DIETARY SUPPLEMENTATION OF CONJUGATED LINOLEIC ACID: MUSCULOSKELETAL INVESTIGATIONS IN MEN AND SELECTED ANIMAL MODELS

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April 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Human Nutrition).

This thesis is dedicated to my beautiful wife Amanda,
for her continual love throughout the course of my doctorate
and to our son Gavin, for whom, there could not be a prouder father.

“Research is what I'm doing when I don't know what I'm doing.”

- Wernher von Braun

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ABSTRACT

Rationale: Conjugated linoleic acid (CLA) in the human diet is derived primarily from ruminant-based foods such as beef or cow's milk and exists mainly in the cis-9, trans-11 isoform (c9, t11) within triglycerides (TG). Common CLA supplements, have an equal ratio of c9, t11 and trans-10, cis-12 (t10, c12) CLA isomers and are predominantly found in free fatty acid (FFA) form, but are also available in TG form. Whether CLA in TG or FFA forms has distinct biological activity in musculoskeletal metabolism is unknown. Prior to engaging in human trials and to optimize studies designed to investigate CLA, it is necessary to clarify if: 1) CLA forms exhibit distinct biological effects on bone and biomarkers of bone metabolism, such as parathyroid hormone (PTH), and 2) PTH assessment requires standardization based on gender, fasting and type of PTH assay. To date, no study has examined the effects of CLA on PTH concentration in humans. Also, whether these observations are maintained into advanced aging, when osteoporosis and sarcopenia are prevalent, is unknown. A longitudinal study in humans to determine the long-term benefits is not feasible necessitating an animal model of accelerated aging.

Objectives: The overall objective of this thesis was to investigate: if there are differences between the FFA and TG forms of CLA regarding incorporation in liver and biological musculoskeletal responses; if assessment of PTH is affected by gender, fasting and the type of PTH

assay used; if CLA status, as measured by red blood cell CLA content, in men is positively related to body composition and bone mass; if CLA supplementation in healthy men reduces PTH concentration; and if CLA can prevent decreases in bone and muscle mass typically observed in advanced aging.

Methodology: ***Study 1*****:** Sprague-Dawley rats (18 male and 18 female) were randomized at weaning to receive a control AIN-93 diet or the same diet supplemented with 0.5% c9, t11 CLA + 0.5% t10, c12 CLA in FFA or TG form. Liver fatty acid profiles were assessed using gas chromatography to evaluate tissue incorporation of both forms. Plasma PTH, ionized calcium (iCa) and biomarkers of bone were measured along with bone density and body composition using dual-energy x-ray absorptiometry (DXA) after 4, 8, 16 wk of supplementation. ***Study 2:*** Sprague-Dawley rats (30 male and 30 female) were randomized at weaning to receive a control AIN-93 diet or the same diet supplemented with 0.5% c9, t11 CLA + 0.5% t10, c12 CLA in FFA for 16 wk. At wk 16, a blood sample was collected, between 0800 and 1000h, from the tail vein in both a fed state (ad libitum intake) and a non-fed state (food removed for 12 h). PTH was assessed using both a second generation intact PTH and third generation bioactive PTH assay. ***Study 3*****:** Healthy men 19-53 y (n=31) were randomized in a double-blind, placebo controlled clinical dose-response trial to receive either: 1.5 g/d or 3.0 g/d of c9, t11 CLA (4:1 c9, t11 to t10, c12 isomer ratio) or placebo (olive oil 3 g/d) for 16 wk. DXA was performed to assess body composition at baseline and end of study

and blood samples were obtained monthly to evaluate changes in PTH concentration, 25-hydroxy vitamin D (25(OH)D), iCa, phosphate and lipid profile. Dietary intake of CLA was examined using a 174 item semiquantitative food frequency questionnaire at baseline and bimonthly 24 h recalls thereafter. ***Study 4*****:** As an advanced aging model, pigmented guinea pigs (n=40) were block randomized by weight at 70 wk of age to 4 groups: 1) SHAM+Control diet, 2) SHAM+ CLA diet (1.0% 4:1 c9, t11 to t10, c12 isomer ratio), 3) Orchidectomy (ORX)+Control diet, 4) ORX+CLA diet. DXA scans for bone density and body composition in addition to blood samples to measure testosterone, estrogen, interleukin-6 (IL-6), were performed at baseline and wk 16. At 16 wk, iCa and 25(OH)D were assessed as well as, bone microarchitecture using micro computed tomography, bone strength and acute protein fractional synthesis rate in skeletal muscle using a flooding dose of 40 mol% of L-[ring-$^{2}H_{5}$]-phenylalanine (1.5 mmol/kg *ip*).

Results: ***Study 1*****:** There were no differences among groups for growth, bone biomarkers or mass nor mineral balance. Liver enrichment of c9, t11 CLA in FFA form was greater than TG form and AIN-93 (FFA: 0.05±0.01 vs. TG: 0.02±0.01 vs. AIN-93 0.001±0.001 % total fatty acids, P<0.0001). However, t10, c12 CLA liver enrichment did not differ among groups (P=0.11). ***Study 2***: Females had a lower iCa compared to males (F: 1.43±0.01 vs. M: 1.46±0.01 mmol/L, P=0.03). In males and females, there was no difference between fed and non-fed groups when PTH was assessed using the INT PTH assay (M Fed: 21.58±1.34 vs. M Non-fed:

22.64±2.27 pmol/L, P=0.59; F Fed: 19.33±1.49 vs. F Non-fed: 22.80±2.92 pmol/L, P=0.39). However, in females only, PTH measured using the BIO PTH was significantly lower in the fed group versus the non-fed (Fed: 8.44±0.77 vs. Non-fed: 16.66±3.37 pmol/L, P=0.05). ***Study 3***: Men with red blood cell (RBC) c9, t11 CLA status above the median had higher whole body bone mineral density (BMD) (1.359±0.024 vs. 1.287±0.023 g/cm^2; P=0.04) and whole body lean mass % (WBL) (78.8±0.9 vs. 75.3±1.0 %; P=0.01), whereas body mass index (BMI) (24.8±0.5 vs. 27.3±0.9 kg/m^2; P=0.01) and whole body fat mass % (WBF) (17.3±0.9 vs. 21.3±1.1 %; P=0.007) were lower. In regression analysis, RBC c9, t11 CLA status accounted for a significant proportion (r^2=0.10) of the variation in whole body BMD (P=0.03). There were no time or treatment differences among any bone or biomarkers of bone metabolism including PTH. ***Study 4***: CLA prevented an increase in Tb.Sp and a decrease in vBMD, Tb.N and Conn.D in metaphyseal regions of ORX compared to SHAM CTRL. CLA also decreased porosity in ORX compared to SHAM. ORX decreased free testosterone (wk 0: 0.14±0.02 vs. wk 16: 0.06±0.01 nmol/L, P<0.0001) whereas interleukin-6 increased (wk 0: 0.014±0.001 vs. wk 16: 0.089±0.006 pmol/L, P<0.0001). ORX decreased trabecular bone density and volume in the metaphysis and increased intracortical porosity in the bone, CLA prevented ORX-induced loss of metaphyseal vBMD and bone volume as well as enhanced diaphyseal porosity. No differences in bone strength were detected. Also, no differences in quadriceps mixed muscle sarcoplasmic and myofibrillar protein fractional synthesis rate were

detected.

Significance: These studies provide advanced understanding of the biological response of bone and muscle to CLA. However, the evidence provided in this dissertation, do not provide conclusive results concerning dietary supplementation of CLA and the assessment of outcomes associated with bone and muscle in men and selected animal models. Overall, small benefits of CLA on the musculoskeletal system were observed, which potentially could have been greater given a longer study duration and larger sample sizes for the human and guinea pig studies.

RÉSUMÉ

Justification: L'acide linoléique conjugué (ALC) dans l'alimentation humaine provient principalement des aliments à base de ruminants tels que le bœuf et le lait de vache et, existe principalement en isoforme cis-9, trans-11 (c9, t11) au sein des triglycérides (TG). Les suppléments de l'ALC sont communément fabriqués en proportion égale des isoformes c9, t11 et trans-10, cis-12 (t10, c12) et ils se trouvent principalement dans les acides gras libres (AGL), mais ils sont également disponibles sous forme de TG. Que l'ALC soit sous forme de TG ou AGL, leur activité biologique concernant le métabolisme musculo-squelettique est inconnu. Avant de s'engager dans des essais humains et afin d'optimiser les études visant à étudier l'ALC, il est essentiel d'évaluer si: 1) les formes de l'ALC présente des effets biologiques distincts sur les os et les biomarqueurs du métabolisme osseux, tels que l'hormone parathyroïdienne (PTH), et 2) l'évaluation de la PTH nécessite une normalisation selon le sexe, l'état de jeûne et le type d'analyse utilisée pour mesurer la PTH. À ce jour, aucune étude n'a examiné les effets des l'ALC sur la concentration de la PTH chez l'homme. De plus on ignore quels sont les effets de l'ALC chez le sujet âgé en présence d'ostéoporose et de sarcopénie. Une étude longitudinale chez les humains afin de déterminer les avantages à long terme n'étant pas possible, nous étudierons un modèle animal de vieillissement accéléré.

Objectifs: L'objectif général de cette thèse est d'étudier: s'il existe une différence dans l'incorporation hépatique et les réponses biologiques musculo-squelettiques entre les formes de l'ALC (AGL et TG) ; si la mesure de la PTH est influencée par le sexe, le jeûne et le type de test utilisé pour mesurer la PTH ; si le statut de l'ALC, tel que mesuré par le contenu de l'ALC dans les globules rouges, chez les hommes est positivement liée à la composition corporelle et la masse osseuse, si la supplémentation en ALC chez les hommes en bonne santé réduit la concentration de la PTH, et si l'ALC peut empêcher une diminution de la masse osseuse et musculaire généralement observées dans le vieillissement avancé.

