ND | 0.63 ± 0.22$^{b}$ | 0.18 ± 0.01 | 0.22 ± 0.05 |
| | 50 | 0.24 ± 0.05$^{ab}$ | 0.34 ± 0.11$^{ab}$ | 0.28 ± 0.13 | 0.14 ± 0.07 |
| | 100 | 0.34 ± 0.08$^{b}$ | 0.25 ± 0.12$^{a}$ | 0.55 ± 0.53 | 0.09 ± 0.01 |
| C20:4 n-6 | 10 | 11.82 ± 1.56$^{a}$ | 2.16 ± 0.21$^{a}$ | 2.35 ± 0.06$^{a}$ | 2.09 ± 0.14$^{a}$ |
| | 25 | 22.39 ± 2.65$^{b}$ | 1.97 ± 0.20$^{a}$ | 1.90 ± 0.07$^{ab}$ | 1.62 ± 0.09$^{a}$ |
| | 50 | 37.99 ± 0.37$^{c}$ | 1.44 ± 0.13$^{ab}$ | 1.45 ± 0.12$^{b}$ | 1.01 ± 0.03$^{b}$ |
| | 100 | 53.05 ± 0.70$^{d}$ | 0.77 ± 0.51$^{b}$ | 0.48 ± 0.37$^{c}$ | 0.32 ± 0.33$^{c}$ |
| C20:5 n-3 | 10 | 0.32 ± 0.04 | 11.91 ± 0.42$^{a}$ | 4.34 ± 0.15$^{a}$ | 0.63 ± 0.01$^{a}$ |
| | 25 | 0.30 ± 0.04 | 24.55 ± 0.48$^{b}$ | 6.86 ± 0.32$^{bc}$ | 0.93 ± 0.09$^{b}$ |
| | 50 | 0.26 ± 0.01 | 35.15 ± 0.83$^{c}$ | 7.71 ± 0.40$^{c}$ | 0.72 ± 0.02$^{a}$ |
| | 100 | 0.29 ± 0.01 | 50.68 ± 0.09$^{d}$ | 6.64 ± 0.46$^{b}$ | 0.47 ± 0.01$^{c}$ |
| C22:5 n-3 | 10 | 0.48 ± 0.07$^{a}$ | 1.99 ± 0.08$^{a}$ | 11.31 ± 0.27$^{a}$ | 0.48 ± 0.01$^{a}$ |
| | 25 | 0.46 ± 0.06$^{a}$ | 2.85 ± 0.06$^{b}$ | 22.14 ± 0.79$^{b}$ | 0.67 ± 0.06$^{b}$ |
| | 50 | 0.37 ± 0.01$^{ab}$ | 2.45 ± 0.25$^{c}$ | 32.54 ± 1.23$^{c}$ | 0.46 ± 0.01$^{a}$ |
| | 100 | 0.29 ± 0.06$^{b}$ | 2.06 ± 0.02$^{a}$ | 43.13 ± 3.51$^{d}$ | 0.35 ± 0.02$^{c}$ |
| C22:6 n-3 | 10 | 0.78 ± 0.12$^{a}$ | 0.83 ± 0.03 | 0.91 ± 0.01 | 15.30 ± 0.26$^{a}$ |
| | 25 | 0.68 ± 0.07$^{ab}$ | 0.62 ± 0.37 | 0.72 ± 0.01 | 27.28 ± 1.08$^{b}$ |
| | 50 | 0.59 ± 0.00$^{b}$ | 0.59 ± 0.06 | 0.50 ± 0.01 | 34.31 ± 1.18$^{c}$ |
| | 100 | 0.43 ± 0.01$^{c}$ | 0.35 ± 0.01 | 13.26 ± 2.37 | 29.65 ± 0.42$^{c}$ |

Data are mean ± SD. Differences were determined using one-way ANOVA with post-hoc Tukey comparisons. Different superscript indicates significant differences among treatment concentration ($p<0.05$). All treatment was performed in triplicates and repeated 3 times

**Supplementary Figure S5.1** *Ex vivo* μCT measurements of the trabecular micro-architectural properties at the lumbar vertebrae 3. Data are mean ± SD. Differences were determined using MIXED model ANOVA with post-hoc Tukey comparisons. Different superscripts indicate significant differences among diet groups ($p<0.05$). CTRL, n=14, HFD, n=14, HFD+AA, n=13. BV/TV, bone volume fraction; BS/TV, bone surface density; Conn.D, connectivity density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; vBMD, volumetric bone mineral density



# CHAPTER 6

## General Discussion

The principle objective of this dissertation was to advance understanding of the role of long-chain polyunsaturated fatty acids (LCPUFA) *in vivo* on bone mass and architecture in children with obesity. The important mediators in this relationship were examined using an animal model of diet-induced obesity. Studying obesity during childhood presents a unique opportunity to examine the bone-fat relationship during a period of rapid growth and maturation, where physiological changes and nutrient requirements are distinct from that in infancy or adulthood. This chapter presents additional results and specific details pertaining to these physiological needs and discusses additional considerations as they relate to the study findings. Despite several limitations, this dissertation contributes to filling important knowledge gaps particularly with respect to the health impact of omega-6 (n-6) LCPUFA in childhood and early adolescence; and is positioned to extend recommendations for future related studies.

**6.1 Study 1**

***Aim 1:*** The first study aimed to describe the diet of children with obesity, and to determine if dietary intakes of LCUPFA status relate to body composition. The hypothesis stated that children with obesity would have suboptimal LCPUFA intake, and that omega-3 (n-3) LCPUFA status measured in red blood cell (RBC) membranes would inversely relate to adiposity, due in part to lower availability in the diet. Median dietary intakes of n-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) combined (32.3 mg/d; IQR 12.0 - 86.7) were substantially below recommendation. Fish and seafood were not common constituents of the diet. Adiposity was negatively related to α-linolenic acid (ALA), EPA and DHA proportions in RBC. The results supported the hypothesis that children with high adiposity had low n-3 LCPUFA status ascribed, in part, to suboptimal dietary intakes.

### 6.1.1 Diet quality and food sources of n-3 LCPUFA

The increased prevalence of childhood overweight and obesity, coupled with knowledge that childhood food intake patterns track through to adulthood [365], has led to an increased need in characterizing the dietary intakes of children. The present study focused specifically on the intakes of LCPUFA, but the inherent complexity of dietary intakes may be missed by analyzing the effects of a single nutrient category. Therefore, the study also explored analysis of foods and/or food groups from which the fatty acids were derived from, offering the benefits of integrating nutrients and insight on the quality of the diet as a whole. Analyses of dietary information collected from Canadian adults and children (n=611, 1.5-66 y) showed that dietary EPA, DHA, and arachidonic acid (AA) were positively associated with protein, but not fat intake as a percentage of energy, while only linoleic acid (LA) and ALA intakes were positively associated with dietary fat intake (% kcal) [366]. Consistently, eggs and poultry meats represented the most common sources for EPA and DHA of children in the present study, and ALA was consumed in the form of margarine, salad dressings, and commercial baked products. These findings, hence, raise the question if recommendations for dietary EPA and DHA should be directed to the choice of foods that are rich in EPA and DHA (e.g., fatty fish), rather than considering their intakes together with n-6 and n-3 fatty acids found primarily in vegetable oils. The distinction of ALA or LA from their longer chain derivatives may be especially important as children with obesity are often counseled to select lower fat alternatives [367-369], but the decrease in overall fat intake might be attained at the expense of the physiologically active LCPUFA.

An important drawback in the modelling of Canada's Food Guide (CFG, 2007) was the use of composite foods based on food choices of Canadians and food popularity (*Provincial*

*Nutrition Surveys*). The problem with such is that as Canadians' eating habits deviate significantly from a healthy diet, recommendations based on the then food choices are not reflective of the appropriate choices for the role of diet in chronic disease prevention. The revised version (2019) has acknowledged this limitation and provides visual representation, in addition to specific statements that speak to high-quality food choices. As such, specific recommendations to "consume foods with healthy fats" and "try different fishes such as trout, salmon, herring, mackerel" are added to the update. While the dietary data in this thesis was not classified into foods based on food quality, it can be predicted that a significant proportion of foods consumed by children in the present study does not align with CFG 2019 recommendations; nor those set in 2007. Specifically, on the occasions where fish intake was reported, about 20% was contributed by fried fish sticks/burgers. Despite the best efforts of Health Canada to promote specific fishes, the public may still assume all fish provide heart-healthy n-3 LCPUFA and do not distinguish among species [370]. For example, cod, tilapia and shrimp remain good choices relative to most terrestrial meats when baked or broiled, but not when breaded or fried. Children with obesity are also more likely to exhibit fussy eating patterns and unwilling to consume unfamiliar foods, as a result parents may offer foods based on children's tastes and preferences [371]. In addition, common barriers including cost of fish, accessibility, the lack of inspiration or cooking skills, and differences in cultural background remain to be important determinants of fish consumption [260]. Hence, guidelines and education strategies developed for families and children with obesity may need to be adapted depending on the availability and acceptability of the healthier alternative.

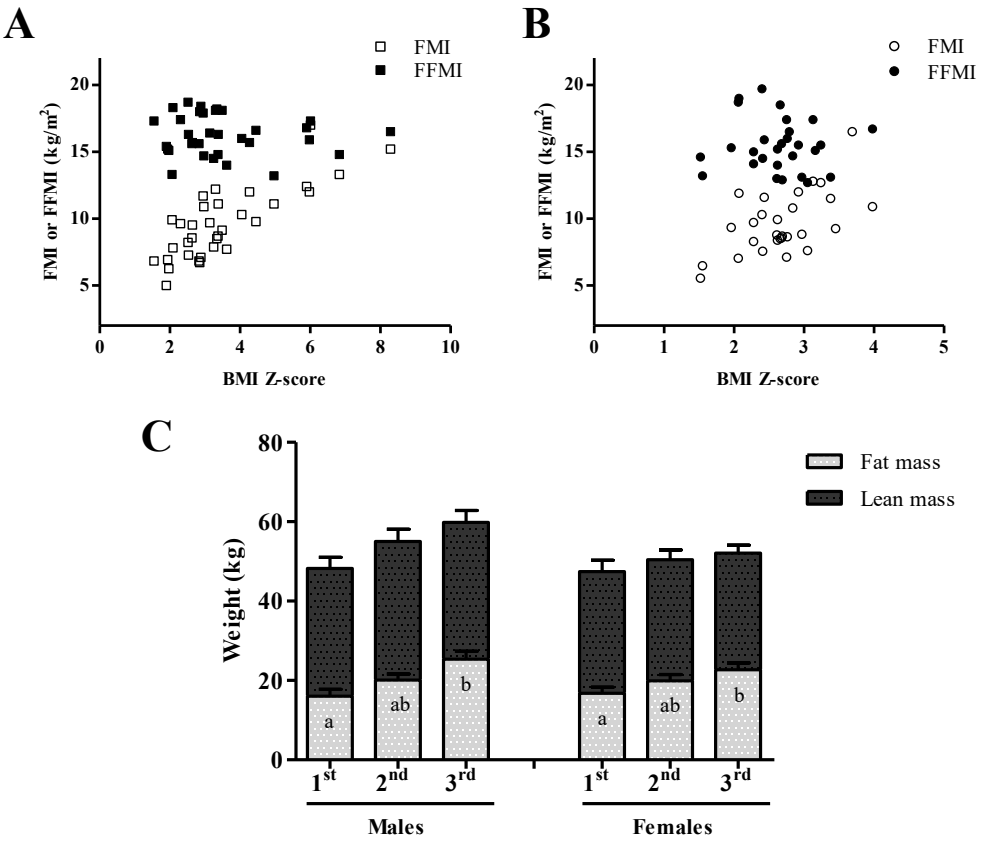
### 6.1.2 Lean body mass in children with obesity

The cross-sectional design of the present study precluded the tracking of changes in body

composition with maturation status in children with obesity. We observed that only fat-mass index (FMI), but not fat-free mass index (FFMI) was associated with body mass index (BMI) Z-score. The relationship was sex-dependent and stronger in males, where the increase in FMI for each unit of higher BMI Z-score was 1.75-fold greater than that in females (**Figure 6.1**). This suggested that an increase in adiposity was not coupled with a proportionate lean mass accrual; and in girls, lean mass is notably under-developed (i.e. reduced FFMI) compared to reference data on normal weight age-matched peers. Children grow taller and heavier as they mature, hence excess energy intake beyond daily activities is essential for growth. There is however, a constraint on the size of lean body mass, as there is for stature. Therefore, the accelerated weight gain in children with obesity mostly consists of body fat and not lean mass [372].

The sex dimorphism in the relation between adiposity and BMI Z-score may also be explained by differences in physical activity level. Although there was no interaction between sex and age in time spent (minutes /day) on physical activity in the present study, the limitations in the data collected have been discussed in section 4.5 and must be interpreted with caution. A longitudinal study in a representative sample of children found that while daily total energy expenditure in males increased continuously between ages 5 and 10 y, that of females declined from 1800 kcal at age 6 to 1600 kcal at 9 y. This reduction was explained by a 50% lower time spent on physical activity between ages 6 and 9 y [373]. The benefits of encouraging physical activity in children with obesity may extend beyond changes in energy expenditure by providing additional stimuli for lean mass accrual and possibly benefit skeletal health.

**Figure 6.1** Correlation between FMI and FFMI with BMI Z-score in (A) males and (B) females; (C) absolute lean mass and fat mass by tertiles of % body fat measured by DXA




The top panel shows the simple correlation between BMI Z-score, Fat mass index (FMI) and Fat-free mass index (FFMI) in **(A)** males and **(B)** females. BMI Z-score positively related with FMI in both sexes (males: $\beta = 1.36$, $r^2 = 0.64$, $p<.0001$; females: $\beta = 2.38$, $r^2 = 0.35$, $p=0.0009$). There were no significant association between BMI Z-score and FFMI. **(C)** The bottom panel shows the differences in absolute lean mass and fat mass by tertiles of % body fat measured by DXA. Groups were compared using a MIXED model ANOVA, data are shown as mean ± SEM, and were adjusted for age, sex, Tanner stage and race (white vs non-white). Fat mass was significantly higher in tertile 3 compared to tertile 1 ($p=0.0002$); there was no interaction with sex. Lean mass did not differ across tertiles or between sexes. n=63

## 6.2 Study 2

***Aim 2:*** The second study aimed to examine the relationships between LCPUFA status, bone mass and geometry in children with obesity. The hypothesis was that higher n-6 LCPUFA status in children with obesity would be negatively related to bone health outcomes, particularly at sites where fractures are common. Children with the highest RBC AA status (expressed as the proportion of total fatty acids) had deficits in distal forearm bone mass, cortical bone geometry, muscle mass and predicted strength. AA status was also positively associated with biomarkers of insulin resistance (IR). The results supported the hypothesis that an inverse relationship exists between AA and bone, and may have further influences on glucose homeostasis.

### 6.2.1 Requirement of AA during growth

The balance between these LCPUFA is important to the quality of growth. Much of the research has evolved from studies in infancy. For example, preterm infants fed a marine oil-supplemented formula containing 0.3% w/w EPA and 0.2% DHA had significantly lower Z-scores for weight, length and head circumference [374]. The growth restriction observed was related to the decline in AA status [375] and can be corrected when the n-3 and n-6 LCPUFA are provided in balance. A recent study comparing infant formulas with 17 mg/100 kcal DHA and 25 mg or 34 mg/100 kcal AA (DHA:AA ratios of 1:2 or 1:1.5 respectively) in healthy term infants showed no effects on growth nor DHA availability for central nervous system development through 120 days of age [376]. Beneficial outcomes may even be observed; early life (4 to 12 wk) supplementation of AA (0.13 % w/w) and DHA (0.09 % w/w) in ApoE*3Leiden mice limited high-fat diet (HFD)-induced body weight and fat mass gain later in life (12 to 20 wk). The AA/DHA supplement was more efficient in reducing plasma lipids and adipose tissue macrophage infiltration compared to the clinically applied n-3 LCPUFA mixture of EPA (0.3%

w/w) and DHA (0.25% w/w) (OMACOR®) [377].

Few explicit dietary recommendations for AA exist to date; possibly based on the understanding that AA insufficiency is unlikely as it is plentiful in the diet, and it can be synthesized endogenously from LA. The Early Nutrition Academy recommended 140 mg AA/day during the first months of life [378]. Contrarily, the European Food Safety Authority has stated that "there is no necessity to add AA to infant formula even in the presence of DHA" [379]. This statement has been heavily challenged as endogenous synthesis of AA in early life is low [380, 381], and the requirement for AA cannot be met by the low quantities found in complementary foods alone [382]. A recent review of dietary LCPUFA (AA, EPA and DHA) intakes in adults surveyed from 175 countries reported that AA intakes ranged from 101 to 351 mg/d and 44-331 mg/d in advanced and developing countries, respectively [383]. In contrast to infancy, childhood and adolescence represent a life stage that is markedly under-represented in the field of LCPUFA. It is uncertain if high n-6 LCPUFA intake and/or status during this period will show similar associations as observed in adults, or if children have a greater requirement and/or tolerance for high n-6 intake given the crucial role in growth. The following studies aimed to fill this knowledge gap through investigating the relationship between n-6 fatty acids and growth in children and adolescents.

### 6.2.2 Influence of AA on bone

While a wealth of evidence exists on the association between n-3 LCPUFA and bone health, to our knowledge no reports to date have focused primarily on the role of AA in human children. Previous studies that have focused on periods of growth were limited to those in animal models. Maternal supplementation with dietary AA and DHA during lactation enhanced whole body areal bone mineral density (aBMD) in rat pups with reduced birth weight [384]. Dietary

supplementation with AA (0.6-0.75% fat) and DHA (0.1% fat) elevated whole body bone mineral content (BMC) in male piglets (5 d) compared to those supplemented with 0.3-0.45% AA [385]. In older adults recruited in the Framingham Osteoporosis Study, plasma AA status was protective against hip fractures in women with high EPA+DHA intakes, but was detrimental in men with the lowest intake of EPA+DHA [44]. These results suggest that the effects of AA may be dependent on EPA+DHA intakes. Consistently, supplementation of 840 mg/d AA in healthy Japanese adults (26-60 y) with high habitual EPA and DHA intakes had no effect on blood lipids or platelet aggregation [386]. Earlier AA supplementation trials in populations with low fish intake (USA and UK) reported increased synthesis of vasoactive eicosanoids [387] and modified immune responses [388, 389], but there was no evidence for a directly promotive role of AA on cardiovascular disease risk [390, 391]. Even though bone outcomes were not measured, results from the trials largely suggested that the adverse effects associated with increased AA intake, at least up to 1.5 g/d, are null [392]. Results from the present study are therefore useful for guiding future interventions investigating influence of high dietary AA complemented with adequate EPA and DHA while monitoring bone health outcomes during growth.

### 6.2.3 AA and skeletal muscle growth

AA makes up to 15-17% of total fatty acids in skeletal muscle in humans [393], *in vitro* evidence has demonstrated a positive influence of AA on lean mass accrual [394]; therefore, an increase in AA status can be speculated to affect skeletal muscle growth. Contrarily, we observed approximately 15% deficit in forearm muscle area at the radial diaphysis, but not in the whole body of children with higher AA status. There were no differences in muscle density observed among groups, suggesting the lower muscle area is not related to intramuscular lipid accumulation. The anabolic effect of AA (3-25 μM) is largely mediated through stimulating

prostaglandin $E_2$ ($PGE_2$) and $PGF_{2\alpha}$ release via cyclooxygenase (COX)-2 activity, resulting in cell hypertrophy (myotube size) and myonuclear accretion in C2C12 murine skeletal myocytes [395]. Interestingly, the net effect of AA may depend on the relative quantities of individual eicosanoid synthesized. Rates of protein synthesis and degradation in murine striated muscle cells were increased by $PGF_{2\alpha}$ and $PGE_2$, respectively [396]. While we were unable to obtain muscle biopsies from children for the assessment of tissue AA content and local $PGE_2$ concentrations, circulating plasma AA are known to reflect levels of AA in skeletal muscle [397, 398]. The inverse relationship observed in the present study suggested the anabolic effect of AA/$PGE_2$ on lean mass accrual is blunted in obesity. In addition, the clinical relevance of this difference in muscle area at the forearm needs to be further considered in relation to whole-body lean mass and future risk of fracture at forearm sites. New studies will be able to build upon the data brought forth in this thesis.