Méthodologie: ***Étude 1:*** Des rats Sprague-Dawley (18 mâles et 18 femelles) ont été randomisés au sevrage pour recevoir le régime de contrôle AIN-93 ou le même régime supplémenté avec 0,5% c9, t11 + 0,5% t10, c12 d'ALC en forme d'AGL ou de TG. Les profils d'acides gras du foie ont été évalués par chromatographie en phase gazeuse pour évaluer l'incorporation de l'ALC dans les tissus de ces deux formes. La PTH, le calcium ionisé (iCa) et les biomarqueurs osseux ainsi que la densité osseuse et la composition corporelle ont été mesurés après 4, 8, 16 semaines de supplémentation. ***Étude 2:*** Des rats Sprague-Dawley (30 mâles et 30 femelles) ont été randomisés au sevrage pour recevoir le régime de contrôle AIN-93 ou le même régime supplémenté avec 0,5% c9, t11 + 0,5% t10, c12 d'ALC en AGL pendant 16 semaines. À la seizième semaine, un échantillon de sang a été recueilli, entre 0800 et

1000h, de la veine de la queue à la fois dans un état nourri (ad libitum) et à jeun (nourriture retirée pendant 12 h). La PTH a été évaluée à l'aide de test de la PTH intacte (PTH INT) de deuxième et de troisième génération (PTH bioactive). ***Étude 3:*** Des hommes en bonne santé âgés de 19 à 53 ans (n = 31) ont été randomisés en double aveugle, lors d'un essai clinique contrôlé pour recevoir soit: 1,5 g / j ou 3,0 g / j de c9, t11 (4:1 c9, t11 à t10, c12 rapport d'isoforme) ou un placebo (huile d'olive 3 g / j) pendant 16 semaines. L'absorptiométrie biphotonique à rayons X (DXA) a été réalisée pour évaluer la composition corporelle au départ et à la fin de l'étude. Des échantillons de sang ont été obtenus mensuellement pour évaluer les changements dans la concentration de la PTH, la 25-hydroxy-vitamine D (25(OH)D), l'iCa, le phosphate et le profil lipidique. L'apport alimentaire de l'ALC a été examiné à l'aide d'un questionnaire de fréquence alimentaire semi-quantitatif comportant 174 questions au départ de l'étude et de rappels alimentaires tous les deux semaines par la suite. ***Étude 4:*** En tant que modèle de vieillissement avancé, des cochons d'inde pigmentés (n = 40) on été randomizés en bloc à 70 semaines d'âge à 4 groupes: 1) chirurgie fictive (SHAM) + Régime de contrôle (CTRL), 2) SHAM + régime d'ALC (ALC) (1,0% 4:1 c9, t11 à t10, c12 rapport d'isoforme), 3) orchidectomie (ORX) + CTRL, 4) ORX +ALC. L'analyse de la densité osseuse et la composition corporelle (par DXA) ainsi que des échantillons sanguins recueillis pour mesurer la testostérone, l'estrogène, l'interleukin-6 (IL-6), ont été effectuées au départ et à la seizième semaine. À 16 semaines, l'iCa et la 25(OH)D ont été évaluées ainsi que,

la microarchitecture osseuse utilisant la microtomodensitométrie, la force des os ainsi que le taux de synthèse protéique fractionnaire dans les muscles squelettiques en utilisant une dose d'inondation de 40% en moles de L-[ring-$^{2}H_{5}$]-phénylalanine (1,5 mmol / kg *ip*).

Résultats: ***Étude 1:*** Il n'existe aucune différence entre les groupes pour la croissance, les biomarqueurs et la masse osseuse, ni l'équilibre minéral. L'enrichissement du foie en c9, t11 sous forme AGL était plus grande que sous forme de TG et AIN-93 (AGL: 0,05 ± 0,01 vs TG: 0,02 ± 0,01 vs AIN-93 0.001 ± 0,001% des acides gras totaux, P <0,0001). Cependant, l'enrichissement du foie en ALC t10, c12 ne différait pas entre les groupes (P = 0,11). ***Étude 2:*** Les femelles ont un niveau de iCa plus bas comparativement aux mâles (F: 1,43 ± 0,01 vs M: 1,46 ± 0,01 mmol / L, P = 0,03). Chez les mâles et les femelles, il n'y avait pas de différence entre les rats nourris et à jeun lorsque la PTH était évaluée en utilisant le test PTH INT (M nourris: 21,58 ± 1,34 vs M à jeun: 22,64 ± 2,27 pmol / L, P = 0,59; F nourris: 19,33 ± 1,49 vs F à jeun: 22,80 ± 2,92 pmol / L, P = 0,39). Cependant, chez les femelles seulement, la PTH mesurée à l'aide de la PTH Bioactive, était significativement plus faible dans le groupe nourri par rapport au groupe à jeun (Nourri: 8,44 ± 0,77 vs à jeun: 16,66 ± 3,37 pmol / L, P = 0,05). ***Étude 3:*** Les hommes avec un statut d'ALC c9, t11 dans les globules rouges au dessus de la médiane avait une densité minérale osseuse (DMO) du corps (1,359 ± 0,024 vs 1,287 ± 0,023 g / cm^{2}, p = 0,04) et un pourcentage de masse maigre du corps plus élevé (78,8 ± 0,9 vs 75,3 ± 1,0%, p = 0,01), alors que l'indice de masse corporelle (IMC)

(24,8 ± 0,5 vs 27,3 ± 0,9 kg/m^2, p = 0,01) et le pourcentage de masse grasse corporelle étaient plus bas (17,3 ± 0,9 contre 21,3 ± 1,1%, p = 0,007). Dans l'analyse de régression, le statut d'ALC c9, t11 dans les globules rouges représentait une proportion significative (r^2 = 0,10) de la variation de la DMO du corps entier (P = 0,03). Il n'y avait pas de différence temporel et de traitement pour n'importe quel autre mesures d'os ou de biomarqueurs du métabolisme osseux, y compris la PTH.

Étude 4: L'ALC a empêché une augmentation de la séparation trabéculaire (Tb.Sp) et une diminution de la densité minérale osseuse volumétrique (DMOv), du nombre de travées (Tb.N) et de la densité de connectivité (Conn.D) dans les régions métaphysaires ORX par rapport à SHAM CTRL. L'ALC a également diminué la porosité dans ORX par rapport à SHAM. L'ORX a diminué la testostérone (sem 0: 0,14 ± 0,02 vs sem 16: 0,06 ± 0,01 nmol / L, p <0,0001) alors que l'interleukine-6 a augmenté (sem 0: 0,014 ± 0,001 vs sem 16: 0,089 ± 0,006 pmol / L, P <0,0001). L'ORX a diminué l'os trabéculaire dans la métaphyse et a augmenté la porosité intracorticale. L'ALC a empêché la perte de la densité osseuse et du volume osseux métaphysaire ainsi que l'augmentation de la porosité diaphysaires observés après l'orchidectomie. cependant, aucune différence dans la force des os n'a été détectées. Également, aucunes différences dans les taux de synthèse fractionnaire des protéines myofibrillaires et sarcoplasmiques n'ont été détectées.

Signification: Ces études améliorent notre compréhension de la réponse biologique des os et des muscles à l'ALC. Toutefois, les résultats obtenus

dans cette thèse, ne fournissent pas des preuves concluantes concernant la supplémentation de l’ALC et les bienfaits associés aux os et les muscles chez les hommes et des modèles animaux sélectionnés. Dans l'ensemble, des petits avantages de l'ALC sur la perte d'os et de muscles ont été observés. Avoir eu une durée prolongée de l’étude et un échantillonnage plus élevé, ces avantages auraient potentiellement été accentués.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the contribution of many individuals who have played a role in some shape or form in the culmination of my doctoral thesis. First and foremost, I would like to thank my supervisor Dr. Hope Weiler for giving me the opportunity to work under her supervision and for her constant help throughout this process. I also would like to thank my research committee members, Dr. Suzanne Morin and Dr. Réjeanne Gougeon for their support and feedback. Moreover, I would like to thank Sherry Agellon, Paula Lavery, Sandra Dell'Elce, Evan Nitschmann, Dr. Linda Wykes for their assistance and expertise in the laboratory. Also, Dr. Tom Hazell, Dr. Andre Piccolomini, and Dr. Patrick Owen for being motivators and constant sources of encouragement. I also would like to thank Catherine Vanstone for helping coordinate and collect data for the human study. I would like to acknowledge all the participants of the conjugated linoleic acid randomized clinical trial, your dedication to the study was unparalleled and much appreciated.

I would like to thank all my fellow graduate students, especially, Ivy Mak, Nour Makarem, Sonia Jean-Phillipe, Negar Tabatabaei, Johanne Naylor, Valerie Taschereau, Lia Mosconas for their help in the lab and at the clinic. I am grateful to Francine Tardif and Lise Grant for their help with administrative needs. Finally, my deepest gratitude goes out to my friends and family for their unequivocal support.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Original contribution to knowledge

The novel contributions stemming from the thesis have brought forth clarification regarding the physiological response to dietary supplementation with conjugated linoleic acid (CLA) in that:

1) There is isomer-specific liver CLA enrichment where the c9, t11 CLA isomer in free fatty acid form (FFA) is preferentially incorporated in liver tissue compared to c9, t11 CLA isomer in triacylglycerol (TG) (**Manuscript 1**).

2) There are no differences in bone and biomarkers of bone metabolism between FFA and TG forms of commercially produced CLA (**Manuscript 1**).

3) Selection of parathyroid hormone (PTH) assays is important depending on the purpose of the study, PTH assays [2nd generation (intact) versus 3rd generation (bioactive)] appear to be unable to differentiate differences incurred based on sex and fasting duration, which reinforces the need for standardization of fast duration, sex effects, and measurement of PTH using a bioactive PTH assay (**Manuscript 2**).

4) Red blood cell c9, t11 CLA status, a reflection of long-term (~ 4 mo) dietary CLA intake, positively relates to bone mineral density (BMD) and whole body lean mass percentage (WBL%) and is negatively associated with body mass index (BMI) and whole body fat mass percentage (WBF%). However, c9, t11 CLA supplementation does not appear to affect PTH in healthy men (**Manuscript 3**).

5) Using a model of accelerated aging, CLA had no effects on sex hormones (testosterone and estrogen), inflammatory cytokines (IL-6), body and bone composition, bone strength, and skeletal muscle protein fractional synthesis rate. CLA prevented loss of metaphyseal vBMD and bone volume as well as improved diaphyseal porosity otherwise observed with orchidectomy. (**Manuscript 4**).

2. Research articles in peer-reviewed journals

1. **DeGuire JR**, Makarem N, Vanstone C, Morin S, Duque G, Weiler HA: Conjugated linoleic acid is related to bone mineral density but does not affect parathyroid hormone in men. Nutrition Research, 2012, 32:911-920.

2. **DeGuire JR**, Weiler HA: Effects of feeding on serum concentration of endogenous bioactive (1-84) and intact parathyroid hormone in Sprague-Dawley rats and humans. Open Journal of Nephrology, 2013, 3:61-65.

3. **DeGuire JR**, Weiler HA: Free fatty acid and triacylglycerol forms of CLA isomers are not incorporated equally in the liver but do not lead to differences in bone density and biomarkers of bone. Prostaglandins, Leukotrienes and Essential Fatty Acids (In Press) 2013 (10.1016/j.plefa.2013.01.006).

3. Research article in preparation for submission to peer-reviewed journals

1. **DeGuire JR**, Lavery P, Mak I, Agellon S, Weiler HA: Does CLA prevent muscle and bone loss during accelerated aging using the orchidectomized (ORX) guinea pig model? (Unpublished at time of submission).

2. **DeGuire JR**, Weiler HA: Sexual dimorphisms in bone mineral density in Sprague-Dawley rats from 8 to 20 wk of age. (Unpublished at time of submission).

3. Makarem N, **DeGuire JR**, Vanstone C, Morin S, Weiler HA: Middle-aged men with dietary intake of omega-3 long chain polyunsaturated fatty acids above the median have higher bone mass for age. (Unpublished at time of submission).

4. Abstracts at professional meetings

1. Mak IL, **DeGuire JR**, Lavery P, Agellon S, Weiler HA (2012): High resolution micro-computed tomography is preferential over dual energy x-ray absorptiometry for detecting bone loss in the orchidectomized guinea pig. Réseau de recherche en santé buccodentaire et osseuse.

2. **DeGuire JR**, Lavery P, Mak I, Agellon S, Weiler HA (2012): Conjugated linoleic acid protects against appendicular bone loss in orchidectomized middle-aged guinea pig. FASEB J 26:244.6.

3. **DeGuire JR**, Weiler HA (2011): Feeding reduces serum concentration of endogenous bioactive (1-84) but not total intact parathyroid hormone in female Sprague-Dawley rats. FASEB J 25:778.5.

4. **DeGuire JR**, Weiler HA (2011): Sexual dimorphisms in bone mineral density in Sprague-Dawley rats from 8 to 20 wk of age. J Bone Miner Res 26(S1):MO011

5. **DeGuire JR**, Makarem N, Vanstone C, Morin S, Weiler HA (2010): The effect of cis-9, trans-11conjugated linoleic acid on parathyroid hormone in middle-aged men. J Bone Miner Res 25(S1):MO0417.

6. Makarem N, **DeGuire JR**, Vanstone C, Morin S, Weiler HA (2010): Middle-aged men with dietary intake of omega-3 long chain polyunsaturated fatty acids above the median have higher bone mass for age. J Bone Miner Res 25(S1): SA0329.

5. Oral conference presentations

1. **DeGuire JR**, Lavery P, Mak I, Agellon S, Weiler HA (2012): Conjugated linoleic acid protects against appendicular bone loss in orchidectomized middle-aged guinea pig. Experimental Biology (EB) Annual Meeting, San Diego, April 2012.

2. **DeGuire JR**, Weiler HA (2011): Feeding reduces serum concentration of endogenous bioactive (1-84) but not total intact parathyroid hormone in female Sprague-Dawley rats. Experimental Biology (EB) Annual Meeting, Washington, April 2011.

CONTRIBUTION OF AUTHORS

For manuscripts one and two, the candidate was responsible for posing the research questions, performing the statistical analyses, contributed to the interpretation of the data, wrote and updated the drafts and revisions, and was the primary author for both manuscripts.

Dr. Weiler provided the data from previous research, contributed to the writing of the manuscripts and the editorial revisions.

For manuscript three, the candidate was involved in the approval of the dietary supplement by Health Canada Natural Health Products Directorate and the development and ethical approval of the consent forms (English and French), and was responsible for recruiting the participants, collecting the data and information (dietary and questionnaire interviews, anthropometry and body composition (DXA) measurements, blood sample processing and analysis for vitamin D and parathyroid hormone), as well as the statistical analyses, interpretation of data and writing/publication (as primary author) of the manuscript.

Nour Makarem was a master's thesis student who assisted in the data collection from the food frequency questionnaire and anthropometry. She was involved in some of the blood sample processing, and revision of the manuscript.

Catherine Vanstone was the registered nurse who performed the blood sampling and collected blood pressure and heart rate on the participants. She was involved in obtaining ethical approval from the

institution and getting the clinical trial authorization approved by Health Canada. She also developed many of the data record sheets, and was involved in the revision of the manuscript.

Dr. Suzanne Morin, research committee member, was the internist consulted regarding bone related issues with recruitment criteria and participant results. She was involved in the revision of the manuscript.

Dr. Gustavo Duque was involved in the development and grant writing for the project. He was also a consultant for diverse issues encountered throughout the study. He was involved in the revision of the manuscript.

Dr. Hope Weiler, the senior author, was involved in the development and grant proposal stages of the project. She supervised all aspects of the study, conducted monthly meetings with the staff and students involved, and ensured the proper functioning of the clinical trial from project development to manuscript publication.

For manuscript four, the candidate was responsible for developing the research project, obtaining ethical approval, welfare of the animals, blood sample collection and processing, body and bone composition analysis (DXA, micro computed tomography (μCT)), bone strength testing, protein fractional synthesis rate, statistical analysis, writing, revision and publication (as primary author) of the manuscript.

Paula Lavery was involved in the welfare of the animals, blood sample collection, body composition collection (DXA), and assisted with the development of μCT analysis methods. She was involved in the revision of the manuscript.

Ivy Mak was a Natural Sciences and Engineering Research Council of Canada (NSERC) Undergraduate Student Research Award recipient who helped with the gas chromatography, μCT and bone strength testing analyses. She was involved in the revision of the manuscript.

Sherry Agellon was the laboratory technician who helped with the blood sample processing, and ordering of the disposable goods needed throughout the study. She was involved in the revision of the manuscript.

Dr. Linda Wykes shared her expertise regarding the protein fractional synthesis rate aspect of the study. She organized meetings to teach and ensure proper technique was used. She was involved in the revision of the manuscript. Moreover, all the protein fractional synthesis work was performed in her laboratory.

Dr. Hope Weiler, the senior author, was involved in the development and grant proposal stages of the project. She supervised all aspects of the study, conducted monthly meetings with the staff and students involved, and ensured the proper functioning of the animal study from project development to manuscript publication.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$; 1,25-dihydroxyvitamin D