### 6.2.4 Modulation of insulin resistance by AA and effect on bone metabolism

The possible interaction observed between LCPUFA status, IR, and bone are intriguing; yet the single measurement collected in the present study requires confirmation using more specific clinical tests and does not offer inference on causality. Plasma and adipose tissue concentrations of AA positively related to IR in both children [289] and adults that are healthy [399]. Exposure of mature 3T3-L1 adipocytes to AA lowered GLUT4 mRNA and protein content; and AA treatment diminished the response of GLUT4 transporters to insulin-mediated glucose uptake [400], suggesting AA, at least *in vitro*, has a direct role in the development of IR.

Insulin-like growth factor-1 (IGF-1) may be an important mediator in the relationship between IR and skeletal growth during the pre-pubertal years. The role of IGF-1 as a crucial determinant in stimulating appositional bone growth is well-characterized [401]. Plasma AA

proportions positively related to IGF-1 levels in piglets [385]. Measurements of IGF-1 levels were not available in the present study, but given insulin and IGF-1 share a common cellular signaling cascade, it can be speculated that children with higher AA status and increased fasting insulin would also have higher IGF-1 levels [402]. In support of this, children (n=315, 9-13 y) with high Homeostasis Model Assessment for Insulin Resistance (HOMA-IR, ≥ 4.0) also had greater circulating IGF-1, and IGF-1 levels positively predicted cortical bone size and bending strength at the 66% radius. However, path analyses demonstrated that such relationships were moderated by high HOMA-IR, implicating IR as a potential suppressor of IGF-1-dependent cortical bone development [305]. HOMA-IR also negatively predicted dual-energy X-ray absorptiometry (DXA)-measured total body bone area and mass (β=-0.10 and -0.08 respectively), as well as trabecular volumetric BMD (β=-0.21) and bone strength index (β=-0.18) at the 4% tibia in girls (9-12 y) [403]. The negative relationship between fat mass and lumbar spine BMC in children (n=240, 7-15 y) was thought to be mediated by HOMA-IR [290]. It is noteworthy that although AA status positively related to HOMA-IR in the present study, all observed values were relatively low and considered to be within the norm (range: 0.4 – 2.8). It would be of interest to examine the longitudinal changes in fatty acid composition with HOMA-IR during growth, and at what threshold, beyond the transient increase of IR during puberty, does excess IR begin to impact bone metabolism.

### 6.2.5 Assessment of bone mass and body composition

DXA represents a safe, rapid, and clinically suitable reference method to analyze bone mass/density, and body composition among children and adolescents [404]. However, the two-dimensional assessment of DXA is prone to projection errors and can be confounded by adiposity and bone size [405]. While scans of the lumbar spine and total body in general can

provide information about the status of trabecular and cortical bone, respectively [294], assessment of bone mass at the spine or whole body do not adequately reveal risk of fracture in a long bone. In addition, assessments of the spine will not be feasible in individuals with obesity who may exceed the scanner's weight limit (135 – 205 kg), which could be the case if the study participants were to extend beyond the pre-teen years (upper range of body weight at baseline: 118 kg). The distal forearm offers a more practical and representative site for estimating fracture risk, especially given that children with obesity have a disproportionate rate of forearm fractures [22]. An additional advantage of the forearm scan is that it is conducted with the person sitting on a chair and not on the bed; i.e. no concern for bed weight/size limits. In addition, DXA measurements of the forearm are also useful for complementing the cross-sectional images and strength indices obtained by peripheral quantitative computed tomography (pQCT).

The pQCT provides a non-invasive approach to understand changes in cortical and trabecular geometry and strength during skeletal development. pQCT measurements are usually confined to a particular slice of the radius and may not necessarily reflect changes at proximal appendicular sites and central skeletal sites. Despite this potential limitation, emerging research has used pQCT to quantify regional muscle density to gain insight on fat infiltration of skeletal muscle which may have important implications on bone turnover. Reference data for regional tissue distribution at the forearm in children and adolescents have recently become available [406]; and allows for calculation of Z-scores to detect early changes in tissue composition. pQCT also offers the potential for assessing bone marrow adiposity, a fat depot that is increasingly recognized to be an important mediator of the bone-fat relationship. A recent study in healthy children and adolescents [407] reported that whole body adiposity is positively associated with bone marrow adipose tissue content measured by pQCT at the 65% radius. The influence of

marrow adipose tissue on bone during growth is conflicted, with some showing a negative [408, 409] or no effect [410, 411] on trabecular and/or cortical bone in animal studies. Both marrow fat and osteoblast differentiation increase during puberty, suggesting that marrow fat may be necessary for osteoblasts to produce bone [412]. Prospective trials utilizing three-dimensional imaging modalities can evaluate if marrow adiposity is increased in children with obesity, and if it is, what effects it has on bone.

### 6.3 Study 3

***Aim 3:*** The third study aimed to determine the effects of a moderately high-fat diet, with or without the addition of AA on bone mass and LCPUFA status during growth in an animal model of diet-induced obesity. The hypothesis was that the adverse effects of diet-induced obesity on bone mass and strength during growth may be exacerbated by AA. Addition of AA to a HFD promoted increases in adiposity and leptin production than HFD alone. AA lowered whole body bone mass, but improved vBMD and microarchitectural properties at the femur metaphysis without impacting bone strength. The results supported the hypothesis that AA is pro-adipogenic whereas the effect on bone is not uniform and may be skeletal-site specific.

The pathophysiology of obesity-related bone impairment is unclear and there are conflicting results from clinical and basic research trying to explain this phenomenon. Observations in children are complicated by: (1) the possible interaction among nutrients and bioactive compounds in the diet; and (2) maturation timing leading to transient changes in body composition and endocrine environment. As mentioned, the effects of AA may be due, in part, to protein intake, as AA is found in protein-rich animal food sources, including eggs and meats. Although no differences in protein intake were seen among participants, we cannot rule out possible anabolic effects of high-quality animal proteins on skeletal growth. The employment of

a high-fat diet hence allows for singling out the effect of excess fat intake with other macronnutrients remaining constant when the diet is formulated on an energy basis. Secondly, fat mass rises during growth and puberty; the effect of the natural maturational increase in fat mass on skeletal development and microarchitecture may be different compared to the presence of excess fat mass from obesity. In lieu of a control group with children of normal weight, use of Z-scores were applied in this thesis to describe deviation of our participants from the growth trajectory of children with normal weight. The research in the final project, however, did include a control group and offered more in-depth exploration of the early stages in the etiology of diet-induced obesity using an animal model as well as *in vitro* studies of exposure to AA.

### 6.3.1 Site-specificity of influence of HFD and AA on bone

Animal models of diet-induced obesity offer the advantages of allowing the examination of increased fat intake alone (with other nutrients remaining the same) on bone growth. A differential effect on the central versus peripheral skeleton was observed at the end of 6 wk feeding trial, where AA showed adverse effects on bone in the whole body and lumbar vertebrae, but improved trabecular microarchitecture in long bones. In addition, the reduction in cortical bone size, characterized by reduced total and medullary bone volume at the femur diaphysis corresponded to our observations at the 66% radius in children. The skeletal site-specificity may be attributed to differences in mechanical loading. Contrasting associations between adiposity with bone microarchitecture and strength at non-weight bearing (radial) versus weight-bearing (tibial) sites has been reported in children (8 – 15 y) [413]. The relationship between fat mass and bone strength was essentially non-existent at the radius but fat mass positively related to tibial bone strength. It is, however, necessary to highlight that differences exist in skeletal loading patterns between humans (bipeds) and rodents (quadrupeds). The mechanical loads on

the horizontal spine in quadrupeds differ from mechanical loads in human, in which the spine maintains an upright (vertical) position [414]; biomechanical data describing structural bone changes as a result of loading requires careful scrutinization before extrapolation to humans.

A role for adipo- or myokines (discussed below in 7.3) in bone metabolism may also contribute to the observations at distant skeletal sites that normally experience relatively low bone strain. Total lean mass is a robust predictor of cortical bone size of long bones, including that of the non-weight bearing radius [415]. This suggests that the correlation between cortical bone size and lean mass at that site might be due to either auto and/or paracrine effects of myokines on the radius, or to systemic endocrine effects of myokines on the whole skeleton. Leptin, as described in section 2.1.3.1, may inhibit bone formation through the central nervous system, or on the other hand, enhance bone formation and inhibit bone resorption peripherally, the latter of which is possibly the dominant way leading to a stronger skeleton [416, 417]. In humans, obesity is associated with central resistance to circulating leptin. Consistently, higher leptin concentrations observed in the HFD+AA group did not result in reduced food intake in the present study. It is possible for differential sensitivity to exist between the central (hypothalamus) and peripheral effect of leptin on skeletal growth in obese animals.

### 6.3.2 AA and glucose homeostasis in HFD-fed rats

While in children a higher AA status is associated with increased HOMA-IR, there was no evidence of impaired glucose homeostasis in HFD-fed rats, indicated by lack of differences in fasting insulin concentrations. The increase in body weight and adiposity over 6 wk in HFD-fed rats was modest. In addition, liver and quadriceps fat content (quantified by gas chromatography) in high-fat fed rats were not different from controls, indicating the absence of lipid accumulation in these tissues. Importantly, AA administration is able to prevent HFD induced IR by

maintaining membrane integrity and normal intracellular signalling [418]. Young rats may also display greater metabolic flexibility than older animals and are able to adapt to the energy excess with minimal impairment in glucose homeostasis. The relatively rapid development of obesity in animals also does not necessarily reflect the physiological changes that occur over time in humans. A delay (> 21 days) in the development of IR and impaired glucose transport in response to rapid (2-3 days) increases in intramyocellular lipids was reported in HFD (60% kcal) fed rats [419]. Finally, human studies frequently examine the relationship of fatty acids with an already established metabolic dysfunction, whereas most animal studies are conducted in healthy states at study inception; which aligns with investigation into the etiology of obesity rather than its management. Hence, the time-frame and dosages required to both cause harm, as observed with children in the present thesis, and to subsequently correct the defect in glucose homeostasis is likely different in animal feeding trials. An oral glucose tolerance test was not performed, given that the primary goal of the animal study was to test for bone health outcomes in the early stages of diet-induced obesity, and in light of the normal ranges of HOMA-IR of children in study 2. However, early consequences of adipocyte metabolism in response to exposure to LCPUFA were tested *in vitro*.

### 6.3.3 Effects of LCPUFA on adipocyte metabolism *in vitro*

An *in vitro* model of 3T3-L1 adipocytes was used to derive possible mechanistic explanation for the observations where AA stimulated leptin production despite no differences in visceral adiposity in the HFD versus HFD+AA group. The employment of a model system allows for the potential to identify cellular mediators to explain the influence of AA on adipocyte metabolism. While differentiated 3T3-L1 adipocytes exhibit differences from *in vivo* adipocytes such that they contain multilocular lipid droplets, compared to the single / unilocular droplets

seen in white adipocytes from humans, primary cells are physically and chemically fragile. 3T3-L1 adipocytes offer the additional advantage as a "pure" fat cell model that is free from contamination of stromal vascular cells (e.g. macrophages, T cells) found in adipose tissue.

Given the observations in this thesis, further studies are suggested regarding mechanisms. The predominant adipogenic pathway of AA is suggested to be exerted through the production of prostacyclins (**Figure 6.2**). Prostacyclin acts externally via the cell surface prostacyclin receptor (IP-R), triggering cAMP production and activating the PKA pathway [62]. IP-R is transiently expressed in preadipocytes and synthesis of prostacyclin ceases in mature adipocytes [420, 421]. The requirement of the IP-R system in mediating the adipogenic effect of n-6 LCPUFA was confirmed *in vivo* using an IP-R knockout mouse model, where IP-R deletion prevented the increase in body weight and fat mass induced by HFD enriched in corn oil (15% kcal) or both corn and perilla oil (10 and 5% respectively) [422].

Other pathways that might also be further explored include AA and some of its metabolites generated through COX and lipoxygenase (LOX) that are activators/ligands of the peroxisome proliferator activated receptors (PPAR) [423]. Thus, AA can stimulate adipogenesis through both cell surface IP-R and nuclear PPAR in early and late events of adipogenesis respectively. What remains uncertain is to what extent does AA rely on each of these pathways to stimulate adipogenesis. Tight regulation at the level of membrane esterification exists as unesterified AA is cytotoxic [424]. AA reacylation is very efficient where a large portion of AA released by phospholipase $A_2$ ($PLA_2$) is rapidly incorporated back into phospholipids [425]; only a minor portion is released from cell membrane and converted into oxygenated metabolites [426]. Inhibition of calcium-independent $PLA_2$ by small interfering RNA lowered AA availability and impaired late events of adipogenesis in 3T3-L1 adipocytes [427]. Pancreatic $PLA_2^{-/-}$ mice were

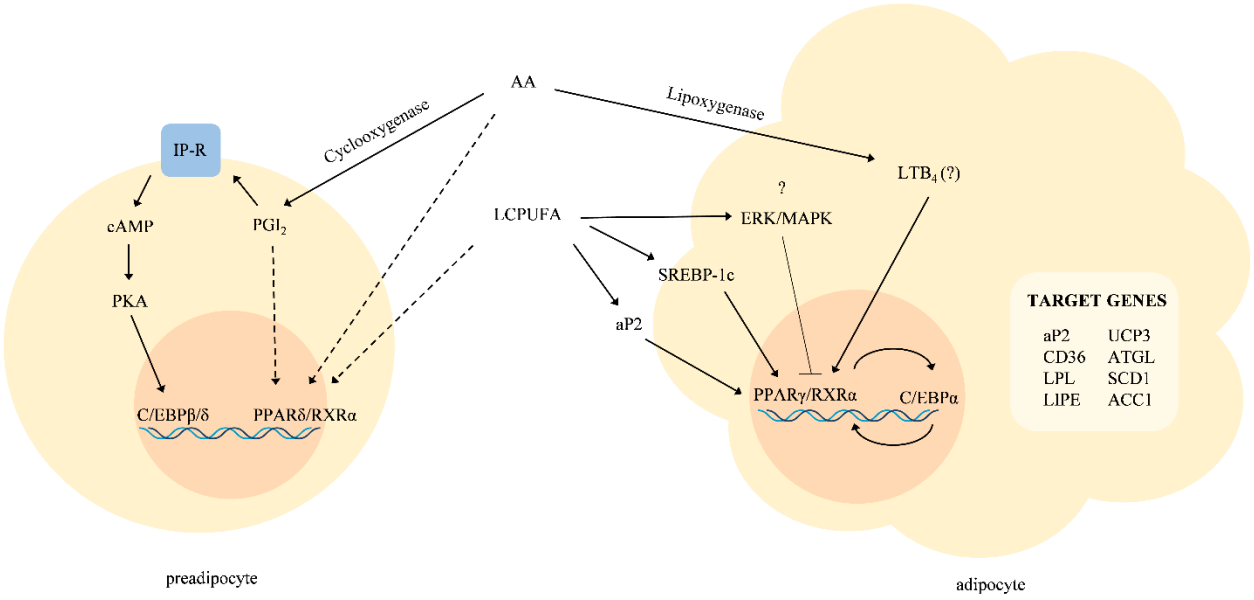
protected against HFD-induced obesity, in part due to decreased lipid absorption [428]. Future studies utilizing stable-isotope labeled fatty acid tracers will provide insight into the uptake of exogenous AA into the cell, incorporated and/or released from the membrane, and subsequently transformed into metabolites that have potent adipogenic properties.

While the present study has focused on the isolated effects of LCPUFA on adipogenesis in adipocytes, the influence of similar treatment on the differentiation of primary bone marrow stem cells, or bone formation / resorption in osteoblastic cells lines should also be explored. PPARγ acts as the master switch between the differentiation of mesenchymal stem cells (MSC) into adipocytes or osteoblasts. It would therefore be expected that activation of PPARγ by excess LCPUFA would lead to an unfavorable balance towards adipogenesis. In bone, PPARγ activation with thiazolidinedione promoted bone resorption through combined effects on osteoblast suppression and osteoclast activation [429]. PPARγ overexpression in MC3T3-E1 osteoblasts reduced ALP activity [430], and osteoblastic cells treated with LCPUFA underwent trans-differentiation into adipocytes [431]. Human MSC differentiated in the presence of AA, but not EPA or DHA, showed reduction in osteoblasts formation and mineralization along with higher PPARγ expression [323]. The activation of PPARγ by LCPUFA may be highly context-dependent, where different tissues and cell types may display varying sensitivities to the presence of LCPUFA. The action of LCPUFA on the bone-fat interaction via PPARγ activation must also be considered beyond the cell-autonomous effects in isolated model systems such as that employed in the present thesis, but also in concert with the systemic effects via other tissues such as the brain, adipose tissue and the gut (**Figure 6.3**).

The beneficial effects of n-3 LCPUFA include modulation of both adipocyte hypertrophy and hyperplasia in rodents [52] An increase in adipocyte number in inguinal fat depots and

decreased plasma leptin concentrations in rats (9 wk) was observed when menhaden oil (3.33 – 6.67%) was added to the diet for 22 wk [432]. Large lipid droplets function more for storage [433], whereas smaller lipid droplets provide a larger surface-area-to-volume ratio, thus favoring lipolysis [434]. Furthermore, hypertrophic adipocytes with large and uniloculuar lipid droplets exhibited impaired insulin-dependent glucose uptake compared to cells with multiloculuar lipid droplets [348]. Histology of adipocytes isolated from visceral fat depots would allow the comparison of the effect of AA on adipocyte morphology *in vivo* relative to our observations *in vitro*. In addition, analysis of gene expression markers specific to preadipocytes (Pref-1, PPARβ/δ, C/EBPβ, and aP2) or mature adipocytes (PPARγ, C/EBPα, and M-CSF) would provide further insight on whether the increase in adipose tissue mass is due to increased recruitment of adipocyte precursor cells, or hypertrophy/hyperplasia of existing cells. Finally, assessment of metabolic outcomes such as lipolysis, or insulin-stimulated glucose uptake will determine if small, multilocular droplets formed following n-3 LCPUFA treatment are metabolically healthier.

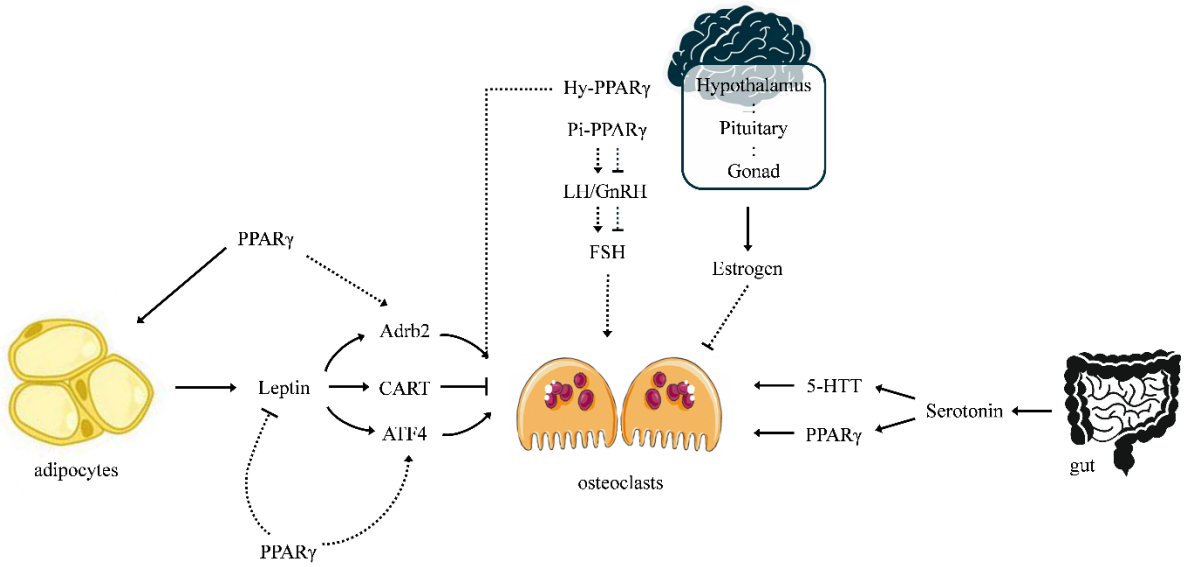
**Figure 6.2** Adipogenic gene regulation by LCPUFA




Adipocyte differentiation is regulated by two families of transcription factors, CCAAT/enhancer-binding proteins (C/EBP) and peroxisome proliferator activated receptor (PPAR)γ. C/EBPβ and C/EBPδ are involved in the early stages of adipogenesis. Once activated, C/EBPβ trans-activates C/EBPα and PPARγ expression, which positively regulate each other and cooperate to promote adipogenesis [435, 436]. Arachidonic acid (AA, C20:4 n-6) up-regulates the expression of C/EBPβ and C/EBPδ via the prostacyclin/IP receptor system and the protein kinase A pathway. AA may also act through prostacyclin as activator/ligand of PPARβ/δ which, in turn, up-regulates the expression of PPARγ. Other LCPUFA also acts as activators / ligands of PPARβ/δ or PPARγ [437, 438]. Activation of PPARγ promotes terminal differentiation through the induction of a variety of differentiation-dependent target genes important for triglyceride update and storage, including *aP2, CD36, LPL, PEPCK*, and others [55]. Upon terminal differentiation, production of prostacyclins and other prostaglandin ceases and is accompanied by reduced expression and loss of functional IP-R [439, 440]. The involvement of AA metabolites synthesized through LOX as ligands of PPARγ are also implicated at a later step(s) of adipocyte maturation [441]. It is noteworthy that LCPUFA activate all three members of the PPAR family in the micromolar range [442], whereas fatty acid metabolites exhibit a more subtype-selective pattern of activation in the nanomolar range [443]. The ERK/MAPK-pathway has been suggested to be involved in FA signaling pathways. Inclusion of AA enhanced ERK1/2 activation in 3T3-L1 adipocytes and prolonged the period during which ERK2 remains in the active state [444]. Inhibition of MAPK-activation restored adipogenesis in the presence of AA and $PGF_{2\alpha}$, thus MAPK activity is necessary for AA-mediated inhibition of adipocyte differentiation. LCPUFA can also alter the expression of lipogenic genes by interfering with SREBP-1 expression. LCPUFA decreased SREBP-1c mRNA levels and inhibited the proteolytic maturation that is necessary for SREBP to exert transcriptional activity. Forced expression of SREBP-1c induced expression of adipocyte specific genes and increased differentiation of fibroblast cell line NIH-3T3 [445]. SREBP-1c is also able to directly transactivate C/EBPα and induce PPARγ expression

Abbreviations: AA, arachidonic acid; aP2, fatty acid binding protein; cAMP, cyclic adenosine monophosphate; C/EBP, CCAAT/enhancer-binding proteins; ERK, extracellular signal-regulated kinase; IP-R, prostacyclin receptor; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; SREBP, sterol response element binding protein 1-c

**Figure 6.3** Context-dependent effects of PPARγ on bone resorption




The regulation of osteoclastogenesis by PPARγ depends not only on cell-autonomous effects but also systemic effects via other tissues including the hypothalamic-pituitary-gonadal (HPG) axis, fat and the gut. **HPG axis**: PPARγ activation in the hypothalamus and/or pituitary inhibits luteinizing hormone secretion and gonadotropin-releasing hormone (GnRH) signaling and subsequent increase in FSH levels; FSH may enhance ostoeclastogenesis independent of estrogen action [446]. Estrogen decreases osteoclastic resorption put in bone by regulating transcription factors (c-fos, c-jun). inflammatory cytokines (IL-1R) decrease osteoclastogenesis [447]; and promote osteoclast apoptosis [448, 449]. PPAR-induced osteoclast activation and bone loss may involve the suppression of estrogen function and/or production. **Gut**: gut-derived serotonin and its transporter (5-HTT) promotes osteoclastogenesis by activating NF-κB. Serotonin metabolites act as endogenous PPARγ agonists to regulate adipogenesis. Gut-derived serotonin may inhibit bone formation and stimulate bone resorption, at least in part, via PPARγ activation. **Adipocytes:** PPARγ and leptin have been suggested to be mutually inhibitory. PPARγ activation represses leptin expression in adipocytes [205], and leptin downregulates PPARγ mRNA levels in macrophages [450]. PPAR$^{-/-}$ mice exhibited higher serum leptin level that WT littermates [451]. Ligand activation of PPARγ represses Adrb2 expression [452], suggesting that also be contributed by suppressing leptin function and the sympathetic tone. PPARγ may also promote osteoclastogenesis by inducing activating transcription factor 4 (ATF4) in osteoclasts, where it is required for induction of RANK, NFATc1 and MAPK activation [453]. Together, these suggest that PPARγ regulation of osteoclastogenesis may also cross-talk with the leptin/CART/Adrb2/ATF4 pathway via a complex signaling network

Abbreviations: 5-HTT, serotonin transporter; Adrb2, β-2 adrenergic receptor; ATF4, activating transcription factor 4; CART, cocaine amphetamine-regulated transcript; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PPAR, peroxisome proliferator activated receptor

### 6.4 Strengths and limitations

The research encompassed within this thesis has strengths and limitations, some as already discussed. Significant advancements in the measuring and reporting of trials involving fatty acids have been made. As new findings regarding the beneficial effects of LA, the primary dietary n-6 LCPUFA have been observed [392, 454, 455], as well as the emerging conceptual problems and flawed assumption of the n-6: n-3 ratio are known, calls to abandon the use of this ratio have become more frequent [456, 457]. The present study was concepted based on the knowledge that fatty acids of the n-3 and n-6 families are not equipotent in various biological functions in the body, and the absolute quantity consumed in the diet and/or in circulation may be a more important determinant than the ratio alone. Relationships with bone health outcomes were therefore assessed with individual fatty acids (or lipid classes), and in the experimental studies, the use of an AA-only exposure instead of combined AA/DHA was a strength of this research.

Assessments of dietary fatty acids intake alone may be misleading due to physiological and genetic variability in achieving a given blood level. The direct measure of fatty acids in RBC thus provides a more accurate reflection of habitual fatty acids intake compared to that measured in plasma or serum due to its longer half-life (120 days) [243]. The use of a biomarker thus overcame the limitations of dietary recalls in capturing food sources of n-3 LCPUFA due to infrequent intake, nor is it subjected to recall bias associated with dietary assessment methods. Further, whole serum / plasma contains an undefined mixture of 4 lipid classes (phospholipids, triglycerides, cholesteryl esters and free fatty acids), each with its own FA proportion unique to that pool [244]. Except for the free fatty acids, these are all carried in unique proportions in 3 different lipoprotein particles (high-density lipoproteins, very-low density lipoproteins and low-density lipoproteins) and will be influenced by dyslipidemia as well as feeding status (i.e. fasted

vs post-prandial states). RBC membranes, on the other hand, are composed predominantly of phospholipids, hence offer the distinct advantage where they reflect the fatty acid profile in the cell membranes of biological tissues [244]. The inclusion of a biomarker in addition to dietary intake is a strength of the present studies. The analytical and reporting methods employed are compliant to international guidelines [298], yet variation in methodologies and equipment may contribute to the discrepancy in fatty acid profile compared to those previously reported [254]. Despite so, a recent report from Health Canada showed that average omega-3 index in a representative sample of Canadian adults (n=1984, 20-79 y) averaged 4.5% [458], compared to 6.1% reported in US adults [232]. The deviations in EPA+DHA proportions in RBC of the results presented herein therefore may not be as wide given the population mean is in general low.

Although the goal was to design an animal model of diet-induced obesity that mimics obesity in human children, we acknowledge that the standard growth diet (AIN-93G) is comprised of 14% energy as fat, hence on a relative scale the HFD utilized in the present study does not translate to humans. Rats become sexually mature at around 6-8 wk of age, whereas humans do not reach puberty until on average 11.5 y [459]. The exposure duration of animals to the HFD is therefore expected to be longer than children in the MYLIFE study. In addition, the quantity of AA provided in the rodent diet (1% w/w) averaged to 0.3 g/d, which exceeds the average AA consumption of approximately 100 mg/d [378] in adults in both absolute quantity and on a body weight basis. However, taking into account the short time frame (6 wk) of the study and rapid growth pattern of rodents, the modest (6%) difference in body weight beginning at 7 wk of age (peri-adolescent), in the absence of impaired glucose homeostasis is consistent with patterns in children where obesity development is gradual. In addition, the expected effect size of the differences in metaphyseal vBMD is approximately 20% with n=14 per group

(compared to the observed 25%). The magnitude of differences in bone mass is also comparable to previous studies in growing rodents fed a HFD for longer duration (≥ 10 wk) [91, 92, 460]. Therefore, the intervention diet utilized demonstrated usefulness in inducing early manifestations of HFD and AA on bone, even under the constrains of the short time frame without substantially increasing body weight. The present study has also utilized standard commercial pellet control diet (AIN-93G) that were otherwise identical from the HFD except for the fat content. This allows for comparison with other animal trials employing the identical control diet and enhances the ability for the current research to be replicated and/or validated.

The important role of AA in relation to the bone-fat relationship was highlighted, yet the investigation of the associations with individual fatty acids is complex, as fatty acids are highly correlated through common dietary sources and metabolic processes. Associations may be weak when exposures to single fatty acids are considered in isolation, and effects may be obscured if they are adjusted for correlated confounders (i.e. other fatty acids present in the sample). In addition, fatty acid precursors and/or products are linked through well-defined metabolic pathways; important synergistic effects may be missed by examining single fatty acids. Employment of a pattern analysis approach should overcome this limitation by considering the combined effects of multiple fatty acids acting simultaneously in the body [461]. The associations between changes in anthropometry (weight or waist circumference) and n-3 LCPUFA content in adipose tissue in adults has been shown to vary depending on the rest of the fatty acid pattern [462]. It is likely that the patterns of fatty acids are more representative of the true endogenous exposure after digestion, metabolism, and storage in adipose tissue, as well as providing new hypotheses regarding potential health effects of emerging fatty acids (e.g. very long-chain PUFA e.g. C ≥28) [463].

# CHAPTER 7

## Future Directions

### 7.1 Sex-specific response to LCPUFA

Most studies on the health effects of LCPUFA to date have been conducted without specific attention to differential response between sexes, but recent work indicated sex-specific effects of n-3 LCPUFA on bone mass [464] and cardiometabolic risks in children [465, 466] exist. While examination of sex differences was not one of the principle objectives of this dissertation, it remains an important concept threading all studies. The sole use of male rats in the present thesis represents one of the limitations, hence the results presented herein cannot be extrapolated to females. The influence of sex steroids, particularly estrogen, on the up-regulation of FADS1 and FADS2 expression are known [467]. Despite so, sex-specific outcomes in response to n-3 LCPUFA supplementation exist early before adolescence [466], and it occurs independent of the increase in sex steroid levels during puberty. A recent study in adult mice (14 wk) with existing HFD-induced obesity showed that even though long-term (15 wk) AA treatment (1% w/w) aggravated existing obesity in both sexes, only male mice displayed the adverse effects of AA on obesity-induced complications. AA-fed females had improved IR and enhanced GLUT4 expression in adipose tissue; and this is possibly mediated via rescuing anti-inflammatory gut microbiota [468]. Collectively, these results highlight the importance for future clinical studies to address all LCPUFA-related outcomes separately for males and females or, alternatively, noting that sex differences are absent.

In addition to the biological differences between sexes, there are gender-related social and cultural influences on food choices, physical activity levels and body satisfaction. Studies analyzing dietary pattern in adolescents and young adults (12-24 y) found that vegetable and fruit intakes of girls were markedly higher in comparison to boys [469], presumably because girls tend to pay attention to foods as a means to influence health and meet nutritional

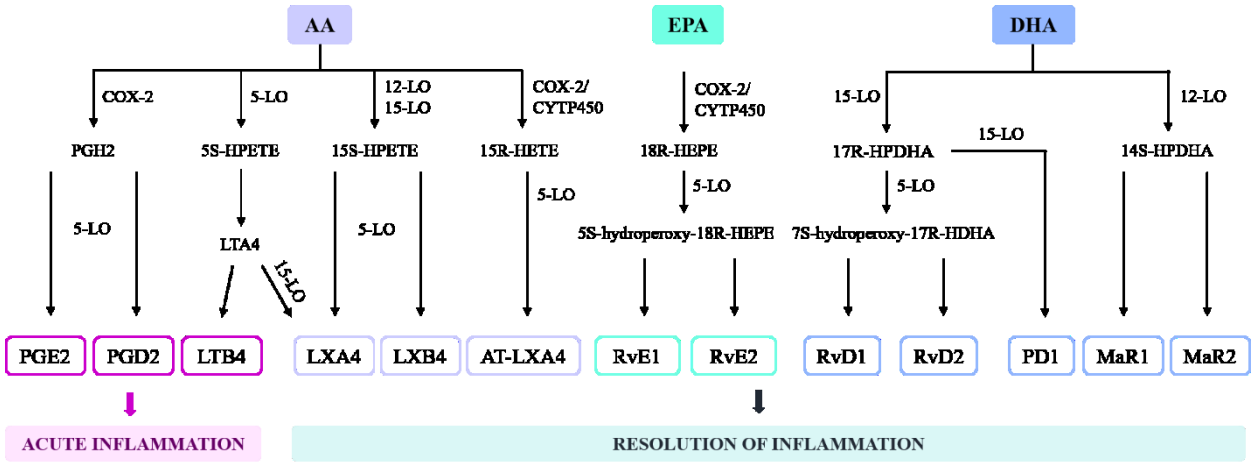
recommendations [265]. Factors contributing to differences in food choices at younger ages, such as culture-bound conventions, societal expectations and stereotypes of males and females have not been identified. In addition, obesity clusters in families, and parental obesity remains the most important risk factor for obesity in children [470]. It was noteworthy that, in study 1, only the proportion of mothers and not fathers with BMI ≥ 25 kg/m$^2$ is greater in children in the highest tertile of % body fat. Parents, and particularly maternal behavior may play an important role as children's food environment is usually influenced more by mothers than fathers [471]. Hence, gender effects in children may be routed in gender-mediated parental behavior to the child. Intervention strategies for childhood obesity may need to consider the relative parental influence on dietary habits and physical activity of the child.

**7.2 Pro-resolving lipid mediators**

Despite the central role of AA in the initiation and progression of inflammation through inducing $PGE_2$ synthesis, it has recently become evident that the resolution phase of inflammation is an active process, mediated by specialized lipid mediators involving lipoxins, resolvins, protectins and maresins [472]. The recognition of the proactive nature of inflammation resolution has revealed alternative therapeutic paradigms based on resolving, rather than preventing the onset of acute inflammation [473]. Indeed, a number of endogenous lipid mediators are capable of switching from the initial proinflammatory to anti-inflammatory actions [474]. The biosynthetic and signaling pathways of these pro-resolving molecules, as well as their relationships with selected pro-inflammatory mediators are outlined in **Figure 7.1.** Notably, AA is prominently involved in the synthesis of the lipoxins $LXA_4$ and $LXB_4$ through the actions of lipoxygenases [475, 476] and may downregulate inflammatory cytokine gene expression [474]. The prostaglandin-type pro-resolving lipid mediator 12d-PGJ2 has been shown to stimulate

collagen synthesis in human osteoblast cells [477, 478]. As aforementioned, an important limitation is the lack of $PGE_2$ measurement in all studies, yet the discovery of pro-resolving mediators suggest that the overall effect of pro-inflammatory lipid mediators should be considered in the context of the presence of pro-resolving mediators. Measurements of $PGE_2$ alone may not provide sufficient information on inflammatory states in the bone milieu; targeting the synthesis of pro-resolving mediators may represent a novel mean to stimulate return to homeostasis, rather than the traditional approach of reducing synthesis of pro-inflammatory mediators, for example, through decreasing n-6 LCPUFA.

**Figure 7.1** Biosynthetic cascades and actions of selected lipid mediators derived from AA, EPA and DHA




LCPUFA serve as substrates for the formation of specialized pro-resolving lipid mediators involving lipoxygenases (human 5-LO, 12-LO and 15-LO) and cyclooxygenases (COX), particularly the inflammation-induced isoform COX-2. EPA-derived mediators are known as E-series Resolvins (RvE1-3) and DHA-derived lipid mediators are named D-series Resolvins (RvD1-6), Protectins (PD1) and Maresins (MaR1-2). AA is involved in the initiation phase of inflammation by forming pro-inflammatory leukotrienes (LTB4) and prostaglandins (PGE2 and PGD2). The most prominent pro-resolving mediators formed from AA are the lipoxins (LXA4 and LXB4), which are formed via sequential actions 5-LO and 12-LO with LTA4, as an intermediate or 15-LO-initiated interactions with 5-LO-bearing cells. The epimeric aspirin-triggered lipoxins (AT-LXA4) [479, 480] have similar actions as the lipoxins but are formed from COX-2 in the presence of aspirin or via the cytochrome 450 (CYTP450) pathway

Abbreviations: AT-LXA, aspirin-triggered lipoxins; COX, cyclooxygenase; LO, lipoxygenase; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HPDHA, hydroperoxydocosahexaenoic acid; HPETE, hydroxyperoxyeicosatetraenoic acid; LT, leukotrienes; LX, lipoxins; MaR, maresins; PD, protectins; PG, prostaglandin; Rv, resolvins

### 7.3 Adipo-myokines

The influence of adipokines on bone metabolism was reviewed in section 2.1.3. Likewise, secretory factors from skeletal muscles, known as myokines, are believed to mediate important interactions between adipose tissue, skeletal muscle and bone. Considerable overlap between adipokines and myokines (termed "adipo-myokine") exists; among the best described members of this family are IL-6 and TNF-α [481], where they induce bone resorption through enhancing RANKL-dependent osteoclastogenesis [482, 483]. Irisin is an exercise-induced myokine derived from the cleavage of fibronectin type III domain-containing 5 (FNDC5) protein, and functions to induce switching of white adipocytes to brown fat-like cells [484]. Interestingly, adipose tissue not only constitutes a target for irisin, it also expresses *FNDC5* and secretes irisin [485, 486]. Leptin dually increased and decreased Fndc5/irisin expression in skeletal muscle and adipocyte respectively, confirming the crosstalk between both tissues. Low-dose recombinant-irisin improved cortical bone mass and geometry *in vivo* [487] through increasing osteoblast differentiation and reducing osteoclast maturation [488]. Studies examining the association of irisin concentrations with BMI and/or adiposity in children are emerging, but results are inconsistent where both positive [489, 490] and negative [491] relationships have been found. Interestingly, LA proportions in cholesteryl ester negatively associated with plasma irisin level among children with obesity (6-9 y) [492]. The discovery of myokines and adipokines have provided a new basis to understand the molecular mechanisms underlying the connection between muscle, adipose and bone tissues. Future efforts are needed regarding the impact of obesity on adipo-myokine secretion such as irisin, myostatin [493] and IGF-1 [494], and how they relate to the muscle-adipose-bone axis.

### 7.4 Interaction between genotype and LCPUFA intake

The characterization of genetic variants that contribute to inter-individual differences in nutrient metabolism is a topic of recently recognized importance in the design of clinical studies. Studies have indicated that the effects of n-3 LCPUFA or oily fish intake is highly heterogenous, and likely influenced by genetic variants which impact on LCPUFA metabolism and tissue partitioning, transcription factor activity, or physiological end-point regulation [495]. Polymorphisms exist in genes coding for proteins involves in the metabolism and signaling of LCPUFA including *FADS*, *PPAR*, *COX* and *PPAR* [496]. For example, genetic variation in the *FADS* gene cluster (*FADS1*, *FADS2*, and *FADS3*) are important genetic determinants of LCPUFA status [497, 498]. Genome-wide association studies identified significant association between plasma AA levels and the SNP rs174537 near *FADS1*, where it accounted for 18.6% of the addictive variance in AA [499]. A recent study in Chinese women showed that maternal genetic variants in *ELOVL2* and *ELOVL5* interacts with DHA intake to affect LCPUFA levels in breast milk [500]. Until now, most studies are performed in adults and little is known about the variation across life stages. DNA methylation of the *ELOVL2* promoter region showed a progressive increase with age (r=0.92) and may be a promising marker of aging [501]. Hence, the interaction among age and sex, among other factors may further contribute to the genetic variation of LCPUFA metabolic pathways. The FiSK (Fish, children, health and cognition) Junior study is a randomized controlled trial that examines the effect of consuming 300 g/wk of oily fish versus poultry on cardiovascular risk markers, cognitive function, behavior and emotion in Danish children (8-9 y); special attention is given to the sex- and genotype-specificity of outcomes [502]. Results from this trial will advance the understanding on the importance of dietary fish while considering the interaction of sex and genotype in childhood.

# CHAPTER 8

## Conclusion

Increasing intakes of fish and seafood is the most realistic and practical way to raise dietary quantities of n-3 LCPUFA. Important questions then arise regarding the impact of aquaculture in changing the consumption pattern of key fatty acids known to affect human health. The rising demand and prices of fish oil are forcing the industry to reduce the reliance on fish oils and increasing the use of vegetable oils in aquaculture feeds [503]. However, fatty acid profile of the fish fillets reflected the fatty acid profile of the feed. Fish given feeds low in fish oil had a low level of EPA and DHA in their fillets [504]. A study published in the *Journal of the American Dietetic Association* suggested that certain farmed fish species, such as tilapia and catfish "have low levels of n-3 fatty acids along with levels of AA so high they can be considered detrimental" [505]; and argued that the inflammatory potential of an average serving of farmed tilapia (134 mg AA/100g) is greater than hamburger, pork bacon and donuts given their lower AA content (34 mg, 4 mg and 191 mg AA/100 g respectively). This opinion was met with considerable resistance [506, 507] and supported by the lack of evidence to provide justification for vilifying low n-3 fish (even if they might contain higher amounts of AA) as "pro-inflammatory" and "pro-atherogenic" [507]. Nonetheless, this demonstrated that the belief that the presence of AA, regardless of quantities, in food is considered harmful, is common, even among health professionals.