1.5CLA; 1.5 g/d c9, t11 CLA

25(OH)D; 25-hydroxy vitamin D

3.0CLA; 3.0 g/d c9, t11 CLA

σ_{max}; Stress

ε_{max}; Strain

µCT; Micro computed tomography

AA; Arachidonic acid

aBMD; Areal bone mineral density

Akt; Protein kinase B

ALA; Alpha linolenic acid

AR; Androgen receptor

BAP; Bone specific alkaline phosphatase

BIO PTH; Bioactive 1-84 PTH assay

BMC; Bone mineral content

BMD; Bone mineral density

BMI; Body mass index

BV; Bone volume

BV/TV; Bone volume fraction

c9, t11; cis-9, trans-11 CLA isomer

C/EBP; CCAAT/enhancer-binding proteins

Ca; Calcium

CLA; Conjugated linoleic acid

Conn.D; Connectivity density

COX; Cyclooxygenase

Ct.Po; Cortical porosity

CTRL; Control

CTx; C-telopeptide of Type 1 collagen

CV%; Coefficient of variation

DA; Degree of anisotropy

DBP; Diastolic blood pressure

DHA; Docosahexaenoic acid

d_{max}; Maximum extension at maximum flexure load

DXA; Dual-energy X-ray absorptiometry

E; Young's modulus

EPA; Eicosapentaenoic acid

ER; Estrogen receptor

FFA; Free fatty acid

FFQ; Food frequency questionnaire

F_{max}; Maximum flexure load

FSR; Fractional synthesis rate

HDL: High density lipoprotein cholesterol

HR; Heart rate

HSC; Hematopoietic stem cell

iCa; Ionized calcium

IGF; Insulin-like growth factor

IL-1β; Interleukin 1-beta

IL-6; Interleukin-6

INT PTH; Total intact PTH assay

K; Extrinsic stiffness

L; Left

LA; Linoleic acid

LDL; Low density lipoprotein cholesterol

LS 1-4; Lumbar spine 1-4

MOI; Moment of inertia

MG; Monoacylglycerol

Mg; Magnesium

mTOR; Mammalian target of rapamycin

MV; Medullary volume

NF-κB; Nuclear factor kappa B

NTX; Collagen type 1 cross-linked N-telopeptide

OC; Osteocalcin

ORX; Orchidectomy

P; Phosphorus

PGE_2; Prostaglandin E2

P1NP; Type 1 procollagen

PI3K; Phosphoinositide 3-kinase

PKD; Polycystic kidney disease

PO_4; Phosphate

PPAR; Peroxisome proliferator-activated receptors

PTH; Parathyroid hormone

PUFA; Polyunsaturated fatty acid

RA; Rumenic acid

RANKL; Nuclear factor kappa-B ligand

RBC; Red blood cell/Erythrocyte

Runx2; Runt-related transcription factor 2

SBP; Systolic blood pressure

SHAM; Sham surgery

SHBG; Sex hormone binding globulin

SMI; Structure model index

t10, c12; trans-10, cis-12 CLA isomer

Tb.N; Trabecular number

Tb.Sp; Trabecular separation

Tb.Th; Trabecular thickness

TC; Total cholesterol

TG; Triacylglycerol

TNF-α; tumor necrosis factor alpha

TV; Total volume

vBMD; Volumetric bone mineral density

W; Energy at break

WB; Whole body

WBF%; Whole body fat percentage

WBL%; Whole body lean percentage

Chapter 1

Introduction, objectives and hypotheses

1.1 Rationale and statement of purpose

People worldwide are becoming increasingly persuaded that the foods they consume can affect their risk of developing various chronic diseases [1]. The World Health Organization projected that in 2005, chronic diseases accounted for 89% of all deaths in Canada and that these were responsible for an estimated loss of 500 million Canadian dollars in national income [2]. Among the suggested solutions was a preventative approach by means of achieving a healthy diet. This has continued to motivate research regarding foods that have potential health benefits by altering pathophysiological processes and reducing the risk of chronic degenerative diseases such as osteoporosis and sarcopenia [3].

Osteoporosis is characterized by a low bone mass and bone mineral density (BMD) accompanied by a disruption in the microarchitecture of bone; all of which result in decreased bone strength and increased risk of fracture [4]. This disease affects 1 in 4 Canadian women and more than 1 in 8 men over the age of 50 years approximating over 2 million Canadians [5]. Muscle mass and strength are important to skeletal health, which affect bone integrity by exerting mechanical strain and promoting an adaptation following muscular contractions via a net gain in bone tissue. Hence, age-associated losses of skeletal muscle mass and strength, known as sarcopenia, have detrimental effects on bone [6]. Based on cohort studies, at least 20% of men and women 70-

years of age are affected by sarcopenia and the prevalence approaches 50% or greater for those over 75 years [7-9].

Previously thought to be common in post-menopausal women, osteoporosis is now commonly recognized as a disease seen in the growing population of elderly men [10]. Furthermore, men after the age of 50 years lose whole body muscle mass and bone mineral content (BMC) and BMD at a combined rate ranging between 1 to 2% per year [11-14]. The loss of BMD and skeletal muscle mass is associated with age-related endocrine and inflammatory changes. [15, 16]. An increased cell mediated inflammatory response known as chronic low-grade inflammation occurs with aging. Low-grade inflammation is associated with multiple chronic diseases, however, it is also observed in healthy individuals otherwise free of disease [17]. High concentrations of inflammatory markers (including cytokines such as interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α)) are associated with increased mortality [18] and are hypothesized to play a role in the musculoskeletal decline of older people, which leads to decreased physical performance and disability [19-24].

To date, the mechanisms explaining the role of inflammation in decreased muscle and bone tissue have not been fully explained. A dual mechanism is suggested as being responsible for these age-related changes. An endocrine-immune dysregulation occurs where decreases in serum estrogen and androgen levels contribute to increases in musculoskeletal levels of cytokines leading to the development of a

catabolic stimuli via increased systemic inflammation [25]. In men, from the age of 23 to 90 years, there is an age-related decrease in circulating bioavailable testosterone (64%) and estrogen (47%) as well as an increase in sex hormone binding globulin (SHBG) (124%), the primary chaperone protein for gonadal hormones, which inhibits the biological function of sex hormones [26-28]. Given the effects of sex hormones observed with aging, the proportion available to the tissues (bioavailable testosterone/estrogen) specifically refers to the free (unbound) fraction [29]. Several studies found an inverse correlation between serum SHBG levels, which has a higher affinity for testosterone than estrogen, with muscle mass and BMD in both men and women [30, 31]. The age-related sex hormone deficiency states and inflammation in muscle and bone are reviewed in detail in chapter 2.

While pharmacological treatment modalities for osteoporosis and sarcopenia are available, prevention and changes in lifestyle, such as healthy eating and exercise, are considered safe and effective ways to reduce the severity of these diseases [32]. A greater understanding of how conventional foods and nutritional interventions can provide health benefits against disease similar to pharmaceutical prescriptions, have created a niche for functional foods and nutraceuticals. Long chain polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and conjugated linoleic acid (CLA) are found in functional foods and provide physiological benefits through reduction of the risk of certain chronic diseases beyond basic nutritional functions.

CLA gained popularity for its anticarcinogenic properties [33, 34] and its capacity to decrease fat mass as shown in several randomized placebo controlled clinical trials [35-38]. In addition, it was observed that CLA could benefit the musculoskeletal system by preventing bone and skeletal muscle loss incurred with aging [39-41]. Thus, as a functional food, CLA could be used to promote musculoskeletal health by positively modulating independently or synergistically bone and muscle and perhaps be used as part of a healthy diet or in conjunction with current pharmacological treatments to help prevent or treat osteoporosis and sarcopenia.

CLA is naturally found primarily in meat and dairy products of ruminant animals in triacylglycerol (TG) form. Naturally occurring CLA contains cis-9, trans-11 isomer (c9, t11) and trans-10, cis-12 isomer (t10, c12) in a ratio of ~4:1. Synthetic CLA is commonly available in free fatty acid (FFA) form, but also in TG form. There is no evidence of physiological or functional differences in animal (mice and rats) and humans between FFA and TG forms of CLA for body composition, energy balance, cardiovascular, hepatic, hormonal, immune and cancer assessments [36, 42-47]. However tissue incorporation and bone outcome differences between these forms have not been well documented and warrant further investigation to determine if one form results in superior outcomes versus the other and hence provide a basis for supplement form selection for future studies.

Typical commercially made mixtures of CLA contain ~1:1 ratios of the c9, t11 CLA and t10, c12 CLA isomers and the most common dosage is 3 to 4.5 g/d CLA supplement [48, 49]. These CLA mixtures have been investigated for potential health benefits including body weight management, body composition, immunomodulation and inflammation. It is well documented that CLA inhibits inducible inflammatory events [50]. The two proposed mechanisms of the health benefits of CLA are based on: 1) activation of the peroxisome proliferator-activated receptors (PPAR) and 2) inducible eicosanoid suppression (primarily prostaglandin E_2 (PGE_2)) [51].

First, PPAR-γ ligands include arachidonic acid derivatives, prostaglandins, and polyunsaturated fatty acids (PUFAs). PUFAs and eicosanoids can bind to multiple isomers of PPAR. Both CLA isomers c9, t11 and t10, c12 have a high affinity for PPAR-α [52] and a moderate affinity to PPAR-γ [53]. Thus, CLA acts as an antagonist to PPAR-γ, which can promote osteoblastic differentiation and repress osteoclastic differentiation by inhibiting hematopoietic stem cell (HSC) differentiation and potentially resulting in net gain in bone mass. Second, PGE_2 is involved in bone physiology where high levels of PGE_2 (10^{-6} M) are found to inhibit bone formation whereas lower levels (10^{-10} - 10^{-8} M) promote bone formation [54]. Low concentrations of PGE_2 enhance osteoblast activity (41% increase in osteocalcin) and reduce (200% decrease versus control) urinary calcium excretion [55]. PGE_2 is synthesized from arachidonic acid via the cyclooxygenase pathway. The enzyme

cyclooxygenase (COX) is induced by factors such as 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), PTH, IL-1β, and IL-6 [56], which are argued to indirectly increase PGE_2 and consequently promote osteoclastogenesis [57]. CLA decreases bone PGE_2 and thus may prevent bone loss as previously observed in rat bone organ culture [58]. PGE_2 is produced in bone primarily by osteoblasts and acts as a stimulator for bone resorption [59]. It is argued that CLA may compete with arachidonic acid in the phospholipid fraction, resulting in diminished prostaglandin production and/or CLA may directly inhibit COX [60] particularly COX-2 production [61], thus decreasing the production of PGE_2. Furthermore, PGE_2 is involved in the release of PTH [62]. Sustained elevated levels of PTH have been reported to decrease bone mass by increasing osteoclastic activity [56]. Hence, due to CLA's inhibitory effects on PGE_2 synthesis, CLA could potentially decrease osteoclastogenesis through a decrease in PTH expression, which may be explained by an upstream decrease in PGE_2 levels, which ultimately would yield positive bone turnover [63]. By inhibiting COX-2 with CLA treatment, this could influence PTH (endocrine) and as well as prostaglandin-dependent osteoclastic bone resorption and PGE_2 (paracrine) receptor-mediated action on bone cells by osteoblasts [64-67].

In support of the mechanisms outlined above, PTH is responsive to CLA in humans and rats and this might explain, in part, the responses observed in bone. PTH is a primary determinant of the extent of bone remodeling activity in the skeleton by regulating bone metabolism and

mineralization in addition to maintaining calcium homeostasis. PTH follows a diurnal and circadian rhythm [1-4], is affected by feeding state [68]. Very few human and animal studies have investigated the effects of CLA on PTH and whether c9, t11 CLA affects PTH in humans has not yet been studied.