The idea has developed that, since AA-derived mediators are involved in pathologies of many diseases, AA itself and its eicosanoid derivatives must be harmful. This is compounded by the observation that many health benefits of n-3 LCPUFA frequently involve an "antagonism" of AA, where n-3 LCPUFA partly replace AA in cell membranes and inhibit AA metabolism to pro-inflammatory eicosanoids. Despite so, studies in humans have yet to demonstrate definite adverse health effects related to high AA intake. A study in healthy Japanese adults with

habitually high EPA+DHA intakes demonstrated that AA supplementation (840 mg/d) for 4 wk had no effect on blood pressure, serum lipid and glucose concentrations, or markers of liver function [386]. Consistently, an earlier cross-over study in US adults using 1.5 g AA/d showed no effects on blood lipids [391]. Interestingly, bleeding time tended ($p=0.06$) to be longer, not shorter, with higher AA intakes. There is also no evidence of an inverse relationship between AA supplementation (ranging from 240 to 1500 mg/d) and plasma composition of EPA and DHA [386, 389, 391, 508]. It appears appropriate then to conclude an increase in AA intake by healthy adults, up to an intake of 1.5 g/d is unlikely to pose any adverse effects. Unfortunately, no similar data on AA supplementation exists in children and adolescence, nor is there any information on the impact of increased AA supply in individuals with pre-existing low-grade inflammation, such as that in obesity. It is uncertain to what extent does exogenous AA beyond average intake (84 ± 0.3 mg/d) [509] exacerbates the inflammatory process.

Overall, in the human study, the negative relation in bone size and mass in the forearm, and possibly IR is manifested in children with the highest AA status. However, no evidence of harm was observed with AA at intermediate amounts based on the cross-sectional analyses. AA was not associated with adiposity or BMI Z-score in children. Further, dietary n-3 LCPUFA intake and consequently EPA and DHA status were low; and declined with increasing adiposity. It remains uncertain if these outcomes would be modulated if intakes of EPA and DHA were to increase in this population. In the animal study, AA accentuated the increases in body weight and leptin production in early stages of diet-induced obesity. The influence of AA on bone is opposite in the central versus appendicular skeleton, and further evidence was provided *in vitro* that AA increased number of lipid droplets in adipocytes and leptin production, possibly involving PPARγ. Given the scarcity of reports on the relationship between AA and bone health in children,

conclusions drawn regarding a strictly beneficial or adverse effects are premature. This thesis adds to the existing literature by providing specific investigation on AA and pointed out important outcomes and aspects related to study design to consider in future studies. Most importantly, the biological importance of AA in childhood and adolescence remain largely unknown, this work aimed to fill this knowledge gap by evaluating effects of AA in children with obesity on well-defined and quantifiable outcomes of public health significance.

The emphasis of the health impacts of AA in adults appears to differ vastly from that in children. High long-term intakes of n-6 fatty acids in adults, especially that of LA and AA have been related to pro-inflammatory states, increased risks of cardiovascular diseases and type 2 diabetes [510]. The North American diet is heavily criticized for excessive total and relative n-6 fatty acid intakes compared to n-3s, hence fixing an upper limit to n-6 LCPUFA consumption has been a recurrent issue in the international scientific agenda for dietary guidelines for fat. Regrettably, the body of literature that showed either neutral or beneficial effects of dietary intakes or blood levels of LA on chronic diseases risk [392, 454, 511-513] have received relatively little attention compared to studies that reported adverse outcomes. In addition, at what age and threshold of intake does excessive n-6 LCPUFA begin to cause harm is largely undetermined. Until more systematic research is available to provide a sound scientific basis for further encouraging the reduction of n-6 LCPUFA intakes, the priority may be to correct the low n-3 LCPUFA intakes/status beginning in youths, as an effort to minimize future risks of diseases.

Measures of childhood obesity based on objective estimates of excess weight (i.e. BMI) have remained stable over the last 10 y [29], suggesting the public effort to prevent and/or treat childhood obesity may prove to be effective over time. However, it is frequently stated the use of high BMI level to identify children who are obese results in high specificity but moderate (80-

85%) sensitivity [514]. It remains possible that a proportion of children who are obese (i.e. excess adiposity) but not overweight due to low lean mass, are missed in the classification based on the BMI Z-score cutoffs. Childhood trends including the under consumption of key nutrients (calcium, vitamin D, fibre, etc.), lack of physical activity, increased screen time, and disordered eating behaviors, are problems that affect children of all BMIs, and not just those above a certain cutoff level. We must therefore readjust the focus to center on the promotion of optimal pediatric health instead of concentrating on body size and shape.

The concept of achieving an ideal dietary n-6: n-3 ratio may have created unintended harm by distracting attention away from increasing absolute intakes of n-3 LCPUFA. The idea that n-6 LCPUFA, at least in moderate amounts, has a critical and likely beneficial role in human health and disease prevention is slowly gaining ground. Perhaps the issue is not the presence of the n-6 fatty acids, but the absence of the n-3s in the diet. Possible barriers to fish consumption, including the conflicting messages conveyed through the media about benefits and risks associated with eating fish needs to be clarified. Until further risks of increasing dietary intakes of EPA and DHA or of fatty fish, related to methylmercury content or contamination with environmental pollutants [224, 515] are identified, it remains safe to emphasize on raising the consumption of n-3 without intentionally avoiding n-6 LCPUFA as part of targeted obesity prevention effort for all children.

# APPENDIX A

**Published:** ***Docosahexaenoic acid (DHA): properties, functions and health effects.***
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# Docosahexaenoic acid (DHA) and Properties, Function and Health Effects: DHA and Growing Bone

Ivy L Mak and Hope A Weiler

## A.1 Abstract

Benefits of dietary long-chain polyunsaturated fatty acids (LCPUFA) on bone health across the life stages (infancy, adulthood, aging and pregnancy) is apparent in a number of human and animal investigations. Docosahexaenoic acid (DHA, 22:6 n-3), in particular, has been advocated for ensuring optimal development of infant brain and visual systems, modulating inflammatory and immune responses, reducing incidence of cardiovascular vascular diseases, and more recently, in supporting optimal bone health. Childhood and adolescence represent the most critical periods for developing optimal peak bone mass and bone strength. Almost half of adult bone mass is acquired between 12 and 18 years of age, and those with the highest peak bone mass are best protected against bone loss with aging. DHA supplementation in growing animals is associated with increased bone formation and calcium retention, accompanied by reduced resorption. Thereby resulting in improved micro-architectural properties and bone mass that translated into greater bone strength. This chapter seeks to review the literature on the effects of DHA in bone health with focus on the growth period. Herein, a summary of current knowledge on DHA sources, intakes and current recommendations will be provided. This will lay a foundation for examining the function of DHA in growing tissues with bone as an example, including potential mechanisms involved, differences in the various sources of dietary DHA, versus preformed DHA on bone; as well as possible sexual dimorphisms in DHA status and bone growth. Lastly, this chapter will summarize basic and clinical findings on the consequences of DHA supplementation on bone mass as an outcome. This seek to shed light on limitations of existing studies and identify gaps in knowledge and needs for improved methodology and/or technology to facilitate research on the effects of LCPUFA on bone mass during rapid periods of growth.

## A.2 Introduction

A vast body of evidence demonstrates multiple beneficial effects of dietary long-chain polyunsaturated fatty acids (LCPUFA, ≥ 18C, ≥ 2 double bonds) on bone health. Data from both cross-sectional and intervention studies indicate that LCPUFA can increase bone formation, affect peak bone mass (PBM) in adolescents, and reduce age-related bone loss in older populations. In addition to the established roles of LCPUFA in regulating membrane fluidity, cell function and responses, LCPUFA appear to affect bone metabolism in multiple pathways. These include modulation of fatty acid metabolites, direct regulatory effects on bone cells of the basic multicellular unit (BMU), or via receptors or sensors that influence patterns of gene expression.

Docosahexaenoic acid (DHA, 22:6 n-3) is an essential fatty acid of the omega-3 (n-3) series that has received substantial attention due to its pivotal roles in biological functions including brain and visual development, cognition, inflammatory responses and with decreasing risk or mortality with cardiovascular diseases [516]. In terms of the emerging role of DHA on bone health, DHA supplementation in growing rodents has been shown to decrease bone resorption, calcium excretion, and enhance PBM accrual in healthy as well as n-3 depleted states [517]. Moreover, DHA may mediate anti-inflammatory effects through its metabolites such as the eicosanoids and resolvins. Overall, DHA demonstrates the ability to influence biological processes by serving as signaling molecules, either indirectly as fatty acid derivatives, or directly regulate gene transcription via binding to specific fatty acid receptors. Childhood and adolescence represent the most critical periods for developing PBM and bone strength. Gains in bone mass are most rapid during adolescence, with 25% of the PBM acquired during the two-year period surrounding peak height velocity [518]. While the timing of PBM remains debatable and varies by skeletal sites, it is generally recognized that about 90% of PBM is acquired by 18

years of age [519, 520]. The remaining 5-12% of gains in BMC are accounted for in the third decade of life (i.e. 20 to 30 y) [3]. Variance in bone mass among individuals at completion of growth is an order of magnitude greater than variance in rates of bone loss during aging (1 SD = 10% and 1% respectively) [8], thus the accrual of PBM during early adulthood is likely to be the dominant determining factor for ones' susceptibility to fracture later in life. This chapter thus seeks to review the literature on the effects of DHA in bone health with a focus on the growth stages of life.

PUFAs are divided into two classes according to their structure. Omega-3 (n-3) and omega-6 (n-6) represent fatty acids with the third and sixth carbon from the methyl end being unsaturated, respectively. Mammals are incapable of synthesizing n-6 or n-3 fatty acids *de novo*, therefore the availability of the 20- and 22-carbon LCPUFA is determined by the conversion of dietary n-6 fatty acid precursor linoleic acid (LA, 18:2 n-6) and n-3 fatty acid precursor α-linolenic acid (ALA, 18:3 n-3) through a series of common and competitive microsomal desaturation-elongation reactions within the peroxisomes (**Figure A1**).

Biosynthesis of LCPUFA requires the introduction of *cis* double bonds by the Δ5 desaturase (D5D) and Δ6 desaturase (D6D) enzymes, that are encoded by the FADS1 and FADS2 genes, respectively. Both D5D and D6D are membrane-bound proteins with amino-terminal cytochrome b5 domains carrying heme-binding motifs, two membrane-spanning domains and three His-box motifs [521]. In humans, D5D shares about 61% amino acid identities with D6D and has 75% similarity to the human D6D [522]. These two enzymes are responsible for the two rate-limiting steps in LCPUFA synthesis, being the desaturation of LA and ALA to 18:3 n-6 and 18:4 n-3, respectively; and desaturation of 20:3 n-6 to arachidonic acid (AA, 20:4 n-6) and 20:4 n-3 to eicosapentaenoic acid (EPA, 20:5 n-3). The expression along with catalytic activity of D5D and

D6D is highest in the liver, but it is also present to a smaller amount in the brain, heart, lungs, adrenal glands, testes and subcutaneous adipose tissue of both rats and humans [521-523]. The synthesis of DHA from its respective 22-carbon precursor docosapentaenoic acid (DPA, 22:5 n-3) involves elongation, desaturation by D6D and peroxisomal β-oxidation that shortens the acyl chain by 2 carbons, resulting in the addition of one double bond at the Δ4 position.

While DHA can be produced *de novo* via the conversion from dietary ALA by D5D and D6D, the extent of conversion is insufficient to increase DHA concentration in tissues to a physiologically significant concentration [524]. On average between 0.01% and 8% of ALA is converted to EPA and less (<1%) to DHA [525, 526]. The rate of conversion also appears to be different between sexes where in women of reproductive age, the fractional conversion rate of ALA into EPA and DHA (n=6, 28 ± 4 y) was approximately 21% and 9.1% respectively [527], as compared to men of 7.9% for EPA, and undetectable DHA production [528]. To date, no Dietary Reference Intake (DRI) recommendations specifically for EPA and DHA have been established by the Institute of Medicine (IOM) for the United States and Canada, owing, in part to a lack of consensus for a clear functional endpoint, and uncertainty whether separate guidelines should be set for EPA and DHA [529]. There is, however, an Adequate Intake (AI) for ALA with values for children 1-3 y being set at 0.7 g/d and 0.9 g/d for children 4-8 y. For those over 13 y, AI values are 1.2 g/d for boys and 1.0 g/d for girls. These AI values are based on the observed median intake in the United States that supports normal growth and neural development and at which no nutrient deficiency is present [296]. Up to 10% of the acceptable macronutrient distribution range (AMDR) for ALA (0.6-1.2% of energy) can be consumed as EPA and/or DHA, which approximately corresponds to the mean intake for EPA and DHA in the United States (~100 mg/day) [296].

Despite the absence of IOM-based DRI values for EPA and DHA, The Dietary Guidelines for America, 2005 reported "there is evidence that suggests that consumption of approximately two servings of fish per wk (approximately 8 oz (224 g) total) may reduce the risk of mortality from coronary heart disease; and that consuming EPA and DHA may reduce the risk of mortality from cardiovascular disease in people who have already experienced a cardiac event" [530]. The Eating Well with Canada's Food Guide also provides similar recommendations of consuming at least two servings of fish per wk (75 g per serving) [531]. Given the potential health benefits associated with EPA and DHA, many organizations and expert committees have recognized the importance of n-3 LCPUFA, and thus have established recommendations for EPA and DHA. Depending on the target population, suggested intake values can range from 250-4000 mg/d (**Table A1**).

Oily fish such as salmon, herring and mackerel represents the richest sources of n-3 LCPUFA, providing approximately 3-3.5 g per 75g serving, with DHA comprising on average 65% of total n-3 LCPUFA. DHA content in fish typically corresponds with overall LCPUFA content, with Atlantic salmon providing the greatest amounts of DHA at an estimated level of 1-1.5 g per 75 g serving. Concentrations in other fish including cod and sole can vary between 100-400 mg DHA per 75 g serving. Other non-fish marine sources including shrimps, mussels and scallops also contain moderate amounts of approximately 250-600 mg DHA. Alternatively, given the limited availability, high cost and low acceptance of fish to some consumers, meats, dairy products and eggs can potentially contribute to n-3 LCPUFA intake, despite in relatively low quantities. DHA is found predominantly as part of membrane phospholipids in land-based foods, providing approximately <40 mg/100 g serving [532]. Enrichment of tissue DHA in such sources appears to be dependent upon dietary DHA instead of *de novo* synthesis. For example, fish oil

[533], linseed oil and rapeseed oil [534] are commonly added to poultry feeds with an aim to manipulate the LCPUFA composition of poultry. Inclusion of EPA and DHA in poultry diets readily increases concentration of these fatty acids in edible tissue, but this is not achievable through supplementation of the precursor ALA [535]. Furthermore, with the growing interest to enrich or fortify foods that traditionally do not contain n-3 LCPUFA such as pasta, bread, milk, dairy products, salad dressings and margarine; these foods have now become the second main contributors to dietary LCPUFA sources in the North American diet following marine sources [536]. While questions have been raised against an optimal ratio between n-6 and n-3 fatty acids since LA and ALA competes for desaturation by D6D, a systematic review on the effect of dietary LA on fatty acid metabolism reasoned that LA supplementation had little effect on DHA concentrations, nor was LA associated with increased inflammatory markers that potentially stimulate osteoclastogenesis [537].

Canadian adults are estimated to consume approximately 80 mg/day of EPA and DHA combined based on the typical male 20-64y [538]. For inland communities (Ontario, Canada), average intakes of 117 mg/d for pregnant women has been reported, although up to 20% of residents in these communities may consume undetectable amounts of DHA [539]. This is in contrast to much higher intakes of 238 mg/day for coastal communities (British Colombia, Canada) with presumably greater accessibility to seafood and fish [540]. Similar observations have also been reported in Australia [541] and Germany [542] where observed EPA and DHA intakes are in general lower than expert committee recommendations, with the exception of France where up to 400-497 mg/d combined EPA, DPA and DHA was consumed [543]. The 2009-2010 NHANES “What We Eat in America” report also placed average DHA intakes of adult males and females at 80 mg/d and 60 mg/d respectively [544]. Hence, the recently

published Dietary Guidelines for Americas 2015-2020 recommend “incorporating seafood as the protein food choice in meals twice per week in place of meat, poultry, or eggs” in light of low average seafood intakes across all age-sex groups [339] .

Although it is generally recommended to employ a food-based approach for achieving nutrient adequacy and preventing and treating diseases, recent data in 600 pregnant and lactating women in the Alberta Pregnancy Outcome and Nutrition (APrON) cohort reported that only 27% of women during pregnancy, and 25% at 3 months postpartum met the European Union consensus recommendation of 200 mg DHA per day by diet alone; while those who took a supplement containing DHA were 10.6 (95% CI: 6.80 – 18.14) and 11.1 (95% CI 6.95 – 16.07) times more likely to meet the recommendation for pregnancy or postpartum respectively [545]. This suggests that supplementation may be beneficial for achieving recommended EPA and DHA intakes in individuals who do not regularly consume fish as part of their diets. Similarly, supplementation or consumption of DHA-enriched foods could ensure an adequate supply of n-3 LCPUFA in children with suboptimal ALA and LA intakes. This is especially relevant to children in light of the established roles of essential fatty acids in growth and development in children, combined with the limited conversion of LA and ALA into longer chains LCPUFA during the first 2 years of life [546].

### A.2.1 Overview of postnatal skeletal development

Despite the astounding range in the sizes and unique morphology of bone both within and between individuals, all bones form through one of the two distinct processes: intramembranous bone formation and endochondral bone formation [547].

Intramembranous bone formation is achieved by the transformation of mesenchymal stem cells (MSC) into osteoblasts where soft connective tissue is replaced directly by bone tissue. This

process forms flat bones of the skull [548], and parts of the mandible and clavicle. Endochondral bone formation, on the other hand, is the process where MSC differentiate into chondrocytes that proliferate to form and enlarge the bone structure. It is responsible for the generation of most bones. Critical events during postnatal bone formation includes the formation of secondary ossification centers, longitudinal growth of long bones at the epiphyseal growth plate, the fusion of secondary ossification centers, diametric bone growth, and vascularization of bone tissue [549].

Ossification is the process in which either membranous fibrous tissue or cartilage is replaced by bone tissue [550]. It begins in a focal area that expands progressively until all of the existing tissue is replaced by bone tissue. While the primary ossification center is the initial site of ossification and develops mostly during the embryonic and early fetal period, the secondary ossification centers are generally formed postnatally, and develop in regions where primary centers do not extend [550].

In long bones, the development of the growth plate results shortly after the appearance of the secondary ossification center in the epiphyseal region. The growth plate is a thin disk of cartilage that creates a barrier between the epiphysis and the diaphysis, and serves as a source of cartilage for conversion to bone [551]. Bone is deposited in the transitional metaphyseal region located directly below the growth plate as long bone lengthens. At the end of puberty where long bones have reached adult length, the growth plate fuses with the diaphysis and longitudinal bone growth ceases at this point [551].

As bone lengthens it also expands in diameter at the diaphysis to improve its ability to support weight bearing loads. In long bones this is achieved by the excavation of a marrow cavity and circumferential expansion of the cortical bone, also known as appositional growth.

The result is enhanced strength and resistance to bending [552]. In contrast to longitudinal bone growth, periosteal apposition can occur at any time during development and is a form of intramembranous bone formation [549].

Optimal vascular supply to the bone is essential for these processes to occur. This is achieved through the hypertrophic chondrocytes in the central diaphysis, which stimulates the invasion of blood vessels of the perichondrium surrounding the future diaphysis, and transforms it into the periosteum [551]. Specific groups of arterial networks are responsible for supplying blood and nutrients to the various portions of long bones to support its function for calcium storage, vitality of bone cells as well as hematopoiesis [553, 554].

Bone is frequently categorized into two main and distinct types: cortical bone and trabecular bone. Different regions of the skeleton contain variable amounts of cortical and trabecular bone. Cortical bone is primarily formed at the diaphysis, complete perimeter of all long bones, as well as the outer layer of nearly all short, flat and irregular bones. It comprises approximately 80% of the skeleton and fulfills mechanical and protective functions. The trabecular compartment accounts for the remaining 20% and has both mechanical and metabolic role, serving as a reservoir for calcium and phosphorus [89]. Trabecular bone density in regions such as the lumbar spine increases during puberty. In contrast, cortical bone density in the appendicular skeleton remains relatively constant across age, gender and race, while cortical thickness increases dramatically [555]. The relatively low turnover rate of 2-3% per year in cortical bone is adequate to maintain biomechanical strength in adults. In contrast, the rate of trabecular bone turnover may be 5-10 times higher, and is observed to be more than required for maintenance of mechanical strength, indicating that trabecular bone turnover is more important for mineral metabolism [556]. Rates of turnover in children are less whereas modeling is highly active and

necessary for bone growth. Most of the gains in bone mineral during growth are due to increases in bone size, rather than density.

In addition to the chondrocytes of the growth plate, actively growing bone is composed of intercellular calcified material called the bone matrix, and four major types of bone cells that function throughout life: osteoblasts, osteoclasts, osteocytes and bone-lining cells; the collective action of these cells is described as the "basic multicellular unit" (BMU). The BMU acts in a highly coordinated manner to reconstruct bone in distinct locations on the three compartments (trabecular, endocortical, and intracortical) of the endosteal envelope, and to a lesser extent the periosteal envelope [557].

In an active remodeling sequence, osteoblasts that arise from osteoprogenitor cells would quickly fill the remodeling space with a collagenous osteoid after osteoclasts resorbs bone. Mature osteoblasts synthesize new collagenous organic matrix and regulate mineralization of matrix by releasing small, membrane-bound matrix vesicles directed toward the bone formation surface that concentrate calcium and phosphate, and enzymatically destroy mineralization inhibitors such as pyrophosphate or proteoglycans [558]. At completion of bone formation, approximately 50-70% of osteoblasts undergo apoptosis, with the remaining becoming osteocytes within bone matrix that support bone structure, or protective lining cells that cover the surface of quiescent bone.

During bone remodeling, resorption by osteoclasts precedes bone formation by osteoblasts. The process of osteoclastogenesis is activated by the release of local factors by osteocytes [559]. While a range of stromal derived factors have been suggested to play a role in initiating osteoclast formation, most converge to interaction between the surface receptor activator of nuclear factor-κB (RANK), its ligand RANKL expressed on osteoblasts, and the decoy receptor

osteoprotegerin (OPG) that can prevent osteoclastogenesis by binding to RANK. Upon activation, osteoclast precursor cells fuse into tartrate-resistant acid phosphatase (TRAP)- and cathepsin K-positive multinucleated osteoclasts. At the resorption site, the osteoclasts form a specialized cell membrane, the ruffled border, and release secretory vesicles containing lyzosomal enzymes, hydrogen pumps and free radicals into a confined space that dissolve minerals and degrade bone matrix [560].

Skeletal acquisition during growth is mediated by a myriad of endocrine factors. Of all, growth hormone (GH) and its downstream effector insulin-like growth factor-1 (IGF-1) are major determinants of bone mass accrual and longitudinal growth, as well as for maintaining bone integrity. IGF-1 is mainly produced in the liver, but is also expressed locally in extrahepatic tissue including skeletal muscle and bone, acting in a paracrine and/ or autocrine manner. IGF-1 synthesis in most extrahepatic tissue is not only under growth hormone control. It appears that locally produced IGF-1 is more important for growth than hepatic derived IGF-1, as selective hepatic deletion of IGF-1 did not result in significant growth impairment despite a 75% reduction in circulating IGF-1 concentrations [561].

A multitude of *in vivo* and *in vitro* model systems have indicated that IGF-1 positively regulates osteoblast and chondrocyte functions. The importance of IGF-1 in embryonic bone development was apparent when chondrocyte-specific knockout of the IGF-1 receptor (IGF-1R) in mice resulted in 10% reduced growth, reduced bone size and decreased skeletal mineralization that was associated with reduced proliferation, delayed differentiation and hypertrophy, as well as increased apoptosis of chondrocytes [562, 563]. Given the poor postnatal survival in models of global IGF-1 deficiency, transgenic line expressing tamoxifen-dependent Cre recombinase was developed to allow for conditional knockout during embryogenesis and postnatally [564].

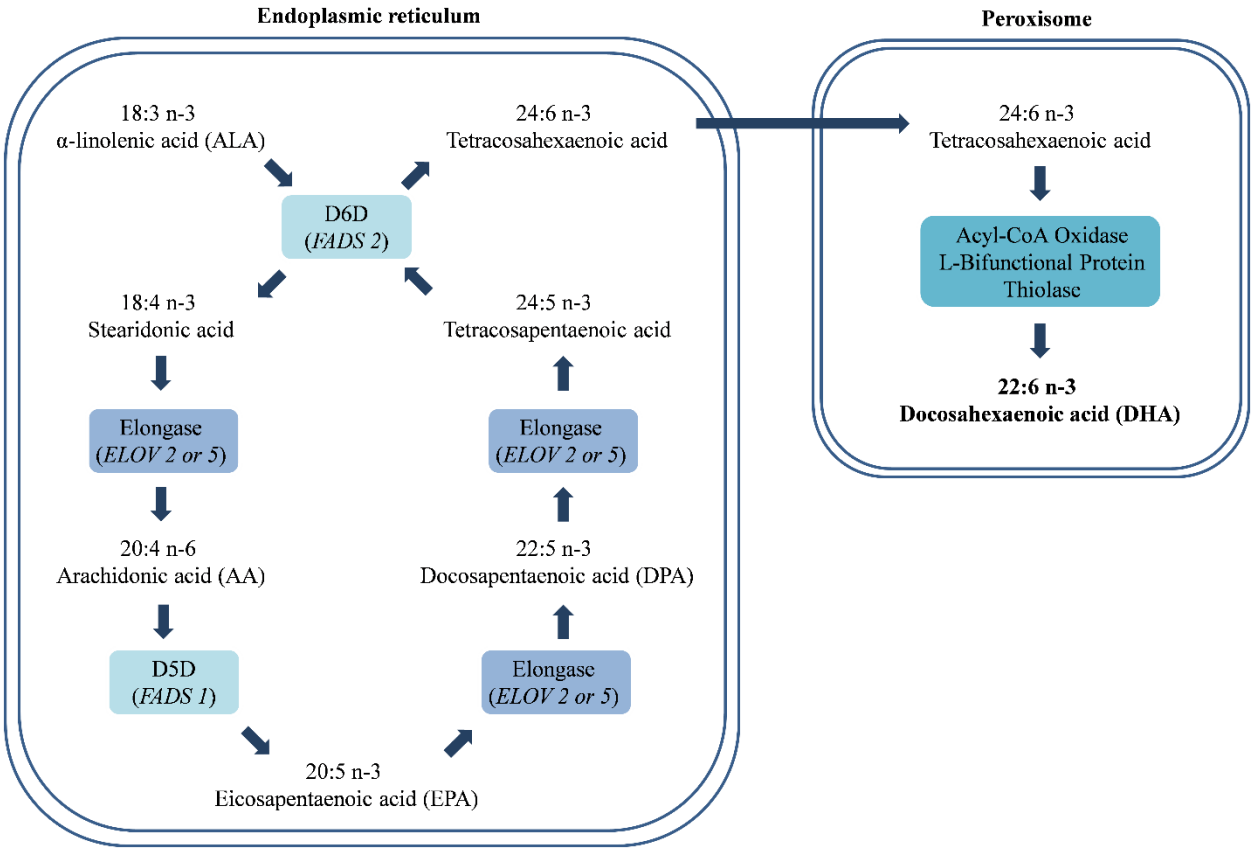
Chondrocyte-specific deletion of the IGF-1 receptor (IGF-1R) in mice at 2 wk of age resulted in significant growth restriction with a body weight about 70% of controls, and severely disorganized growth plates [562], supporting a central role for locally produced IGF-1 in optimal development of the epiphysis.

IGF-1 is the most abundant growth factor is that deposited in the bone matrix [565]. IGFs are released during osteoclastic bone resorption, producing an osteogenic microenvironment to stimulate new bone cell formation from MSC and matrix production, linking bone resorption with formation [566]. Receptors for IGF-1 are expressed in osteogenic cells including osteoblasts, osteoclasts and osteocytes, where IGF-1 signaling directly regulates bone cell function. IGF-1 stimulates RANKL expression and osteoclastogenesis in bone MSCs [567, 568], and deletion of IGF-1R from osteoclast precursors inhibits osteoclastogenesis [569]. Osteoblasts from IGF-1 knockout mice have decreased RANKL expression and are poor stimulators of osteoclastogenesis [569], suggesting that both osteoblasts and osteoclasts produce and are targets of IGF-1, and exhibit anabolic as well as catabolic effects on bone. IGF-1 has also been suggested to in part, mediate the anabolic effects of parathyroid hormone (PTH) on bone. Osteoclast precursors lacking IGF-1R blocked the ability of PTH to stimulate the expression of osteoblast differentiation markers including RANKL in coculture [569]. Mice lacking IGF-1R from osteoprogenitor cells also showed blunted responses to PTH that was associated with reduced osteoblast numbers, proliferation and differentiation resulting in decreased bone volume fraction (BV/TV) in the proximal tibia [570]. Taken together, IGF-1 actions in bone tissue are crucial for maintaining normal interaction between bone cells in some measure through regulating RANK and RANKL expression [569].

**Table A1** Summary of the recommended EPA + DHA intake from expert organizations. Adapted from Flock *et al.* [249]

| Source | Recommended daily dose of EPA + DHA (mg) | Population |
|---|---|---|
| Academy of Nutrition and Dietetics (United States) | ≥ 500 | General Population |
| International Society for the Study of Fatty acids | ≥ 500 | General population |
| | ≥ 500 (≥ 200 DHA) | Pregnant/lactating women |
| British Nutrition Foundation Task Force | 500-1000 | People at risk of cardiovascular diseases |
| U.K. Department of Agriculture | ≥ 250 | General population |
| American Heart Association | Fatty fish ≥ 2 times/wk (~500) | Adults without CVD |
| | ~ 1000 | Adults with CVD |
| | 2000-4000 | Adults with high triglycerides |
| European Food Safety Agency | ≥ 250 | General population |
| | ≥ 250 (100-200 DHA) | Pregnant/lactating women |
| | ≥ 100 DHA | Children 7-24 months |
| | ≥ 250 | Children 2-18 years |
| World Health Organization | 1-2 servings of fish/wk (200-500 mg of EPA +DHA / serving) | General population |
| Dietitians of Canada | ≥ 500 n-3 LCPUFA | General population |
| Ministry of Health, Labor and Welfare (Japan) | > 1000 | General population |
| | 1700 - 1900 total omega-3 | Pregnant/lactating women |

**Figure A1** Metabolism of α-linolenic acid into longer chain omega-3 polyunsaturated fatty acids in the endoplasmic reticulum and peroxisome via a desaturase/elongase enzyme system



## A.3 Epidemiological and clinical studies linking DHA to bone mass

### A.3.1 Human studies

Studies in adults have repeatedly reported positive associations between n-3 LCPUFA levels and bone mineral density (BMD) in adults, as well as its beneficial effects in preventing age-related bone loss in aging populations. However, those that have investigated the possible role of DHA in periods of bone acquisition in humans are sparse.

Some evidence for the benefits of LCPUFA in bone health of children comes from studies designed to treat various clinical conditions. For example, in comparing the incidence of fractures in neonates with congenital cholestasis receiving either a fish oil or soybean oil-based intravenous emulsion, those maintained on the fish oil formula had a lower incidence of fractures compared with soybean oil (5.3% and 12% respectively) [359]. Another study in girls aged 8.2-16.5 y (n=190), dietary LCPUFA correlated positively (r=0.167) with gains in ultra-distal radius areal BMD (aBMD) over a year [571]. In a 6-y follow-up study of a cohort of 78 healthy young men between 16 and 22 y of age, concentrations of serum phospholipid n-3 fatty acids were also associated with changes in whole body aBMD (r=0.27) and spine aBMD (r=0.25). This same study also showed an independent positive relationship between DHA and aBMD (r=0.32 and r=0.30 for whole body and spine respectively), thus favoring peak bone mass development in adolescents [358]. Similar results have also been reported in 34 cystic fibrosis (CF) children (male 13.2 ± 3.8 y, female 11.6 ± 3.4 y), where AA: DHA was negatively associated (r=-0.39) with spine BMD z-score [572]. Moreover, endosteal circumference of the radius increased with greater serum phospholipid DHA concentrations (r=0.79) in men with CF (n=14, 21.6 ± 2.2 y) relative to healthy controls [573]. In younger children (8.2 ± 0.34 y), however, neither n-3 PUFA nor DHA was related to whole body BMC or aBMD [285]. In slightly overweight adolescent

boys (n=78, BMI 23.1-25.2 kg/m$^2$, 13-15 y) randomly assigned to breads enriched with fish oil (1.1g of n-3 LCPUFA) or control for 16 wk, fish oil significantly increased red blood cell (RBC) membrane EPA and DHA levels, and reduced n-6 PUFAs LA and AA. However, no significant differences in bone mass measured by dual-energy X-ray absorptiometry (DXA) was found, nor was DHA status correlated with whole-body aBMD, BMC and bone area. A weak (β=0.24), but significant positive association between DHA status and IGF-1 concentrations was reported, suggesting more sensitive measures for changes in bone mass, and studies over longer time spans should be required for determining the potential bone-accumulating consequences exerted by IGF-1 [214] Moreover, observations in healthy girls (n=351, 8-12 y) include inverse associations between total dietary PUFA (β=-0.13) and trabecular BMC or predicted bone strength at distal femur in pre-pubertal girls (≥1 y pre-menarche 9.8 ± 0.5 y). These observations were likely due to high proportions of n-6 fatty acid intakes (89 % of total PUFA) in this population. In contrast, in early pubertal girls (<1 y pre-menarche, 11.7 ± 0.5 y), dietary EPA intake was a positive predictor (β=0.14) of proximal tibial trabecular BMC, femoral periosteal circumference and cortical bone strength estimated by peripheral quantitative computed tomography (pQCT) [574]. These separate analyses suggest, that associations between dietary fatty acid intake and bone health may vary by stages of maturation, and that trabecular and cortical bone may respond differently to n-3 PUFA in the diet.

### A.3.2 Animal studies

There is a wealth of animal feeding trials investigating the effects of LCPUFA using different ratios of n-6: n-3 fatty acids, specific fatty acids, or combined with other dietary factors such as boron and inulin to promote bone mass accrual during growth (**Table A2**). While purified forms of n-3 fatty acids are the preferred source in these experiments, oils from vegetables or

marine origins are more often employed to provide polyunsaturated fats for practical reasons. There are, however trade-offs in using purified fatty acids, as these are in the form of free fatty acids or not necessarily positioned within the triglyceride as are dietary fatty acids [575] and hence metabolism could vary.

Early studies in newborn rats reported pathological fractures induced by essential fatty acid deficiency [576]. Maternal-fetal transfer represents one of the most important means for infants to obtain sufficient amounts of LCPUFA. Maternal supplementation of high n-3 LCPUFA has the potential of imposing imbalances in maternal-fetal transfer of n-6 LCPUFA in the fetus [577]. In both rats and guinea pigs, when maternal diets during lactation were supplemented with 0.5% of the fat as AA and 0.2% DHA, increased BMC of the lumbar spine and tibia were observed in the pups [384]. Consistently, offspring born to rat dams fed a LCPUFA-deficient diet during late gestation and lactation, and continued with diets equivalent to maternal intake had diminished femur structural integrity. Administration of a LCPUFA-sufficient diet restored body weight and accelerated increments in tibia length, mid-diaphyseal cross-sectional geometry and led to two-fold greater gain in areal moment of inertia, which predicts the resistance of long bone to bending around a given axis [578]. In a pregnancy model where female dams and their offspring were fed menhaden oil (65g oil/kg diet) until 21 wk of age, maximum force and bending moment of the femur was significantly improved, confirming that fish oil is beneficial to cortical bone strength [579]. Supplementation of the maternal diet with fish oil also resulted in early changes in longitudinal bone development. Offspring of Japanese quail hens fed 5 g/100g menhaden oil had wider proliferative and hypertrophic zones in the tibial growth plates as early as 1 d old, and consequently modified the conversion of cartilage to mineralized bone, resulting in a greater cortical thickness by 3 wk of age [580].

Benefits of fish oil supplementation in young growing animals are also apparent. The majority of studies performed in growing rodents reported positive effects feeding diets made with fish oil on bone mass measured by DXA in the femur [581-583] as well as the vertebrae [215, 581]. However, the limitations of DXA in accurately assessing bone mass, particularly in growing bone is well-recognized [584]. The reliance on two-dimensional projection for calculating BMD poses the difficulty to detect surface-specific changes in bone mass deposition or resorption [585]. It is not uncommon for bone size and amount of bone mineral within it to change independently and in opposite direction during growth. Areal BMD measurements only reflect less bone mineral mass of a specific bone or region without distinguishing whether its due to a smaller bone, less mineral in the bone, or both [405]. Using high-resolution micro-computed tomography (μCT), a three-dimensional imaging technique that allows for characterization of bone mass, geometry and micro-architectural properties, female mice fed DHA-supplemented diet (2.1% kcal DHA) show greater BV/TV, trabecular number and connectivity, alongside decreases in structure model index (SMI) values at the femur distal metaphysis [359]. This suggests that DHA also exerts a positive influence on micro-architectural properties in addition to bone mineral acquisition. Reports from studies where bone strength was assessed *ex vivo* appeared to be in agreement with such observations. Japanese quails fed 50 g/kg diet of menhaden oil for 7 mo from 4 wk of age had significantly improved shear force and stress in the tibia [586]. Likewise, increased peak load, stiffness and the energy required to cause bone failure have been reported in bending tests performed in long bones of rodents [215, 587, 588], indicating that the benefits of DHA in bone mass acquisition and structural properties also translate into building stronger bones that are more resistant to fractures.

*In vivo* observations suggest that DHA influences both bone formation and resorption.

Male piglets born small for gestational age (1-1.2 kg, 5 d) and fed a diet supplemented with AA: DHA (6:1, 0.1-0.6g/100g DHA) for 15 d demonstrated significantly higher lumbar spine BMC, and had lower rates of bone resorption with no changes in bone formation noted [589]. Alternatively, weaning rats (3 wk) fed greater proportions of DHA-rich menhaden oil (30:70 safflower: menhaden oil) had greater bone formation rates in the proximal tibia and alkaline phosphatase (ALP) activity, a surrogate for osteoblast activity [287]. Furthermore, DHA concentration in RBC membranes was positively associated with BMD, bone calcium content, and calcium absorption in young rats (5 wk) consuming a diet with 5% tuna oil [581], possibly due to increased cell membrane unsaturation, as well as intestinal calcium ATPase activity that favors calcium transport [590].

Of greater interest is whether beneficial bone adaptations such as increased bone mass and strength from PUFA will persist later in life and reduce risk of fracture. Long term DHA supplementation (0.13 mg DHA/g diet) in mice beginning from 12 wk of age improved trabecular bone volume in the caudal vertebrae by 17.2% at 8 mo of age but not thereafter [215], consistent with observations in young men where DHA supports PBM acquisition during growth. However, DHA did not prevent trabecular bone loss in the tibia beginning from 8 mo of age, and that only EPA, but not DHA reduced age-related decline of osteocalcin, a marker of bone formation [215]. It appears that n-3 LCPUFA influence bone growth and loss with aging differentially depending on the bone compartment and skeletal site studied; and individual actions of n-3 LCPUFA could change across the lifespan. All of these studies, however, used difference sources of DHA, making firm conclusions difficult to draw and to cautiously ascribe the benefits in bone specifically to DHA.

To compare effects of different n-3 PUFA sources on bone metabolism, growing female

rats (35 d) were assigned to a high-fat diet (HFD, 12% fat w/w, ~27% kcal) containing either corn, flaxseed, krill, menhaden, salmon or tuna oil for 8 wk. Rats fed tuna oil with the highest DHA content (2.9 g/kg diet) had higher tibia aBMD, BMC and longitudinal growth, whereas those fed ALA-rich flaxseed or menhaden oil had improved bone microarchitecture at the distal femur metaphysis associated with increased bone formation [582]. It is unlikely that the micro-architectural change is mediated by conversion of ALA to EPA/DHA, given previous reports where rats fed HFD rich in flaxseed oil had negligible amounts of EPA and DHA in the femur marrow and epiphyses [587]. Additionally, tuna oil-fed rats also had 50% higher Ca retention than those fed krill oil despite similar total n-3 LCPUFA concentrations in both oils. This could in part be related to differences in EPA and DHA incorporation in various lipid fractions, where they are preferentially esterified in forms of triglycerides and phospholipids in tuna and krill oil respectively [582]. Therefore, while HFD comprised of n-3 LCPUFA rich oils has positive effects on long bones, a combination of n-3 FA sources may be required for optimal bone acquisition during rapid growth.