Cross-sectional studies have found relationships between CLA and the musculoskeletal system. In postmenopausal women, dietary CLA was related to hip BMD [69]. In addition, women with intakes above the median intake of CLA had higher BMD of the forearm, and a trend for higher mineral density of hip, lumbar spine and whole body [69]. However, no study has investigated, in men or women, the relationship between CLA status, which provides a more appropriate assessment of systemic CLA compliance (versus dietary CLA intake), and measures of body composition and bone metabolism. The possible mechanisms and causal relationships for these observations remain undefined.

A supplemental dosage of 3 g/d CLA has been studied as related to markers of calcium and bone metabolism in healthy men over 8 wk and was well tolerated with no change in bone metabolism [70]. Similarly, higher amounts of CLA at 6.2 g/d for 28 days did not affect bone metabolism in men [71]. In 12 and 24 month studies [36, 42] dosages of CLA at 3.4 to 3.6 g/day in both TG and FFA forms were well tolerated. A transient reduction in whole body bone mass was observed with the FFA form at 12 months that resolved by 24 months following further CLA supplementation in TG form. Nonetheless, regions susceptible to fracture

such as lumbar spine or hip were not examined and thus an explanation for the fluctuation in bone mass associated with CLA is not clear.

The effects of CLA on bone and muscle have also been observed mostly in animal studies, however some human randomized controlled trials have demonstrated beneficial effects [69, 72]. Despite the evidence showing the benefits of CLA and its isomers on bone, the studies used primarily young and female murine models. Since these animals are constantly growing, this may not reflect the hormonal or bone changes seen with aging in the human.

Few studies have investigated effects of CLA using an aging model. A study by Rahman et al. found that 12 month old female mice fed a 0.5% CLA (1:1, c9, t11 to t10, c12) diet for 10 wk had increased BMD and muscle mass, which was accompanied by a decrease in the proinflammatory cytokines and decreased osteoclast function [73]. A subsequent study by the same group using a similar design, investigated the differences in muscle mass by using diets of varying CLA isomer composition (0.5% c9, t11 + 0.5% t10 c12; 0.25% c9 t11 + 0.25% t10 c12). After 6 months, all diets lead to significantly higher lean mass when compared to the control group [74]. Notably, the control group maintained lean mass over the study and therefore CLA has the capacity to elevate lean mass even at older ages. In spite of the current evidence showing the benefits of CLA and its isomers on bone and muscle, there is a scarcity in the number of studies showing these effects. Furthermore, the suggested aging model used females only and represented middle-aged animals,

which could be argued is the period when age-associated bone and muscle loss begin, but osteoporosis and sarcopenia have not yet developed. Hence, to account for these limitations, it is proposed to use a guinea pig model of accelerated aging where mature males beyond age of peak bone mass would undergo an orchidectomy.

In humans, very few randomized controlled trials have observed beneficial effects of CLA on bone or muscle. Furthermore, there are no studies investigating the effects of CLA on bone and muscle in an older or elderly population in men or women or of sufficient duration to observe protective effects that manifest as higher muscle mass.

Whether CLA could prevent muscle loss and diminished protein synthesis and/or elevated protein catabolism during aging in either females or males has not been thoroughly addressed. While the studies summarized above showed enhanced bone and muscle mass or altered metabolism with CLA, no other research group has focused on the bone and muscle mass protective effects of CLA using an aging model in males. There is limited research demonstrating the effects of CLA on the musculoskeletal system and related factors including testosterone, estrogen, proinflammatory cytokines (notably IL-6) and PTH. Furthermore, when assessing PTH, standardization of timing of sampling, food intake patterns prior to sampling and PTH assay used is required in trials designed to assess the physiological response of PTH to diet or supplements. Further research is needed to confirm these results and to

assess possible differences in feeding state, sex, diet and type of PTH assay used.

1.2 Thesis objectives and hypotheses

Objective 1. To investigate the tissue incorporation, bone mass and biomarkers of bone metabolism differences between FFA and TG forms of 1.0% 1:1 mixture of c9, t11 to t10, c12 CLA isomer in rats.

a. *Hypothesis: There will be no differences in tissue incorporation and bone outcomes between FFA and TG forms of CLA.*

Objective 2. To determine sex, fasting, and PTH assay generation effects on measurements of PTH in rats.

a. *Hypothesis: There will be sex, fasting, and PTH assay generation effects on measurements of PTH*.

Objective 3. CLA status, as measured by red blood cell CLA content, in men will be positively related to body composition and bone mass.

b. *Hypothesis: Higher CLA status will be associated with higher lean mass and BMD as well as lower fat mass.*

Objective 4. To assess the effects of CLA on PTH in humans, specifically men.

c. *Hypothesis: 3 g/d of c9, t11 CLA for 16 wk will reduce PTH by 30%.*

Objective 5. To determine if 16 wk of dietary c9, t11 CLA following orchidectomy-induced aging in the guinea pig model will:

1) prevent reductions in BMC and BMD of both trabecular and cortical in long bone and vertebrae;

2) elevate and preserve whole body lean mass despite lower testosterone;

3) prevent elevations in fat mass associated with withdrawal of testosterone;

4) reduce catabolism as indicated by change in lean mass and enhance fractional protein synthesis rate in gastrocnemius muscle;

5) reduce circulating concentrations of inflammatory cytokines (IL-6)

6) prevent losses in bone strength in long bones.

- *Hypothesis: CLA will prevent induced age-related losses in bone density, bone strength and muscle mass by reducing the effects of the withdrawal of anabolic stimuli (testosterone) and by reducing the catabolic effect of increased systemic inflammation (IL-6).*

Chapter 2

Literature review

2 - Introduction

The purpose of this chapter is to review the literature on conjugated linoleic acid (CLA) and its interaction with the musculoskeletal system. To accomplish this objective, a comprehensive review of the history, structure, biosynthesis, content in food and diet, metabolism and safety of CLA is presented in the first section. This is followed by a summary of recent studies describing the effects and potential mechanisms of action of CLA in bone and muscle with an emphasis on hormonal and inflammatory changes observed with aging.

2.1 Background of conjugated linoleic acid

2.1.1 History of conjugated linoleic acid

The discovery of CLA is a result of a series of observations originating in the mid-1930's. Booth et al. noticed that when cows were returned to the pasture after the winter, there was seasonal variability in the ultra-violet light absorptive qualities of milk fat [75], where fatty acids produced in the summer had more intense absorption in the 230 nm UV region. These changes in absorption were related to the hydrogenation of fatty acids, which resulted in two conjugated double bonds [76]. In 1957, the hydrogenation of unsaturated fatty acids by "microorganisms" (later termed biohydrogenation) was demonstrated in the rumen of sheep [77].

Later, in 1961, while using a more sophisticated method of infrared spectroscopy as a more efficient detection method for monitoring the illegal addition of hydrogenated vegetable oils in butter, Bartlet and Chapman found a constant relationship between *trans*-C18:1 and conjugated unsaturation in multiple samples [78]. In 1963, Reil confirmed Booth's earlier observation by demonstrating the seasonal changes in the fatty acid profile of Canadian milk and concluded that the conjugated dienoic acid was twice as high in the summer compared to the winter [79]. In 1966, Kepler et al., while investigating the biohydrogenation process of the rumen bacteria *Butyrivibrio fibrisolvens*, determined the reduction of linoleic acid was not a single step process to stearic acid, but rather a pathway where a conjugated cis-trans octadecadienoic intermediate is formed in the rumen [80]. This intermediate was later identified as *c*9, *t*11 octadecadienoic acid [81]. In 1977, using a specific non-polar phase with liquid-gas-chromatographic analysis of milk fat, Parodi was the first to identify that the *c*9, *t*11 CLA isomer was present in milk fat [82].

Upon the identification of *c*9, *t*11 CLA isomer, research regarding its physiological functions and health benefits emerged. Ha et al. discovered that CLA mixtures isolated from grilled beef or from base-catalyzed isomerization of linoleic acid, inhibited skin neoplasia that were chemically-induced in mice [83, 84]. These observations set the foundation for studies examining the effects of CLA on carcinogenesis, type 2 diabetes, immune function, atherosclerosis, obesity, osteoporosis, sarcopenia and gene expression in various tissues (see detailed review [85]).

2.1.2 Structure of conjugated linoleic acid

The various CLA isomers qualify as polyunsaturated fatty acids because these are characterized by the presence of at least one pair of double bonds separated by only one single bond resulting in a general delocalization, which creates a region where the electrons do not belong to a single bond or atom, but instead, to a group [86]. The electrons are shared across all the adjacent parallel-aligned p-orbitals of the compound and this distribution increases the stability and lowers the overall energy of the molecule [86]. CLA consist of a collection of positional and geometrical isomers of octadecadienoic acid, with conjugated double bonds ranging from $\Delta^{6,8}$ to $\Delta^{12,14}$. For every positional isomer there are four possible geometric pairs of isomers (i.e. *cis,trans; trans,cis; cis,cis*; and *trans,trans*), thus, there are 28 possible CLA isomers [87]. Despite the high number of isomers, only a few are naturally occurring including $\Delta^{7,9}$; $\Delta^{8,10}$; $\Delta^{9,11}$; $\Delta^{10,12}$; $\Delta^{11,13}$ and $\Delta^{12,14}$ [88]. As depicted in **Figure 2.1**, the major CLA isomer found in food is the *c*9, *t*11 CLA isomer, known as rumenic acid (RA) accounting for approximately 90% of CLA intake in the human diet [89]. Moreover, along with RA, the *t*10, *c*12 CLA isomer provides additional health benefits. **Figure 2.2** illustrates the chemical structure of the parent linoleic acid (LA), as well as RA and *t*10, *c*12 CLA isomers.