Excess LCPUFA can also lead to adverse effects on bone mass. High-dose supplementation of either n-6 or n-3 LCPUFA results in impaired bone formation during growth. In weaning rats (21 d) fed a fish oil diet (7% fat w/w, 60 g/kg menhaden + 10 g/kg soybean oil) for 5 wk, significant reductions in length growth and a lower vertebral peak load were observed; these were not explained by changes in urinary calcium excretion, circulating IGF-1 or estrogen. However, this was confounded by the significantly lower food intakes relative to controls (12.2 ± 1.1 vs 13.5 ± 1.2 g/day), that resulted in approximately 10% lower cumulative calcium and vitamin D intakes among females fed fish oil; and the fact that an interaction between sex and diet was not considered by the authors [591]. Similarly, supplementation of fish oil (10 g/100 g

fish oil) in rabbits (28 d) for 40 days led to impaired longitudinal growth of the tibia, alterations in tibial mid-diaphyseal morphology, and decreased structural strength properties but not that of material property. Although rabbits on the fish oil diet had significantly less weight gain than controls, energy-restriction alone did not explain these changes since they were not observed in pair-fed rabbits [592]. It was speculated that this is due to an increase in the total amount of fat in the diet, and resulted in altered production of tissue-specific cytokine and lipid peroxidation products that caused damages to bone tissue. To date, many mechanisms of action of EPA and DHA have been proposed, with inflammatory pathways among those most commonly studied.

**Table A2** Selected animal studies on the effects of LCPUFA on bone growth

**Gestation / Infancy**

| Ref. | Diet | Animals | Biomechanics / Bone ash | Bone Imaging | Biochemistry |
|---|---|---|---|---|---|
| [578] | (1) n-3 adequate<br>(2) n-3 deficient<br>(3) n-3 def-replete: Flaxseed oil (4.8g/kg/diet) + DHASCO (3.0 g/kg/diet) | Long Evans pups born to dams fed n-3 adq or n-3 def diets through pregnancy<br>- maintained on diets equivalent to maternal diets (n=48) or switched from n-3 def to n-3 adq (= n-3 def-replete)<br><br>Duration: 8 wk (15 wk of age) | **3 point bending (tibia)**<br>• ↑ loads at failure (~40%), peak load (~20%) and bending moment (~20%) in n-3 adq vs n-3 def at wk 0<br>• ↑ ultimate stress (~35%) in n-3 def-replete vs n-3 adq at wk 8<br>• ↑ energy to peak load (45-60%) in n-3 adq vs n-3 def-replete and CTRL at wk 8 | | |
| [593] | Isocaloric diets of:<br>(1) n3: 70 g/kg linseed oil, n6:n3=0.4<br>(2) n6 + n3: soybean oil; n6:n3=9<br>(3) n6: sunflower oil; n6:n3=216 | Sprague Dawley dams (d 7 of gestation), fed diet during late gestation + through lactation<br>Female pups (n=30, 3 wk)<br><br>Duration: 27 wk (30 wk of age) | | **Peripheral QCT (femur)**<br>• ↑ cortical BMC (6.6-8.6%) + area (6.6-9.2%) in n6+n3<br>• ↑ cortical thickness (4.0%) in n6+n3 vs n3<br>• ↓ periosteal (6.0%) and endosteal circumference (8.7%), cortical cross-sectional moment of inertia (22.9%), cross-sectional moment of resistance (16.1%) in n6 vs n6+n3<br>**DXA (femur)**<br>• ↑ BMC (8.4-15%) and area (9.7-13.6%) in n6+n3<br>• No difference in aBMD | • ↓ serum IGF-1 (27.7 - 36.5%) in n3 |
| [579] | 2 x 2 factorial:<br>(1) Boron-deprived (0 mg/kg)<br>(2) boron-adequate (3 mg/kg);<br>and<br>(1) SO: 75g | Sprague Dawley dams for 6 wk and male pups (n=15@, 3 wk of age)<br><br>Duration: 19 wk (21 wk of age) | **3 point bending (femur)**<br>• ↑ maximum force to break (4.7-12.2%) and bending moment (3.5 - 10.3%)<br>• ↓ femur length (0.7-3.0%) in FO | ***Ex vivo* µCT (LS4)**<br>• ↓ connectivity density (26.1%) of boron-deficient rats + FO vs boron-sufficient + SO | • ↓ tibia $PGE_2$ release (94.7 - 110.9%) in FO |

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| | Safflower oil/kg or<br>(2) FO: 65g Menhaden oil + 10 g LA/kg | | **Finite-element analysis by µCT (LS4)**<br>• ↑ compression force (4.3 – 48.8%) in FO | | |
| [594] | (1) Control (6% soybean oil w/w)<br>(2) AA+ DHA (0.6% fat as AA + 0.2% fat as DHA)<br>(3) DHA (0.8% fat as DHA)<br><br>AA and DHA in form of single-cell oil | Guinea pig sows (n=21) – experimental diet during pregnancy and lactation<br>Pups at d3 and d21 (n=15, reared by natural mother, 2 pups @ sow) | | **DXA**<br>Diet x sex interaction:<br>Males:<br>• whole body, tibia and LS BMC: CTRL > DHA > AA+DHA<br>• ↓ whole body and femur aBMD (~4-20%) in DHA<br>Females:<br>• ↑ WB and LS aBMD and BMC (~10-30%) in DHA<br>• Tibia and LS BMC: DHA, AA+DHA > CTRL<br>***Ex vivo* µCT (LS3, proximal tibia metaphysis)**<br>Males:<br>• ↑ tibia vBMD (~30%) in CTRL vs DHA<br>Females:<br>• ↓ tibia vBMD (~10%) in CTRL vs AA+DHA<br>• ↓ LS3 vBMD (~20%) in females fed DHA female vs all others except males AA+DHA | • ↑ plasma deoxypyridinoline (Dpd, 29.1 – 43.5%) in DHA vs control and AA+DHA |
| [580] | (1) 5 g/100 g soybean oil (SBO)<br>(2) Hydrogenated SBO (HSBO)<br>(3) Chicken fat (CF) or<br>(4) Menhaden oil (FO) | Japanese quail laying hens fed one of four diets from 4 wk<br>Newly hatched quail fed identical starter diet<br><br>Duration: 2 wk | **Shear test (tibia)**<br>• ↑ shear force (17.9%) and stiffness (30.4%) in FO vs CF at 2 wk | | • ↑ Dpd (33.0 – 52.4%) and total collagen cross-links (35.7-43.2%) in FO vs CF or SBO |

**Growing (non-rodents)**

| Ref | Diet | Animals | Biomechanics / Bone ash | Bone Imaging | Biochemistry |
|---|---|---|---|---|---|
| [589] | (1) CTRL<br>(2) AA:DHA as 0.6:0.1 g/100g<br>(3) AA:DHA as 1.2:0.2g/100g | Male Cotswold piglets low-birth-weight (LBW, 1.1-1.2 kg) and very-low-birth-weight (VLBW, =<1 kg) (n=30, 5 d)<br><br>Duration: 15 d (20 d of age) | | **DXA (LS)**<br>• ↑ BMC (23.2%) in LBW piglets with 0.6% AA + 0.1% DHA<br>↑BMC (35.5 – 42.9%) in VLBW piglets in 1.2% AA + 0.2% DHA | • ↓ urinary n-telopeptide: creatinine (65.3 – 71.7%) in groups 2 and 3 vs CTRL<br>• No difference in OC, urinary calcium, IGF-1 or $PGE_2$ release |
| [592] | (1) CTRL<br>(2) FO (10 g Menhaden oil /100g diet, 31% energy as fat)<br>(3) Pair-fed, 95% energy intake of FO to match body weight of fish oil, 8% energy as fat | Male weaning New Zealand White rabbits (~4 wk, n=50)<br><br>Duration: 40 d (~9 wk of age) | **3 point bending (tibia)**<br>↓ load at proportional limit (23%), maximal load (27%), energy absorbed to proportional limit (36%), energy absorbed to maximum (45%) in FO | **Micro-radiography (tibia mid-diaphysis)**<br>• ↓ periosteal envelop area (15%), intracortical porosities area (35%), moments of inertia (30%), cortical bone area (17%), and tibia length (4%) in FO | |
| [595] | (1)70 g/kg diet SBO<br>(2) Sesame oil (SO, 50g SO + 20g SBO),<br>(3) Fish oil (FO, 50g FO + 20g SBO),<br>(4) DHA (50g DHASCO + 20g SBO), | New Zealand white rabbits (6 wk, n=45, 25 male)<br><br>Duration: 100 d (~20 wk of age) | **Bone ash**<br>• ↑ Ca (13.6-50.4%), Mg (15.9-54.5%), P (5.9-16.1%) and Zn content (22.2-35.3%) in the DHA/AA, DHA, and FO | | • No difference in *ex vivo* $PGE_2$ levels |

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| | (5) DHA/AA (25g DHASCO + 25g ARASCO + 20g SBO) | | |
| [586] | (1) Control: 50g/kg diet SBO,<br>(2) Hydrogenated SBO (HSBO),<br>(3) Chicken fat (CF)<br>(4) Menhaden oil (FO) | Japanese quails (4 wk, n=30@)<br><br>Duration: 7 mo (8 mo of age) | **Shear test (tibia)**<br>• ↑ shear force and stress (24.7 – 29.9%) in FO<br>**Bone ash**<br>↑ % ash (12.7%), calcium content (13.9-15.3%), phosphorus content (9.8-14.3%) and mineral content (5.3-12.7%) in FO |

**Growing (rodents)**

| Ref | Diet | Animal | Biomechanics / Bone ash | Bone Imaging | Biochemistry |
|---|---|---|---|---|---|
| [591] | (1) Control: AIN-93G (7% fat w/w), 70 g/kg SBO<br>(2) FO: 60 g/kg Menhaden oil + 10 g/kg SBO | Sprague Dawley rats (3 wk, n=9-12 rats@ group/gender)<br><br>Duration: 5 wk (8 wk of age) | **Compression test (LS5)**<br>• ↓ peak load (15.7%) among females in FO vs CTRL | **DXA (LS1-6, femur)**<br>• No difference in length, bone area, BMC and aBMD | • No difference in serum IGF-1, estradiol, testosterone or urinary calcium in FO vs CTRL |
| [588] | 2 x 2 factorial:<br>(1) SO: modified AIN-93G with 15% w/w as SBO<br>(2) FO: 1:0.3 (w/w) FO and SO mixture (FSO)<br>With or without 10% inulin-type fructans | Male Wistar rats (n=24, 6 wk)<br><br>Duration: 15 d (8 wk of age) | **3 point bending (tibia)**<br>• ↑ peak load (20.9%), yield load (15.0%) and absorbed energy (28.3%) in FO vs SO | | |
| [287] | Safflower: Menhaden oil in different proportions (wt/wt):<br>(1) AIN-93G<br>(2) 90:10 (SMI)<br>(3) 80:20 (SMII)<br>(4) 50:50 (SMIII)<br>(5) 30:70 (SMIV)<br>Total fat: 70 g/kg | Male Sprague Dawley rats (n=60, 3 wk)<br><br>Duration: 7 wk (10 wk of age) | | | • ↓ $PGE_2$ in liver homogenates + bone organ culture (43-55%) in SMIV vs SMI<br>• ↑ alkaline phosphatase (ALP, 20.3 – 30.3%) in rats fed higher n3 FA vs SMI<br>Total, intestinal, liver and bone ALP rose with |

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| | | | | | decline in dietary n6/n3 ratio |
| [596] | (1) AIN-93G, *ad libitum*<br>(2) SBO<br>(3) Perilla oil (60% ALA)<br>(4) DHA (49.5% DHA, 9.8% EPA)<br>(5) EPA (28.1% EPA, 14.1% DHA)<br>Groups 2-5: 50% food restriction | Female Wistar rats (n=25, 7 wk)<br><br>Duration: 3 wk (10 wk of age | • ↓ femur fracture force (toughness, 21.3-41.5%) in group 2 | • No differences in BMD, density values from bone X-ray images | • ↑ urinary Dpd (7.3-20%) in groups 2 and 3<br>• ↓ serum estradiol (~50%) in groups 2-5 vs group 1<br>• No difference in serum $PGE_2$ |
| [597] | 2 x 2 factorial:<br>(1) modified AIN-93G with SBO at 70g/kg diet<br>(2) MSO (menhaden + safflower in 56:44)<br>With or without 10g/kg CLA | Male Sprague-Dawley rats (n=40, 3wk)<br><br>Duration: 6 wk (10 wk) | **Bone ash**<br>• ↓ bone dry weight (11.1%), ash weight (8.7%) and mineral density (5.0%) in SBO vs MSO | | • ↑ *ex vivo* $PGE_2$ (46.5 – 108%) in bone organ culture in SBO vs MSO<br>• No difference in serum IGF-1<br>↑ serum IGFBP-3 (~45%) and total IGFBP levels in MSO vs SBO |
| [581] | AIN-93G diet<br>(1) CTRL: 5% corn oil<br>(2) Evening primrose oil (EPO)<br>(3) 4% fish oil + 1% corn oil (FO)<br>(4) 4% tuna oil + 1% corn oil (TO) | Male Sprague-Dawley rats (n=40, 6 wk)<br><br>Duration: 6 wk (12 wk of age) | **3 point bending (femur)**<br>• No difference in bone strength | **DXA**<br>• ↑ right femur (8.3%) and lumbar spine (3.7%) aBMD in TO vs CTRL | |

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| [598] | AIN-93G based-diet:<br>2 x 2 factorial:<br>(1) AIN-93G (SBO)<br>(2) Menhaden-safflower oil (56:44 w/w, MSO) at 70g/kg diet<br>With or without CLA (10 g/kg diet) | Male Wistar rats (n=40, 4 wk)<br><br>Duration: 8 wk (12 wk of age) | **Bone ash (femur)**<br>↑ dry weight (2.4-4.0%, p=0.075) and bone mineral mass (2.2-5.4%, p=0.058) in MSO | | • ↓ urinary pyridinoline and Dpd (11.1-29.1%) in MSO<br>• No difference in serum osteocalcin and IGF-1<br>• ↓ *ex vivo* $PGE_2$ production from tibia (~50%) in MSO |
| [359] | (1) CTRL (AIN-93M)<br>(2) Hydrogenated coconut oil (HCO)<br>(3) Menhaden oil (MO)<br>(4) 20:1 DHA: AA (2.0% kcal DHA, 0.1% AA, 7.9% HCO)<br>(5) DHA-rich (2.1% kcal DHA, 7.9% HCO)<br><br>Total fat kcal: 5% | Female mice (n=50, 21d)<br><br>Duration; 9 wk (13 wk of age) | | ***Ex vivo* µCT**<br>Femur distal metaphysis:<br>• ↑ trabecular bone volume fraction (BV/TV, 55%), trabecular number (Tb N, 10%), trabecular thickness (Tb Th, 10%), connectivity density (Conn D, 45%)<br>• ↓ trabecular separation (Tb Sp, 15%), structure model index (SMI, 30%) in DHA<br>Femur mid diaphysis:<br>• ↓ BV/TV (3.84%) in DHA,AA<br>• ↓ cortical thickness (7.81%) in MO and DHA<br>↑cortical vBMD (1.2-1.4%) in DHA | |
| [582] | Modified AIN-93G (12% w/w) with:<br>(1) Corn oil (CO)<br>(2) Flaxseed oil (FO) | Female Sprague-Dawley rats (n=60, 4 wk + 1 wk acclimation) | **Bone ash (tibia)**<br>• ↑ dry weight (~3%) in SO and TO<br>**3 point bending (femur + tibia)** | **DXA (tibia)**<br>• ↑ bone area (16.4%), BMC (23.3%) and aBMD (9.2%) in TO<br>• ↑ bone length (2.4-5.8%) in TO | • ↑ serum osteocalcin (18.6-41.8%) in FO<br>• No difference |

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| | (3) Krill oil (KO, 10g/kg) + 2g/kg CO<br>(4) Menhaden oil (MO,10g/kg) + 2g/kg CO<br>(5) Salmon oil (SO)<br>(6) Tuna oil (TO | Duration: 8 wk (13 wk of age) | • No differences in bone strength | ***Ex vivo* µCT**<br>• ↑ femur distal metaphysis BV/TV (54.5%), Tb N (31.7%), Conn (55.6%) and lower Tb Sp (32.9%) in MO<br>• ↑ tibia proximal metaphysis BV/TV (56.0%), Tb N (46.2%) and Conn (69.9%) in FO<br>No difference in tibia mid-diaphysis cortical bone | in serum ALP or pyridinoline |
| [583] | (1) DR: Dam-reared / AIN-93G (10% dietary fat)<br>(2) LA: n-3 deficient milk<br>(3) DPA: LA with 1% DPA n-6 of total lipids (DPA)<br>(4) DHA: LA with 1% DHA<br>(5) DHA/DPA: LA with 1% DHA-DPA n-6 (2:1) | Male Long-Evans pups (n=50)<br>Postnatal day 2: artificial rearing system<br>Postnatal day 21: pelleted diets of the same treatment<br><br>Duration: 12 wk (15 wk of age) | | **DXA (femur, tibia, LS4)**<br>• ↑ distal (15.7%) and proximal femur (12.1-16.6%) BMC in DR and DHA/DPA vs DPA<br>• No difference in tibia or LS4 | |
| [587] | (1) Control (Prina 5L79) 5.2% fat (w/w)<br>(2) SFA: 18% coconut oil + 2% SBO (w/w)<br>(3) n-3 PUFA: 20% flaxseed oil<br>(4) n-6 PUFA: 20% safflower oil | Male Sprague-Dawley rats (n=31, 40 d)<br><br>Duration: 65d (15 wk of age) | **3 point bending (femur)**<br>• ↑ bone width (2.6%, adjusted for BW) in n-3 PUFA vs CTRL<br>• ↑ stiffness (31.3-34.4%) + peak load (38.5-42.0%) adjusted for BW) in n-3 PUFA and n-6 PUFA vs CTRL | **DXA (femur)**<br>No difference in aBMD, BMC or area | |
| [215] | (1) CTRL (55% SFA, 40% MUFA, 10% PUFA) | Female C57BL/6 mice (n=38, 12 wk) | **3 point bending (femur and tibia)**<br>• ↑ ultimate force, ultimate stress | **DXA (LS)**<br>• ↑ decrease in aBMD in DHA (-15.3%) between 8 and 17 mo of age vs CTRL (- | • ↑ serum osteocalcin (~40%) and |

| | | | | |
|---|---|---|---|---|
| (2) DHA: 0.13 mg DHA + 0.04 mg EPA / g diet<br>(3) EPA: 0.14 mg EPA + 0.02 mg DHA / g diet | Duration: until 17 mo of age | and plastic energy (12.3% - 29.9%) in EPA<br>• ↑ ultimate stress (27.3%) in DHA | 1.4%)<br>***In vivo* µCT**<br>• ↑ increase in vertebral BV/TV and TbN (~3-5%) between 3 and 8 mos of age with EPA and DHA<br>• ↑tibia mid-diaphysis cortical BV (~7%) and cortical thickness (~10%) in EPA<br>***Ex vivo* µCT** (femur)<br>• ↑ cortical total volume (TV, 6.3%), BV (8.1%) and thickness (4.4%) in EPA<br>Increase in cortical TV (6.3%) in DHA | tartrate-resistant alkaline phosphatase (~20%) at 17 mo in EPA and DHA |

***Fat-1* mice**

| Ref. | Diet | Animals | Biomechanics / Bone ash | Bone Imaging | Biochemistry |
|---|---|---|---|---|---|
| [670] | AIN-93G with 10% safflower oil | C57BL/6 x C3H *fat-1* mice (n=50, 3 wk)<br><br>Duration: 9 wk (12 wk of age) | **3 point bending (femur)**<br>• No difference in bone strength | | |
| [349] | Normal chow diet | *Fat-1* mice (2-, 5-, 12- and 24-wk)<br>Exposure to n-3 in gestation and lactation: Offspring (2 wk of age) from *fat-1* mother and WT father (MF) vs *fat-1* father and WT other (MB) | **3 point bending (tibia)**<br>↑ area under curve, ultimate load, failure load (wk 12 only), slope and yield point (30-80%) in fat-1 at 12 and 24 wk | **Ex vivo µCT (tibia)**<br>• ↑ metaphysis BV/TV (40-100%), Tb N (85-90%) and lower Tb Sp (65-120%) in fat-1 at 5 and 24 wk<br>• ↑ mid-diaphysis cortical thickness (15-50%), cortical area (~20%), vBMD (~5-10%) and smaller marrow area in fat-1 at 5 and 12 wk<br>MB vs MF:<br>• ↓ metaphysis BV/TV and Tb N (33-50%) + ↑ Tb Sp (~55%) in MF<br>• ↑ mid-diaphysis cortical thickness, cortical area, vBMD (10-60%) + ↓ total and marrow area (5-10%) in MF | |

## A.4 Selected mechanisms of actions of DHA in growing bones

### A.4.1 DHA has anti-inflammatory properties

Metabolites of the n-3 and n-6 PUFA, including the eicosanoids (prostaglandins and leukotrienes), resolvins, protectins, and lipoxins are important mediators of inflammation. EPA and DHA act as substrates for the 3-series prostaglandins that in general are viewed to have anti-inflammatory properties. The 2- and 4- series of prostaglandins, thromboxanes and leukotrienes, including prostaglandin $E_2$ ($PGE_2$) and leukotriene $B_4$ ($LTB_4$) are derived from the n-6 PUFA AA by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) respectively [599]. These substrates are in direct competition for this key conversion enzyme and its isozymes, but COX exhibits the greatest specificity for AA such that $PGE_2$ is preferentially produced [600].

Prostaglandins act in an autocrine and/or paracrine manner through prostanoid receptors to activate differential signaling cascades [601]. To date, at least four distinct G protein-coupled receptors for $PGE_2$ (EP) have been identified, designated as $EP_1$, $EP_2$, $EP_3$ and $EP_4$, affecting various secondary messengers: EP1 is linked to inositol trisphosphate (IP3) signaling and phospholipase C (PLC) through $G_{q/p}$ activation, resulting in intracellular calcium mobilization and exocytosis. $EP_2$ and $EP_4$ stimulates cyclic adenosine monophosphate (cAMP) production/ protein kinase A (PKA) signaling; and $EP_3$ activated $G_i$ to inhibit adenylyl cyclase, cAMP production and opposed the effects of $EP_2$ and/or $EP_4$ [602, 603].

In growing animals fed moderate levels of n-6 PUFA, low levels of circulating $PGE_2$ appeared to favor bone formation, yet excessive $PGE_2$ production has been associated with increased bone loss [604]. Inclusion of n-3 PUFA in diet significantly decreased *ex vivo* $PGE_2$ production in rat femurs, while also reducing circulating pyridinoline levels, a marker of bone resorption [605]. This suggests an effect of n-3 LCPUFA on lowering bone resorption by

modulating pro-inflammatory $PGE_2$ synthesis. Exposure of MC3T3-E1 osteoblastic cells to AA (20 μg/ml) stimulated $PGE_2$ production [606], potentially due to auto-amplification by inducing COX-2 mediated via the $EP_1$ subtype of PGE receptors expressed in mouse osteoblasts.

$PGE_2$ has also been shown *in vitro* to be a potent modulator of bone modeling and remodeling, exhibiting biphasic properties in affecting both bone formation [607, 608] and resorption [609]. $PGE_2$ at low concentrations (0.01 – 1 nM) stimulated cell proliferation in primary human bone cell cultures through enhancement of PLC, resulting in an increase in PKC activity and intracellular calcium influx. Whereas at higher concentrations (0.1 μM), $PGE_2$ stimulated cAMP production and inhibited human bone cell proliferation [610]. Similarly, time course experiments in murine spleen cell cultures demonstrated that $PGE_2$, in presence of RANKL and macrophage colony-stimulating factor (M-CSF) showed initial inhibitory effects (5-6 days of culture) and later a stimulatory effect (8 and 9 d) on osteoclastogenesis, accompanied by increased osteoclast size and resorption pit formation and reduced osteoclast apoptosis [611]. Incubation with $EP_2$ and $EP_3$ agonists, but not that of $EP_1$ or $EP_4$ mimicked effects of $PGE_2$ in decreasing osteoclast number, indicating inhibitory effects possibly mediated by $EP_2$ and $EP_3$ receptors [611]. Conversely, selective $EP_4$ deletion in fibroblasts abrogated expression of RANKL that is normally induced after $PGE_2$ signaling, inhibiting osteoclastoegensis [612]. Such discrepancy can in part be due to the different expression patterns of prostaglandins receptors by cell type, differentiation status, tissues, and species. MC3T3-E1 mouse osteoblastic cells were reported to predominantly express $EP_1$ and $EP_4$ receptors [613], whilst human mesenchymal stem cells express $EP_4$ receptors [614].

Prostaglandin synthesis is also involved in osteoclastogenesis and bone resorption mediated by pro-inflammatory cytokines including interleukin-1β (IL-1β), IL-6 and tumor

necrosis factor-α (TNF-α) [615, 616]. Addition of the COX-2 inhibitor celecoxib to co-cultures of bone marrow cells markedly inhibited osteoclastogenesis induced by IL-1β, TNF-α, lipopolysaccharides,1, 25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) and PTH. This effect was negated when $PGE_2$ is present in culture, suggesting that COX-2-dependent PG synthesis is essential for osteoclast differentiation [615]. Offering further insight into the mechanism of $PGE_2$ action on the interaction among bone cells, disruption of COX-2 expression resulted in defective RANKL secretion in osteoblasts and in turn impaired osteoclast formation in response to $1,25(OH)_2D_3$ and PTH [617]. Consistently, COX-2 and $PGE_2$ stimulated osteoclast formation through inhibiting OPG secretion and enhancing RANKL production by osteoblasts, as well as stimulating RANK expression in osteoclasts. While IL-6 alone had little direct effect on osteoclast differentiation in monocultures of RAW264.7 macrophages, IL-6 significantly enhanced expression of $PGE_2$ receptors in both osteoblasts and osteoclasts, and demonstrated reciprocal interaction with the COX-2/$PGE_2$ system, inducing each other's production [616].

On the other hand, the less inflammatory eicosanoids $PGE_3$ and $LTB_5$ that are derived from EPA by the same COX-2 and 5-LOX enzymes [618] have functions in bone. Once synthesized, $PGE_3$ is equipotent to $PGE_2$ in bone resorption [619]. However, conversion to PGs from n-3 PUFA is less effective than n-6 PUFAs, resulting in lower $PGE_3$ levels [600]. Induction of COX-2 and IL-6 expression in RAW 264.7 macrophages by $PGE_2$ was significantly greater than that by $PGE_3$; and that $LTB_4$ is more potent chemoattractant at promoting polymorphonuclear cell adherence compared to $LTB_5$ [620], thus the n-3 derived eicosanoids potentially benefit bone, as the otherwise increased differentiation of cells in the granulocytic lineage has been associated with enhanced bone resorption by osteoclasts [621]. Supplementation of n-3 PUFA significantly reduced *ex vivo* $PGE_2$ synthesis in rat femurs as well as serum pyridinoline [605]; whereas higher

DHA in the diet was associated with lower bone resorption based on urinary N-telopeptide [622], another marker of bone resorption. Evidence from cell culture studies supports the underlying mechanism that n-3 LCPUFAs reduce COX-2 protein levels and $PGE_2$ production but increase ALP activity in cultured osteoblast-like MC3T3-E1 cells [287]. Altogether these observations provide support for a protective effect against bone resorption by reducing production of pro-inflammatory prostaglandins [605].

The resolvins is a relatively new family of lipid mediators synthesized from EPA (E-series resolvins) or DHA (D-series resolvins) through the COX and LOX pathways, and are identified to be potent anti-inflammatory, proresolving molecules [623]. Resolvin $E_1$ is generated in healthy humans given EPA and aspirin, suggesting that in scenarios where COX-2 is induced during inflammation, treatment with aspirin could initiate an active process involving production of endogenous chemical modulators (i.e. resolvins) to resolve inflammation. *In vitro*, resolvin $E_1$ is associated with inhibiting dendritic cells migration and pro-inflammatory IL-12 production possibly through terminating NF-κB activation [624]. In primary osteoclast cultures derived from mouse bone marrow, treatment with resolvin $E_1$ resulted in marked decreases in resorption pit formation and the number of multinucleated osteoclasts, as well as the attenuation of RANKL-induced nuclear translocation of the p50 subunit of NF-κB that forms part of the mature protein for activating transcription [625]. Using a temporal metabololipidomics approach with self-limited resolving exudates, resolvin $D_3$ was reported to be uniquely associated within the inflammation-resolution period. This study also demonstrated that resolvin $D_3$ might have specific roles in blocking neutrophil migration, modulating eicosanoid production, and increasing anti-inflammatory IL-10 production [626]. In addition, resolvin $D_1$ and the DHA metabolite protectin $D_1$ inhibited IL-1β and TNF-α production in human endothelial cells [627],

as well as osteoclastogenesis from mouse bone marrow macrophage culture [628]. The mechanisms, as well as physiological effects of resolvins when applied *in vivo* remains to be fully elucidated, however, it appears from available evidence that such fatty acid derivatives have promising anti-inflammatory properties.

### A.4.2 DHA affects osteoblastogenesis and osteoclastogenesis

Depending on the type of fatty acids present in the bone and marrow milieu, key mediators of osteoblast- and osteoclastogenesis including RANKL, its receptor RANK, a decoy receptor OPG, and $PGE_2$ are modulated to various extents, possibly via peroxisome proliferator activated receptor (PPAR) binding (**Figure A2**).

Increasing dietary intake of fish oil has been shown to prevent the ovariectomy-induced increases in membrane bound RANKL expression in murine T cells [629]; and treatment with DHA or EPA *in vitro* inhibited osteoclastogenesis evident by lowering TRAP activity and $TRAP^+$ multinuclear cell formation [629]. In MC3T3-E1 osteoblast like cells, incubation with DHA (5 μg/ml) inhibited $PGE_2$-induced RANKL expression [283]; whereas fish oil feeding in mice prone to autoimmune diseases resulted in significantly reduced RANKL, but enhanced OPG mRNA levels at the lymph node [630], implying that n-3 PUFA may exerts its influence on osteoclasts through its action on osteoblasts by altering the RANKL/OPG ratio. More recently, DHA strongly inhibited RANKL-induced osteoclastogenesis in bone marrow derived macrophages, and was associated with a 40% reduction in dendritic cell-specific transmembrane protein (DC-STAMP) mRNA levels [628], which could impair the normal cell-cell fusion during osteoclastogenesis.

As mentioned previously, the n-3 PUFA may also act upon osteoclasts directly by controlling their inflammatory responses. Osteoclasts are recruited and activated upon

stimulation from osteoclastogenic cytokines such as IL-6 and TNF-α, which leads to eventual bone resorption. Exposure of the murine monocytic cell line RAW 264.7 to DHA reduced pro-inflammatory responses induced by RANKL stimulation, via decreasing NF-κB and p38 MAPK expression which eventually lowered TNF-α synthesis [631]. Aging C57BL/6 mice fed fish oil for 6 months showed decreased IL-6 and TNF-α secretion in concanavalin-stimulated splenocytes [632]; and as well in humans (73 ± 9 y) supplemented with 1.7 g DHA for 6 mo, where IL-6 release is suppressed by about 40% in mononuclear leukocytes [633]. *In vitro* evidence exists for an interaction between IL-6 and $PGE_2$, where the $PGE_2$-induced OPG suppression in osteoblasts was dependent on IL-6 [616].

As aforementioned, osteoblastogenesis is influenced by the balance of chemical mediators present in the marrow that determines lineage commitment into either adipocytes or osteoblasts. Key transcription factors required for differentiation into osteoblasts are core binding factor α-1 (Cbfa-1), also known as Runx2, Wnt/LRP-5, and its downstream mediator β-catenin [634]. In human hetapocellular carcinoma cells, Runx2 translocation into the nucleus was reported to be stimulated by IGF-1 and PTH, but inhibited by TNF-α. Supplementation of DHA or n-3 PUFA in dogs [635] and middle-aged rats [605] suppressed serum TNF-α and enhanced IGF-1, thus allowing for DNA binding of Runx2 in the nucleus to promote osteoblast differentiation. The anti-inflammatory effects of n-3 PUFAs may indirectly enhance osteogenic differentiation of MSCs, as IκK-NF-κB activation by TNF-α and IL-17 promoted β-catenin ubiquitination and degradation through induction of Smurf1 and Smurf 2 [636]. DHA treatment (5 – 60 μM) resulted in a dose-dependent reduction in cell viability through simultaneous inhibition of COX-2 and β-catenin [637], suggesting a possible explanation for the adverse effects of high n-3 PUFA in early development, since loss of β-catenin produced severe osteopenia with substantial

increases in osteoclast activity [638].

Offering a novel molecular target of n-3 PUFAs on regulating bone mass, the free fatty acid receptor 4 (FFA4; also known as GPR120), which is a newly deorphanized G-protein coupled receptor (GPR) has been identified to be a receptor for n-3 PUFAs [639, 640]. Expression of FFA4 is greater in mature osteoblasts and osteoclasts relative to their precursors, and DHA treatment (0.5 μM) of osteoclasts and MC3T3-E1 cells promoted internalization of FFA4 into perinuclear space, confirming that DHA activates FFA4. Using a transgenic mouse model that overexpresses n-3 fatty acids but lacking FFA4 by crossing fat-1 transgenic and FFA4 (*Ffar*4$^{-/-}$) knockout mice, female fat-1 mice were protected against ovariectomy-induced bone loss only when they had the *Ffar4*$^{+/+}$ background but not the *Ffar4*$^{-/-}$ background. Similarly, male fat-1 mice fed HFD displayed higher bone mass relative to their WT littermates, but this was observed only in those with the *Ffar4*$^{+/+}$ background. Higher bone mass in both models was associated with increased osteoblastic bone formation, evident by greater osteocalcin and ALP expression as well as β-catenin activity; alongside decreased osteoclastic resorption through inhibition of NF-κB activity by FFA4 [641]. Given that receptors for PTH, calcitonin and calcium also belong to the GPR superfamily, and the wide spectrum of cellular responses that GPRs initiates, it is of particular interest to further characterize the role of FFA4 in bone metabolism across various life stages and metabolic states.

### A.4.3 DHA acts through PPARs to affect cell differentiation and gene expression

As discussed previously, another important family of transcription factor in directing the fate of MSC into commitment of either lineage is the PPARs. The PPARs are ligand-activated transcription factors of the superfamily of nuclear hormone receptors that contains a hydrophobic ligand-binding site and a DNA binding domain [642]. To date, three subtypes of PPAR (α, β/δ,

and γ) have been identified. All PPARs share the same molecular mode of action via the conformational change upon ligand binding, with subsequent formation of a heterodimer with retinoid X receptor (RXR) and binding to peroxisome proliferator response elements (PPREs) in the promoter region of the target genes. Expression of PPARα is found in many tissues but is predominant in oxidative tissues such as brown adipose tissue, cardiac muscle, and liver. PPARβ/δ is found ubiquitously, while expression of PPARγ is more restricted, with adipocytes and macrophages expressing the highest level [643, 644]. Transcription activation by PPARs is dependent on a series of steps including ligand binding to PPAR, binding of PPAR to the target gene, removal of co-repressors and recruitment of co-activators, remodeling of chromatin structure, and eventually facilitate gene transcription [643]. All 3 PPARs are able to bind fatty acids with a general preference for LCPUFAs including DHA [320, 645, 646].

All PPAR subtypes are expressed in bone tissue. The function of PPARα and –γ are best characterized to date. While PPARα appears to be important in channeling fatty acids towards β-oxidation in the liver, PPARγ has a central role in mediating the balance between adipipogenesis and osteoblastogenesis. in addition to direct effects on osteoclastogenesis [647]. PPARγ expressed in adipose tissue regulates adipocyte differentiation and regulates the metabolic responses of adipocytes, including insulin sensitivity [648]. In inflammatory cells, activation of PPARγ favors an anti-inflammatory state by modulating inflammatory cytokines production [649]. DHA has been found to be a dual ligand for PPAR α/γ [650-652], one can therefore reason that DHA would exert its effect on bone at least directly via these surface or intracellular fatty acid receptors.

The inhibitory role of PPARγ on maturation of various osteoblastic cell lines concomitant with adipocyte differentiation had been well characterized in various *in vitro* models [431, 653].

Activation of PPARγ in cells of the osteoblast lineage resulted in conversion to terminally differentiated adipocytes, and irreversibly suppressed osteoblast-specific signaling pathways including the Wnt, TGF-β/bone morphogenic protein (BMP), IGF-1 and Runx2 [325, 326]. *In vivo*, homozygous PPARγ-deficient embryonic stem cells fail to differentiate into adipocytes, but spontaneously differentiate into osteoblasts; furthermore, PPARγ heterozygous mice display a high bone mass due to increased osteoblast number and bone formation [451]. PPARγ agonists such as pioglitazone lead to bone loss [654], suggesting that PPAR acts as a positive regulator of adipogenesis, but not that of osteoblast differentiation. In a recent study using an osteoblast-specific PPARγ knockout mouse model, suppression of PPARγ activity in osteoblasts significantly increased osteoblast differentiation and distal femur metaphysis trabecular number quantified by *in vivo* μCT [655]. Endogenous PPARγ in MSC and osteoblasts strongly inhibited mTOR/p70S6k activity [655] that has been implicated to have essential roles in osteoblastic differentiation [656, 657], suggesting that PPARγ may modulate bone formation both directly and indirectly through regulation of mTOR pathway and diverting MSC differentiation into adipocytes respectively.

In addition to the potent adipogenic function of PPARγ in bone marrow MSCs, more recent studies suggest that it participates in regulating osteoclastogenic functions. Selective ablation of the PPARγ gene in mouse osteoclasts resulted in osteopetrosis characterized by increased bone mass and a reduced medullary cavity size that is indicative of impaired osteoclast differentiation. Ligand activation of PPARγ by rosiglitazone further exacerbated osteoclast differentiation by directly regulating c-fos protein content [647], a key determinant of osteoclast lineage commitment and RANK development. Supporting evidence stems from a recent study, where addition of rosiglitazone to murine bone marrow stem cells increased differentiation of

multinucleated osteoclasts by about 25%, and may be associated with TRAF6 and downstream ERK signaling pathway indicated by 1.5-2 fold induction in protein and mRNA levels [658]. Taken together, PPARγ and its ligands appear to favor bone resorption through promoting osteoclastogenesis and suppression of osteoblast differentiation.

Only a few studies have evaluated the direct effects of DHA on bone homeostasis via PPARγ signaling. While a recent study in human MSC reported that DHA at a lower concentration (20 μM) had no effect on differentiation into osteoblast/adipocytes [323]; treatment of human primary osteoblastic cells with DHA (30 μM), AA (30 μM) and 15d-$PGJ_2$ (highly specific PPARγ activator) significantly inhibited cell proliferation and viability by 27-51%, mediated through a 4-5 fold induction in PPARγ mRNA expression [324]. Of note is that osteoblast cells express adipogenic transcription factors that are able to respond to adipogenic agents including PUFA, demonstrating that a certain degree of plasticity exists between osteoblasts and medullary adipocytes in cells cultured in an adipogenic medium [653]. In support of this was the observation that DHA (50 μM) appeared to inhibit cell proliferation not by inducing cell apoptosis, but by inhibiting the transition from G1 to the S phase in the cell cycle of osteoblastic cells [659], this may represent the initiation of cell differentiation re-programming and a potential switch into the adipocyte lineage. In bovine calcifying vascular cells incubated with DHA (25 μM), ALP activity and mineralization of vascular cells were significantly down-regulated, partially via the p38-MAPK pathway. Transient transfection of the PPAR response element-luciferase construct in these cells revealed that DHA increased luciferase activity similar to a known PPARγ ligand ciglitazone, indicating the inhibiting effects on mineralization of DHA is mediated together by p38-MAPK and that of PPARγ [660]. Interestingly, EPA and DHA stimulated osteoclastogenesis in bone marrow-derived monocytes (BMM) precursor cells,

evident by increased expression of PPARγ and osteoclast markers including c-fos, TRAP, cathepsin K and nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent-1 (NFATc1); addition of PPARγ antagonists inhibited stimulations of osteoclastogenesis and c-fos expression. However, when EPA and DHA were incubated with bone marrow cells including both BMMs and MSCs, osteoclastogenesis was inhibited through the suppression of expression of RANKL and NF-κB-regulating genes, COX-2, TNF-α and IL-6 in culture. Similarly, the PPARγ antagonists reversed inhibitions of NF-κB transcriptional activity and osteoclastogenesis by EPA and DHA, confirming that effects of EPA and DHA on BMM and bone marrow cells are PPARγ-dependent [661]. Treatment of primary BMM from mice with DHA in the presence of RANKL and M-CSF inhibited osteoclastogenesis at the late stage (after 48h), gene expression analyses with microarray shown that DHA significantly downregulated sets of genes related to cell motility, cell adhesion, cell-cell signaling and morphogenesis, including DC-STAMP following RANKL stimulation [662]. Given the wealth of evidence of a positive association between n-3 PUFA and bone mass in animals and humans, and taking into account the dynamic interaction of bone cells of the BMU, it is possible that n-3 PUFA suppresses osteoclastogenesis by inhibiting NF-κB activation through PPARγ signaling *in vivo*.