2.1.3 Biosynthesis of conjugated linoleic acid

The presence of both major CLA isomers (RA and *t*10, *c*12 CLA) in meat and milk of ruminant animals [e.g. bovine (cow), ovine (sheep), caprid (goat), macropod (kangaroo)] is a result of ruminal fermentation, specifically by microorganisms via biohydrogenation of unsaturated fatty acids [90]. CLA acts as an intermediate in the formation of *trans*-11-octadecenoic acid (*trans* vaccenic acid, C18:1 *t*11) and stearic acid (C18:0). In the rumen, where the majority of the microbial lipid metabolism occurs, free fatty acids are released from dietary glycolipids, phospholipids, and triacylglycerols via lipolysis [91]. First, linoleic acid (C18:2 *c*9, *c*12) is isomerized to RA, which is catalyzed by the anaerobic bacterium *Butyrivibrio fibrisolvens* [92] then two hydrogenation reductions occur producing *trans* vaccenic acid and finally stearic acid [80]. Linoleic acid is also isomerized to the *t*10, *c*12 CLA isomer by *Megasphaera elsdenii* strain YJ-4, a lactate fermenting bacterium [93]. However, the *t*10, *c*12 CLA isomer is a substrate for *Butyrivibrio fibrisolvens* and is biohydrogenated to *trans*-10 C18:1 at approximately 33% the rate of conversion of the RA to *trans*-11 C18:1, which explains part of the isomeric distribution seen in **Figure 2.1**.

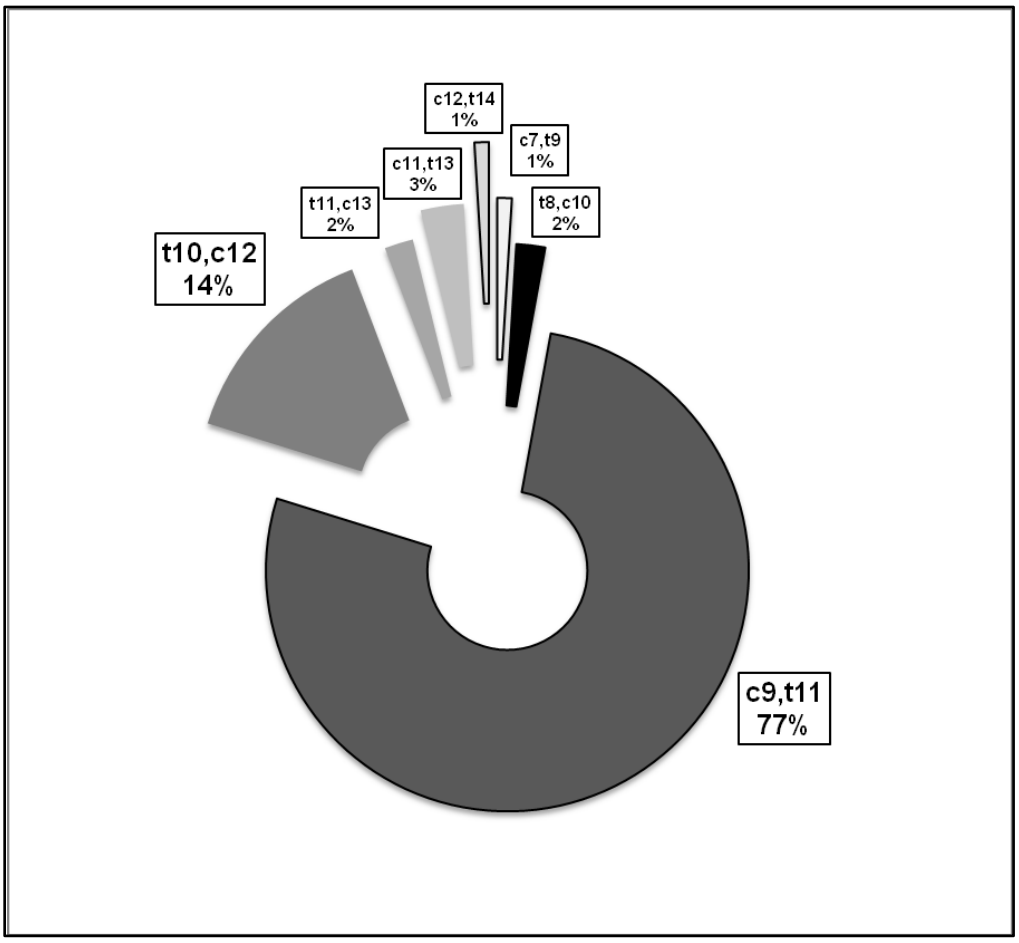

Figure 2.1. Percent distribution of CLA isomers found in the duodenal digesta of cattle. There are predominantly two isomers, the c9, t11 and t10, c12. Of these two isomers c9, t11 is the predominating representing ~80% of total isomers followed by t10, c12 with ~15%. Data source [94].

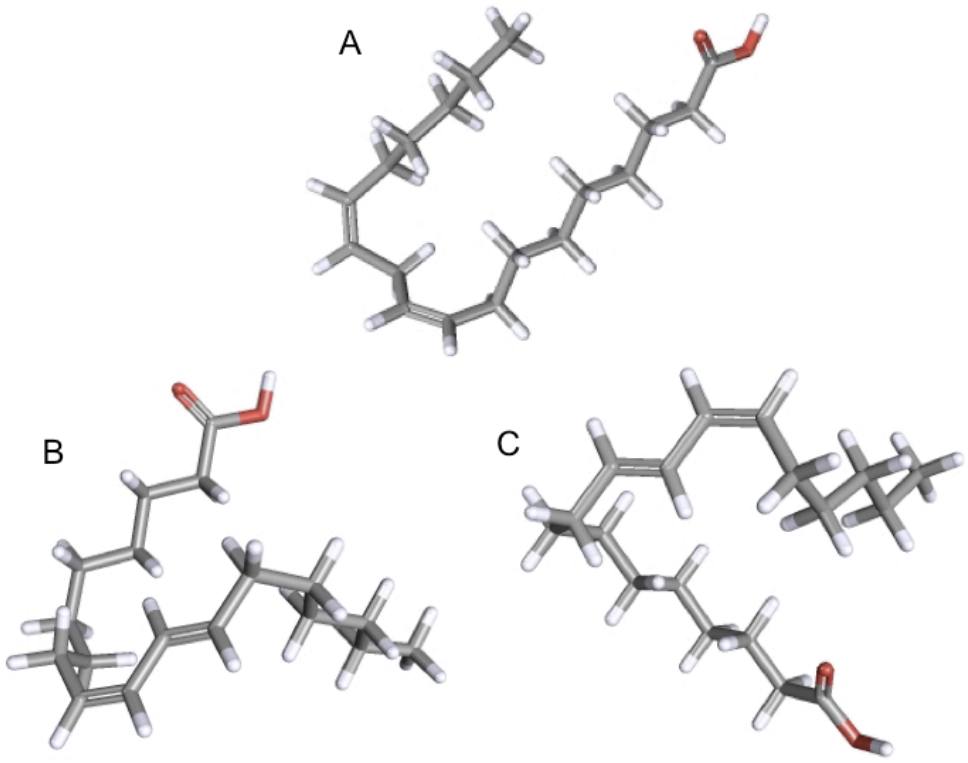

Figure 2.2. CLA is derived from the parent molecule linoleic acid (A) [95]. It contains 18 carbons atoms and two double bonds in the *cis* configuration (hydrogen atoms on the same side of the double bond) at positions 9 and 12 from the carboxyl end. The *c*9, *t*11 (B) [96] and *t*10, *c*12 (C) [97] CLA isomers have the same molecular composition as A, however, the double bonds have differing positions and geometry. The double bonds in B and C are located at positions 9/11 and 10/12 respectively. These double bonds are in *trans* geometry (hydrogen atoms are on opposite sides of the bond). These isomers are qualified as being conjugated since they have a pair of double bonds separated by one single bond. Of note, trans linkages in a conjugated system are not counted as trans fats for the purposes of nutritional regulations and labeling.

Ruminants living in symbiosis with rumen bacteria are not the only source of CLA; pork, poultry and fish also contain CLA but in significantly smaller concentrations [98]. Endogenous synthesis of RA from *trans* vaccenic acid occurs in multiple species including rats, mice, pigs, cows and humans [99]. Dormandy and Wickens hypothesized that endogenous CLA is a result of free radical initiated carbon-centered oxidation of linoleic acid [100]. The consumption of safflower oil, which is high in linoleic acid, the precursor for bioisomerization, did not increase the CLA concentration in total lipids of human plasma [101]. However, Salminen et al. demonstrated that a diet comprised of 25% of *trans* fatty acid leads to higher serum concentrations of CLA (total lipids) in humans when compared to a diet low in *trans* fatty acid [102]. Nevertheless, the corresponding increase of CLA in the serum from low to high dietary *trans* fatty acid was only 0.11% [102]. Thus, it was concluded that the quantity of endogenous production of CLA in human from dietary sources is most likely negligible. In addition to the endogenous production of CLA, Chin et al. identified bacterial synthesis of CLA in the digestive tract of rats [103] as a non-dietary source of CLA. Similarly, bacterial strains of *Butyrivibrio fibrisolvens* (involved in biohydrogenation process) have been isolated from human feces [104].