It appears from *in vitro* evidence that PPARγ signaling adversely influences bone homeostasis, which is contradictory to the observations *in vivo* where DHA is associated with higher bone mass and stimulates osteoblastogenesis. Whether DHA acts directly on bone cells in lieu of PPARγ signaling, along with its regulatory roles in inflammatory cytokines and $PGE_2$ production to produce the final bone phenotype remains to be determined. To date no studies have reported the effects of DHA on bone mass via PPARα signaling. PPARα agonists resulted in elevated bone mass and OPG *in vitro* [654]. Likewise, both the PPARα/δ agonists LA and

bezafibrate up-regulate osteoblast differentiation *in vivo*, resulting in 7-11% and 15-221% increases in trabecular and cortical periosteal bone formation respectively [663]. It is therefore possible that DHA could benefit bone via this pathway. Recently it has also been suggested that the regulation of skeletal homeostasis by PPARγ and its ligand rosiglitazone is age-dependent, since in old mice, rosiglitazone increases bone resorption while sustaining bone formation. In contrast, rosiglitazone decreases bone formation while sustaining bone resorption in young mice [664]. This raises the importance of taking into consideration not only age, but also metabolic state when one attempt to parallel *in vitro* results in *in vivo* models.

### A.4.4 Effects of DHA and prostaglandin metabolites on IGF-1

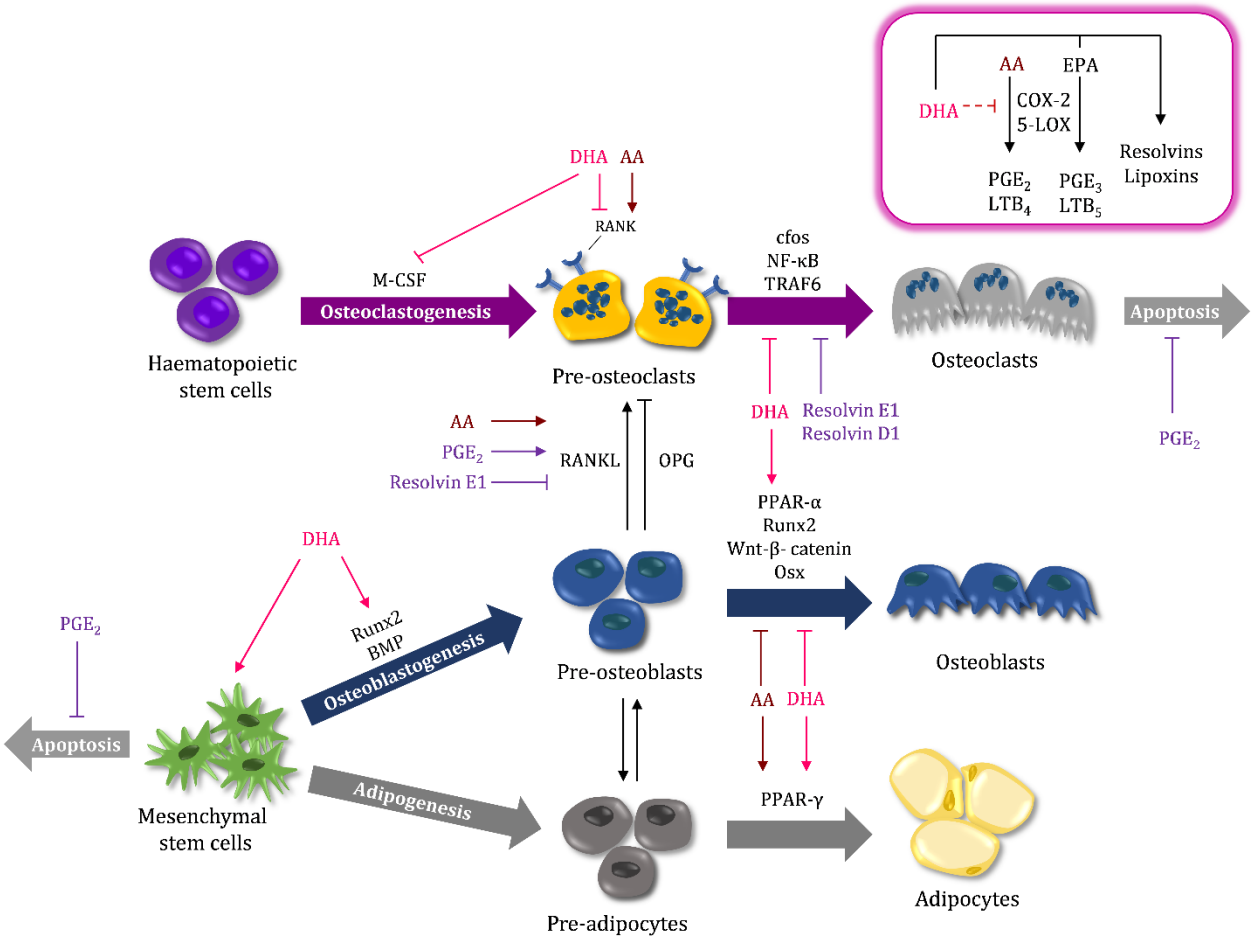
Dietary PUFAs may influence levels of IGF-1 indirectly through $PGE_2$ production. Primary osteoblasts isolated from fetal rat bones exposed to $PGE_2$ had up to 4.7 fold increase in IGF-1 transcript and protein levels. This stimulatory effect of $PGE_2$ was mediated through induction of cAMP signaling, as its effect on IGF-1 production was mimicked by other agents that increase cAMP levels [665]. Treatment of cells with COX-2 inhibitor indomethacin did not alter PTH-stimulated IGF-1 levels, since PTH signaling also increases cAMP. This suggests that $PGE_2$ production contributes to bone modeling and remodeling processes by way of its ability to increase cAMP and IGF-1 synthesis by osteoblasts [666]. The ability of $PGF_{2\alpha}$ to stimulate proliferation of osteoblastic MC3T3-E1 cells was completely abolished by the addition of a neutralizing anti-IGF-1 antibody. While $PGE_{2\alpha}$ decreased IGF-1 transcript levels and secretion into the culture medium, the number of high affinity binding sites for IGF-1 were up-regulated in a dose-dependent manner, suggesting the anabolic effects of prostaglandins may also occur through increasing response of bone cells to IGF-1 [667].

The activity of IGF-1 is modulated by a family of IGF binding proteins (IGFBP). IGF-1

polypeptide degradation may depend on its association with a specific IGFBP, and changes in IGFBP synthesis, degradation, and IGF-1 affinity could play a role in the stability of IGF-1. Treatment of primary osteoblasts-enriched cultures from fetal rat bones with $PGE_2$ elevated transcript levels of IGFBP-3, -4 and -5 (~22-, ~2- and ~4 fold respectively) as well as the 30/32 kDa IGFBP complex that consists of IGFBP-2 and -5. It is thus possible that $PGE_2$ modulates activity of locally produced IGFs in skeletal tissue by altering association of complexes between IGFs and certain IGFBP, storing IGFs for future utilization during bone formation [668].

Limited data exist to date for the direct effect of PUFA on IGF-1. Animal studies also reported mixed data on the effect of LCPUFA on circulating IGF-1 concentrations. Female pups (3 wk) born to dams fed a n-3 rich linseed oil- based diet through late gestation and lactation had 27.7-36.5% lower serum IGF-1 compared to those fed soybean or sunflower oil [593]. Whereas growing male rats (3 wk) fed a fish-oil enriched diet for 7 wk had increased IGFBP-3 levels [597], with more studies showing no effect of LCPUFA on IGF-1 concentrations [589, 591, 598]. *In vitro,* EPA and DHA inhibited Caco-2 cell proliferation compared to cells cultured with LA. This was associated with reduction in IGF-2 and up to 3.5 fold increase in IGFBP-6 transcript levels and secretion, resulting in less free IGF-2 thus contribute to the inhibition of Caco-2 cell proliferation [669].

**Figure A2** Effects of DHA on osteoblastogenesis and osteoclastogenesis via its anti-inflammatory properties and the direct effects on bone cells



## A.5 Knowledge gaps and future directions

### A.5.1 Sexual dimorphism in metabolism of LCPUFA and skeletal growth

It is widely accepted that sex differences exist in bone development, and this becomes apparent during puberty where boys and girls experience periods of rapid growth at different ages with resultant differences in PBM. Bone strength and PBM are greater in adult men than women. However, growth does not produce a denser skeleton in males, rather it is a result of variation in structural properties formed during growth and sexual maturation.

The sex differences in bone strength are established during puberty and largely under the influence of levels of circulating sex steroids. While the contribution of the height of the vertebrae to sex dimorphism in height is modest, differences in appendicular growth are apparent, and in part, is a result from the differences in timing of puberty. In humans, pubertal growth in boys begin approximately 1 year later than girls, yet it lasts longer, and boys experience greater growth velocity than girls [670, 671]. Males grow longer and wider bones that are stronger than female bones. In females, earlier completion of longitudinal growth with epiphyseal fusion, inhibition of periosteal apposition and reduced resorption at the endosteal surface produces smaller bone and marrow cavity. While in males, continual lengthening of bones and periosteal apposition increases cortical thickness, leading to a larger external bone size in men than in women [405]. Pubertal growth in males is associated with adding bone mostly on the periosteal surface, where the effect on bone strength is highest; whereas females deposit bone on the endocortical surface, where bending stress is lowest, and the mechanical advantage of the bone is least. Thus, for a given bone mass, males have stronger bones than females because of placement of bone further from the neutral axis of the long bone. It has been suggested that the purpose of this mechanically inefficient endocortical bone apposition during female puberty is to

create a reservoir of calcium that can be mobilized during reproduction without compromising bone strength [304].

Sexual dimorphisms in circulating plasma concentrations of n-3 LCPUFA, especially DHA has long been reported. While the majority of observational studies vary in their sample population, age range, degree of dietary control exerted, and the range of blood lipids analyzed, the prevailing view is that women have significantly higher circulating DHA concentrations compared to men, and that it is independent of dietary intake [672, 673]. This has been hypothesized to be an evolutionary adaptation to provide to the fetus DHA to support its major roles in cognitive and visual development early in life, and could be mediated through estrogen actions as shifts in fatty acid composition in plasma membrane during the menstrual cycle, pregnancy, and menopause have been reported [674, 675]. The concentration of DHA in maternal blood was also significantly elevated in the third trimester of pregnancy, when circulating levels of estrogen are the highest [676, 677]. This increase in maternal DHA is required, presumably, to support the fetal brain growth spurt in the third trimester, as the fetus' capacity to produce DHA from ALA may be insufficient to meet the extreme demand [678].

Given the modulatory effects of sex steroids on bone development and LCPUFA status that are amplified during puberty, one can anticipate a significant interaction among these three aspects to impact patterns of bone growth. However, studies that evaluated the effect of LCPUFA on bone growth in both genders remain scarce. A gender x genotype interaction was reported in studies involved in fat-1 mice, males at 12 wk of age showed greater incorporation in DPA (C22:5 n-3) and DHA in the femur, as well as approximately 20% greater femur width despite no differences in bone strength [679]. In a study where diets of guinea pig sows were supplemented with DHA alone or combined AA+DHA through pregnancy and lactation, contrasting

observations in bone outcomes measured in males versus female offspring at 3 wk of age was reported. Male pups in both DHA and AA+DHA groups had lower values of whole body aBMD as well as tibia metaphyseal trabecular volumetric BMD (vBMD) than controls; whereas in female pups an opposite trend was observed, suggesting combined LPCUFA may have positive effects in females. Despite so, no differences in circulating biomarkers for bone formation or resorption, nor was LCPUFA status different in males and females [594]. This is in agreement with previous reports in guinea pig pups where maternal LCPUFA supplementation during lactation up to day 21 was beneficial for LS 1-4 aBMD in female but not male offspring [384]. Nonetheless, the relatively young age of offspring when measurements were obtained render it difficult to draw a definitive conclusion on the influence of sex on LCPUFA and bone growth, as it would be reasonable to expect the effect of sex steroids to be minimal during that period. It remains to be determined if this difference in bone outcome observed early in life will persist through puberty and relate with LCPUFA status.

### A.5.2 Dose and type of LCPUFA

The majority of the studies performed in both humans and animals utilized fish oil as the main source of DHA. While common fish oils and their supplements can provide up to 12-20% DHA (or 200 mg DHA per capsule) [680], it could be potentially confounding as these fish oils also contain high concentrations of EPA as well as other LCPUFA including ALA and DPA that have been shown to affect bone [583, 681]. The fatty acid profile of fish oils can also vary greatly depending on the species, location, as well as time of year of the catch [682]. DHA-rich single-cell oi (DHASCO) has emerged as an alternative source of DHA to marine oils, yet relatively few studies to date have utilized DHASCO as supplementation source or vehicle for food fortification. As fish are incapable of synthesizing DHA, they rely on the microalgae at the

bottom end of the food chain that represent the primary source of DHA in the biosphere. DHASCO is derived from the microalgal species *Crythecodinium cohnii*. It contains approximately 40% DHA by weight, and no appreciable amount of any other LCPUFA [683]. It has been designated by the FDA as Generally Recognized as Safe (GRAS), and is employed commercially as the source of DHA in infant formulas and maternal supplements. Use of DHASCO have been favored over marine oils, in part due to reports of reduced growth and decline in measures of AA status in infants that consumed term and preterm infant formula supplemented with fish oil [374]. The presence of n-3 LCPUFA, particularly that of EPA is known to be an antagonist of AA metabolism by downregulating the chain elongation and desaturation of LA to n-6 LCPUFA. This has also been demonstrated *in vivo*, where addition of additional n-3 PUFA in diets of piglets with the sole source of n-6 LCPUFA being LA produced a reduction in tissue AA [684]. Studies in human infants have also documented a reduction in plasma and RBC AA status after administration of a formula supplemented with marine oil [374]. This reduction in AA may result in decreased growth, since plasma AA levels are positively correlated with the growth of premature infants [375]. It would therefore be reasonable to speculate similar effects to be observed in bone given stimulatory effects of AA on bone growth exerted indirectly through $PGE_2$ synthesis.

It is recognized that the proportion of DHA in sn-2 position of triglycerides found in DHASCO is different from human breast milk and potentially affect the extent to which they are absorbed [685]. However, preclinical and clinical studies have indicated that DHASCO is efficiently absorbed and capable of delivering target fatty acids to tissues to similar extents as human milk, since the proportion of DHA at the sn-2 position is well within the range found in human milk [575, 686, 687]. DHASCO thus represents a source of DHA which contains no other

significantly bioactive fatty acids. This oil offers the opportunity for researchers to distinguish for the first time the clinical effects of DHA from those effects generally attributed to fish oil. Future studies in older children are needed to confirm the metabolism of DHASCO and its benefits to bone growth and PBM.

Another important consideration in feeding trials is the dietary consumption necessary to achieve changes in DHA status associated with beneficial effects on bone. While ample data exist on the physiological effects of DHA on bone, its effective dosage remains unknown. It is well recognized that curvilinear relationship exists between dietary intakes of DHA and blood levels of this nutrient [236]. Administration of DHA over daily dose range of 200 – 1000 mg DHA in form of algal oil capsules or fortified foods in healthy adults (n=96, 18-70 y) showed that plasma phospholipids levels of DHA increased at a rate of approximately 0.3 g per 100 g fatty acids, and reached new equilibrium after 2 wk of supplementation, irrespective of dose. The equilibration of RBC DHA levels in blood, on the other hand, occurred at a slower rate and continued to rise throughout the 4 wk supplementation period, indicating that an equilibrium status had not been reached [688]. Others have also reported slower equilibration of RBC DHA levels in blood, which is not unexpected given their 120 d lifespan in the blood [689]. More research should thus focus on dose response relationship both in dietary intakes of DHA, responses in blood and tissue levels and influence on bone metabolism, taking into account the variability in individual's response to DHA supplementation given DHA accretion in plasma have been shown to be highly inversely dependent on baseline DHA levels but is independent of age, weight, gender, or race [688].

### A.5.3 The fat-1 transgenic model as potential model for studying DHA n-3 LCPUFA in bone

Offering an alternative approach to studying the influence of n-3 LCPUFA on metabolism independent of dietary modulation, the fat-1 mouse is a transgenic model that carries a n-3 fatty acid desaturase gene from *C. elegans* that enables endogenous conversion of n-6 to n-3 fatty acids, circumventing practical difficulties with obtaining comparable n-3 LCPUFA sources from the diet [690], and provides an unique tool to study how certain fatty acids influence bone metabolism through different metabolic pathways the ingested fatty acids typically undergo. While no studies to date had directly compared obtainable n-3 FAs levels in bone between fat-1 mice and mice supplemented with a n-3 FAs rich diet, it is commonly accepted that the fat-1 mice is an appropriate model for evaluating roles of n-3 FAs and n-6: n-3 ratios in certain chronic diseases. Fat-1 mice consuming a 10% safflower oil diet showed comparable brain levels of DHA as WT mice fed 2% fish oil + 8% safflower oil diet, and n-3 PUFA levels in brain of fat-1 mice were not augmented with fish oil feeding, suggesting that the fat-1 model can be an alternative or complimentary approach to dietary modulation of brain n-3 PUFA levels [689].

Feeding fat-1 mice AIN-93G diet containing 10% safflower oil from weaning until 12 wk of age resulted in a lower n-6: n-3 in the lumbar vertebrae of fat-1 mice, this was negatively correlated with BMC and peak compression load upon biomechanical testing. On the other hand, total n-3 PUFA, including ALA, EPA and DHA, were positively correlated with BMD and the peak load in the vertebrae. Similar findings where reported for the femur, suggesting that n-3 PUFA have a favorable effect on mineral accumulation and functional measures of bone in growing fat-1 mice [681, 691].

## A.6 Conclusion

The LCPUFAs, especially the n-3 fatty acids including DHA have shown promising benefits for bone metabolism and bone health. Studies in both humans and animals have provided ample evidence that LCPUFAs can increase bone formation, affecting PBM in adolescents [692]. Given the crucial role of DHA in brain and visual development early in life, it would be of particular value to further characterize whether DHA plays an equally important role in skeletal growth, and the relative amounts of DHA partitioned for utilization in bone especially when dietary intake of DHA is suboptimal. It is also of interest to note that in most *in vivo* studies, n-6 PUFA appeared to associate negatively with bone formation or BMD. However, it is also apparent that certain amounts of n-6 PUFA are essential for optimal bone development. The optimal dietary ranges of n-6 and n-3 LCPUFA through life stages and metabolic states thus remains to be determined. Additionally, the role of EPA to support bone formation during growth warrants clarification. Data from animal studies indicate that EPA is a potent n-3 PUFA that moderate actions of AA and other fatty acid metabolites that enhance osteoclastic activity. Thus DHA and EPA appear to both exert supporting roles in bone metabolism throughout the life cycle, but through different mode of actions and mechanisms.

The cellular mechanisms of action of the LCPUFAs are complex and involve modulation of fatty acid metabolites such as prostaglandins, resolvins and protectins, cytokines, growth factors, and other molecular signaling pathways. Mechanisms by which DHA regulate bone metabolism identified to date include modulating $PGE_2$ production and subsequent RANKL production. DHA and other n-3 LCPUFA may elevate or preserve bone mass through increasing MSC number [693], and by enhancing expression of key transcription factors such as Runx2 and osterix. Furthermore, n-3 may modulate the number of pro-inflammatory cytokines, increasing

production of IGF-1 and improving calcium accretion in bone [54, 692]. PPARγ has emerged to be an important nuclear receptor central to effects of DHA on inflammatory response and MSC lineage commitment into osteoblasts. Of equal significance is the dual roles of PPARγ in regulating bone formation and resorption, and that antagonists of PPARγ hold potential for simultaneous anabolic and anti-catabolic intervention of skeletal fragility.

Bone mass acquired through childhood and adolescence tracks into adulthood and may ultimately determine future osteoporotic risk. However, inconsistencies in the sera composition used *in vitro*, methods of analyzing fatty acid profiles, and source of DHA employed in feeding trials have made comparison across studies difficult. Also, the applicability of supplementation either through diet or capsule oils in the pediatric population has not been determined. More data is therefore required before incorporating DHA into recommendations and guidelines for the attainment and maintenance of bone mass in children.

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