It is now known that various organs including mammary glands and adipose tissue synthesize CLA by the endogenous $\Delta 9$ desaturase enzymatic system of dietary *trans* vaccenic acid in multiple species [105-

108]. In humans, it was demonstrated that high levels of dietary *trans* fatty acids for 5 wk increased serum CLA content compared to dietary stearic acid [109] and that deuterated 11-*trans*-octadecenoate (fed as triacylglycerol) was converted to CLA *c*9, *t*11 via the Δ9 desaturase pathway [110]. Moreover, Mosley et al. demonstrated the benefits of using ^{13}C labeled fatty acids to assess the activity of Δ9 desaturase in lactating women by measuring the conversion of vaccenic acid to *c*9, *t*11 CLA [111]. Thus, CLA measurement from various tissues may reflect exogenous and endogenous sources (see **Figure 2.3**).

Additionally, CLA can be synthesized using various biotechnological methods such as microbial synthesis (see detailed review [112]), enzymatic synthesis of structured lipids [113-116] and by means of catalytic hydrogenation using catalysts such as nickel to increase the oxidative and heat stability of the oils [117]. CLA can also be produced by autooxidation. This process is partly responsible for the accelerated generation of CLA during heat processing of meat [118]. Details of these biotechnological methods are beyond the scope of this review, however, they are important to take into consideration since the different production methods can yield CLA in TG forms with varying stereospecificity, which could lead to differences in metabolism.

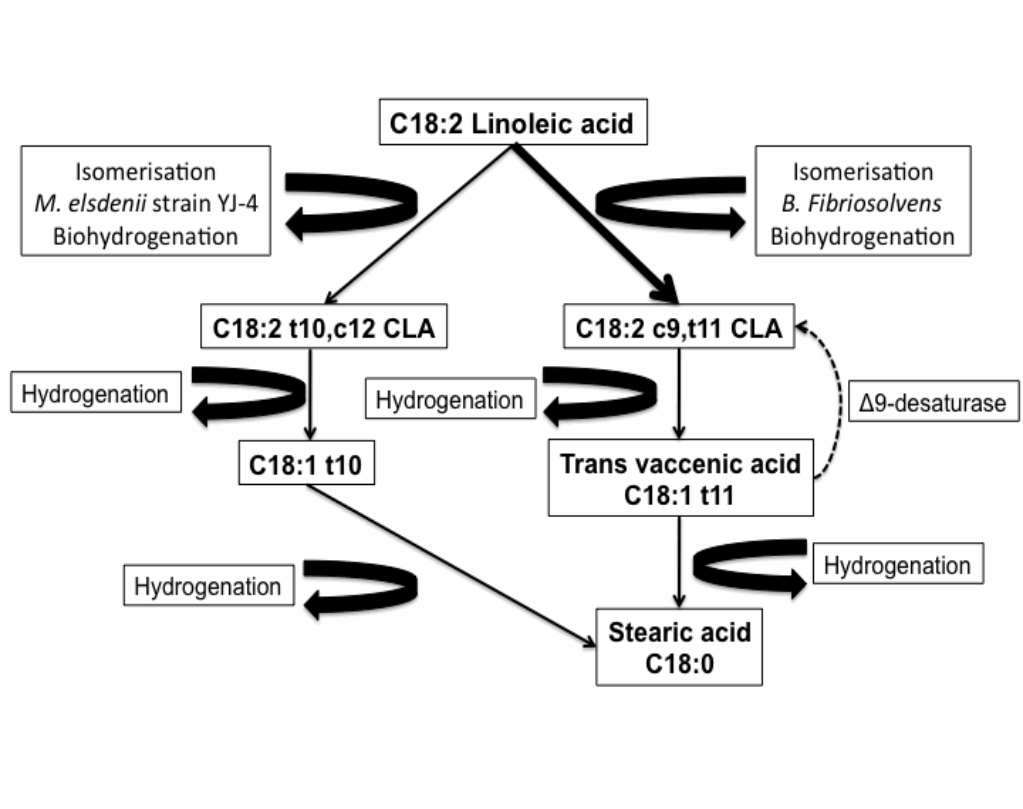

Figure 2.3. Biosynthesis of *c*9, *t*11 and *t*10, *c*12 CLA isomers.

2.1.4 Conjugated linoleic acid in food

In Canada, dietary sources of CLA include primarily dairy and meat products derived from ruminant cattle, sheep, and goats (**Table 2.1**) [119]. The concentration of CLA produced by these animals is dependent on the feeding strategies utilized. Different feeds containing corn, sunflower and/or fish oil are all sources of linoleic and linolenic acid that increase CLA content of milk fat [120] by providing substrates for CLA and *trans* vaccenic acid synthesis or modifying the rumen biohydrogenation process driven by microbial activity [121]. Apart from the animal's diet, CLA content of milk fat also varies according to the animal's age, breed [122] and seasonal changes, where levels are highest in the summer months since during the winter, cows are typically fed corn silages and cereal-rich concentrates, which are poor in polyunsaturated fatty acids creating a substrate deficiency for biohydrogenation in the rumen [123].

Processing or storage can alter CLA content of raw food materials. This is typical in many processed dairy and meat products, which are typically higher (~1.25 fold) in CLA than their unprocessed counterparts [124]. In cheeses, factors that can vary the amount of CLA include incubation temperature, protein quality, starter cultures and period of aging [124]. Food processing methods such as cooking (especially grilling), and the addition of hydrogen donors such as butylated hydroxytoluene, increase CLA content in foods [125]. Since CLA is a fatty acid, its quantity is expressed as a function of the amount of fat found in the food product.

On average, 80 to 90% of the CLA in dairy products is the *c*9, *t*11 isomer. As shown in **Table 2.1**, dairy products contain a greater concentration (more mg CLA/g fat) than foods from non-ruminant animals or plant oils.

2.1.5 Conjugated linoleic acid in the diet

Several studies from various geographical areas have attempted to estimate dietary CLA intake using written dietary assessment methodologies including food duplication, diet records and food frequency questionnaires (FFQ). Using food duplication methodology, Ritzenthaler et al. found that CLA and RA intakes from food duplicates were significantly higher in men (compared to diet records) and women (compared to diet records and FFQ) **(Table 2.2)** [126]. This implies that both diet records and FFQ are limited estimators of CLA including RA dietary intake and can underestimate values for these fatty acids. Nevertheless, the bulk of studies investigating dietary CLA intake have used some form of written dietary assessment due to the unrealistic and costly use of duplicate food analysis in study designs. Regarding CLA and other fatty acids, the accuracy of the dietary assessment technique used can be confirmed by performing fatty acid profiling of plasma (reflects short-term (~48 h) diet intake can confirm 24 h recall) and RBC (reflects long-term (~4 mo) diet intake can confirm FFQ) via gas chromatography. International estimated dietary intake data are summarized in **Table 2.3** whereas **Table 2.4** provides an estimate of CLA consumption based on different population

groups. Estimated international CLA intake values are consistent with North American (United States) intakes.

Table 2.1. Estimated conjugated linoleic acid quantities in food products[1]

	mg CLA /g fat	% c9, t11 of total CLA	Serving[2] Size	CLA per Serving (mg)
Bovine Milk				
Milk General Range	[0.7-10.1]	59-100		
Milk Fat General Range	[2.0-30.0]	90		
Skim Milk (<1.0%)	1.8		250 mL	0.3
Reduced Fat Milk (1.0% - 2.0%)	4.5		250 mL	15.0
Whole Milk (3.3% - 3.7%)	4.3		250 mL	35.7
Fermented Dairy Products				
Sour Cream General Range	[4.1-7.5]	78-100		
Buttermilk General Range	[4.7-5.4]	89-100		
Yogurt General Range	[1.7-9.0]	71-100		
Cream (Whipped or Sour)	4.9		15 g	10.2
Frozen Yogurt, Non-Fat Ice Cream	1.9		125 mL	3.1
Non Fat Yogurt	3.7		250 mL	1.3
Low Fat Yogurt	5.6		250 mL	23.1
Regular Yogurt	4.1		250 mL	44.6
Ice Cream				
Ice Cream General Range	[3.6-5.0]	76-86		
Ice Cream	3.5		125 mL	41.6
Butter				
Butter General Range	[4.7-11.9]	78-90		
Butter	5.5		15 g	63.3
Natural Cheese				
Blue	[0.6-8.0]	15-100		
Cheddar	[1.4-5.9]	18-100		
Cottage	[4.5-5.9]	83-100		
Cougar Gold	[3.2-5.2]	85-100		
Monterey Jack	~4.8	90-100		
Mozzarella	[4.3-5.0]	84-100		
Swiss	[5.5-14.2]	90-100		
Cream Cheese	3.5		30 g	17.8

Non Fat Cheese (0 g fat/svg)	3.5		30 g	0.8
Low Fat (< 3 g fat/svg)	4.8		30 g	9.1
Regular Fat Cheese, (>3 g fat/svg)	8.1		30 g	60.0
Processed Cheeses				
Processed	[1.8-8.8]	18-100		
Cheez Whiz	6.5	21-100	30 g	45.5
Kraft Singles	3.2	58	1 slice	16.0
Beef				
General Range Beef Products (Raw)	[1.2-8.5]	21-61		
General Range Beef (Cooked)	[3.3-9.9]	19-84		
Beef Hotdog	2.7		1 unit	32.0
Hamburger (Lean)	1.4		75 g	13.2
Hamburger (Regular)	2.7		75 g	39.8
Beef (General)	3.7		60 g	47.2
Liver (Beef)	2.0		120 g	12.2
Salami or Bologna	3.5		60 g	48.7
Other Processed Meats	3.2		60 g	37.0
Pork				
General Pork Products	[0.6-2.7]			
Pork (General)	0.6		150 g	8.3
Bacon	1.3		2 slices	6.9
Pork Hotdog	2.7		1 unit	32.0
Liver (Pork)	2.0		120 g	12.2
Lamb				
General Lamb Products	[5.6-5.8]			
Lamb (General)	5.8		60 g	12.8
Poultry				
General Chicken or Turkey	[0.9-2.6]			
Chicken or Turkey (General)	1.4		150 g	25.4
Chicken or Turkey (With Skin)	1.4		150 g	30.1
Chicken or Turkey (Without Skin)	1.4		150 g	15.6
Chicken or Turkey Hotdog	1.1		1 unit	9.7
Liver (Chicken or Turkey)	2.0		30 g	7.7

Fish & Shellfish			
General Fish/Shellfish Range	[0.1-0.6]		
Canned Tuna	0.1	90 g	0.3
Breaded Fish Cakes	0.5	3 unit	10.4
Shrimp, Lobster, Scallops	0.4	90 g	1.3
Dark Meat Fish (Salmon)	0.6	120 g	7.1
Other Fish (Cod, Haddock or Halibut)	0.5	120 g	0.9
Eggs			
Egg (Whole)	0.5	1 unit	2.5
Vegetable Oils			
Safflower Oil	0.7	5 mL	3.2
Sunflower Oil	0.4	5 mL	1.8

[1] Estimated CLA and % c9, t11 isomer values in food consolidated from [89, 127-130].
[2] Serving (svg) sizes obtained from Harvard/Willett food frequency questionnaire (FFQ) [131].

Table 2.2. Comparison of conjugated linoleic acid assessment methodologies[1,2]

	Assessment Method	Total CLA (mg/d)	RA (c9, t11) (mg/d)
Men			
	Food duplication	212±14[b]	193±13[b]
	Diet records	176±20[a]	133±15[a]
	Food frequency questionnaire	197±19[a,b]	151±15[a]
Women			
	Food duplication	151±14[b]	140±14[b]
	Diet records	104±20[a]	79±15[a]
	Food frequency questionnaire	93±11[a]	72±9[a]

[1] Adapted from Ritzenthaler et al. [126].

[2] Differences (versus food duplication) detected using two-way paired *t* tests. Different letters indicate significant differences ($P<0.05$). Data presented in mean ± SEM.

Table 2.3. Estimated conjugated linoleic acid intake in human sub-populations

	Participants	Method	CLA Intake (mg/day)	Ref.
United States				
	Adults Males	Diet Records	127	[101]
	College-Aged Males	Diet Records	137	[132]
	College-Aged Females	Diet Records	52	
	Lact. Women High Dairy	Diet Records	291	[133]
	Lact. Women Low Dairy	Diet Records	15	
Finland				
	Adults High Dairy	Diet Records	310	[109]
	Adults Low Dairy	Diet Records	90	
	Adult Women	FFQ	132	[33]
Australia				
	Adults	N/R	500-1000[1]	[134]
Germany				
	Adults Males	FFQ	430	[135]

[1]High daily intake of CLA is due to high consumption of ghee (butter) in this population.

Table 2.4. Estimated daily intake of conjugated linoleic acid in U.S. population[1,2]

	Age (y)	Mean (mg)	90th Percentile (mg)	Mean (mg/kg)	90th Percentile (mg/kg)
Child					
	3 to 11	360	1170	14.15	45.48
Female Teen					
	12 to 19	300	1050	5.47	20.92
Male Teen					
	12 to 19	330	1160	5.38	19.27
Female Adult					
	20+	290	1070	4.5	16.04
Male Adult					
	20+	300	970	3.55	11.67
Total					
	All Ages	316	1040	5.96	19.97

[1] Adapted from GRAS notification for conjugated linoleic acid (CLA)-rich oil for use in certain foods [136].
[2] Data from 1994-1996, 1998 USDA CSFII.

2.1.6 Conjugated linoleic acid digestion, absorption, metabolism and excretion

The metabolic fate of CLA is similar to that of other fatty acids. Digestion begins in the oral cavity where lingual lipase breaks down TG to diacylglycerol, monoacylglycerol (MG), and free fatty acids (FFA). This hydrolysis continues in the stomach via gastric lipase to a point where the fat entering the upper duodenum is composed of approximately 70% TG [137]. In the intestine, digestion continues where pancreatic lipase breaks down TG into FFA and MG. Moreover, bile salts act as emulsifiers and via a process facilitated by colipase, form micelles for transport and absorption of FFA and MG into the enterocytes, mainly by passive diffusion and/or via fatty acid binding proteins [137]. Within the enterocytes, the absorbed FFA and MG are reacylated to form TG and packaged together with phospholipids, cholesterol and apoproteins to form chylomicrons. Chylomicrons released from mucosal cells circulate through the lymphatic system and reach the superior vena cava by way of the thoracic duct. Transport in the circulatory system is followed by the hydrolysis of TG at the capillaries of adipose and liver tissue by lipoprotein lipase, which releases FFA for entry into these tissues and subsequent metabolism. This leads to the production of TG-depleted chylomicrons remnants, which are readily taken up by the liver by specific remnant receptors and endocytosis [137].

Following cellular uptake, FFA are re-esterified into TG and PL for

energy storage or incorporated into cell membranes. The fatty acid composition of membrane PL and the lipid configuration of the membrane influences the physiochemical characteristics of the membrane affecting the function of various membrane proteins such as hormone receptors, ion channels, and enzymes [137]. The degree of fatty acid absorption varies depending on whether it is ingested as a TG, an ethyl ester or a FFA. Long chain polyunsaturated fatty acids consumed as TG are absorbed more completely than fatty acid ethyl esters, but not as completely as FFAs [138-140]. Using a structured synthetic TG acylated with [^{14}C]-rumenic acid to determine the stereospecific characterization of CLA, RA (c9, t11 isomer) FFA present at the external *sn*-1 and *sn*-3 positions were better absorbed and oxidized whereas c9, t11 FFA located in the central *sn*-2 position lead to greater incorporation in the large intestine and carcass in rats [141]. Valeille et al. demonstrated using low and high RA dairy fat that 87 and 84% of RA FFA were situated in the *sn*-1 and *sn*-3 positions [142]. Furthermore, RA FFA were predominantly positioned at *sn*-3 (low RA fat 52% and high RA fat 65%) [142].

Regarding digestion, the preferential *sn*-3 positioning of RA makes it a target for hydrolysis via gastric (*sn*-3 specific) and pancreatic lipases *sn*-1 and *sn*-3 specific, which can lead to faster release from the glycerol moiety and faster absorption by the enterocytes. Furthermore, because of the *sn*-3 specificity of gastric lipase, synthetic CLA in TG form with equal isomers at the *sn*-1 and *sn*-3 positions could be nutritionally different than naturally occurring CLA in TG form, which could be cause for concern for

studies investigating the effects of CLA [142]. Interestingly, Paterson et al. supplemented weaned ruminant lambs with either safflower oil or CLA for 21 days prior to weaning and observed a 300% increase in CLA content in the *sn*-1 and *sn*-3 positions of TG from diaphragm, rib muscle, and subcutaneous adipose tissue in the safflower fed animals. As for the CLA fed animals, an increase in CLA in the *sn-2* position of TG from subcutaneous adipose tissue was determined suggesting tissue-specific dietary and potential age-related effects on the manner of FFA incorporation into TG [143].

The accumulation of various CLA isomers in animal and human tissues has been well documented [144, 145]. Tissue enrichment of CLA can occur in most tissues including the liver, heart, kidney, adipose, skeletal muscle and also in brain tissue. Since, CLA incorporation in the brain originally appeared minimal, it was believed to be unable to cross the blood-brain barrier [146]. However, a recent study has clearly demonstrated the occurrence of c9, t11 and t10, c12 CLA isomer incorporation in the rat brain tissue [147]. The low detectable concentrations of brain CLA are a result of highly efficient localized beta oxidation of CLA (particularly the t10, c12 isomer) as explained by higher production and concentrations of c9, t11 and t10, c12 metabolites in the brain [147, 148]. CLA incorporation is similar to that of oleic acid and occurs preferentially into neutral lipids, which are more abundant in adipose and mammary tissues than in liver and plasma [87]. CLA (specifically in the cis-trans configuration) interacts with other fatty acids

including linoleic acid and docosahexaenoic acid (except in brain) by reducing tissue enrichment [87, 146]. The enrichment of CLA and altered membrane lipid composition could impact the competition between 18:2 n-6 and 18:3 n-3 for the Δ^5 and Δ^6 desaturase and elongase enzymes [149]. CLA enrichment also impacts upon metabolism of these fatty acids through the inhibition of lipoxygenase and cyclooxygenase by metabolites of CLA [150, 151].

Energy and CLA metabolites are generated by mitochondrial and peroxisomal β-oxidation [87]. Fatty acids are transported across the mitochondrial membrane in the form of acyl-carnitine and afterward undergo sequential removal of 2-carbon units until acetyl CoA is formed and enters the tricarboxylic acid cycle for adenosine triphosphate production. CLA isomers have a very similar metabolic fate as linoleic acid [87]. Essentially, desaturation and oxidation pathways metabolize CLA. Desaturation of CLA has been studied more thoroughly than β-oxidation, though both are well-known metabolic pathways of CLA [152]. In animal and human tissues, the c9, t11 and t10, c12 CLA isomers undergo Δ6 desaturation, elongation and further Δ5 desaturation forming C18:3, C20:3, and C20:4 fatty acids as shown in **Figure 2.4**. Both isomers can also be oxidized by peroxisomal β-oxidation to C16:1 and C16:2 for ATP production upon activation of the peroxisome proliferator-activated receptor (PPAR) α [87, 153]. To determine the pathways of excretion of CLA, Sergiel et al. used radiolabeled metabolites of CLA in rats. They

demonstrated that over 24 hours, 71.8 and 70.3% of c9, t11 and t10, c12 CLA respectively was recovered in expired CO_2 compared to 60.3% for linoleic acid. Furthermore, it was observed that the extent to which CLA metabolites were recovered in expired CO_2 was time-dependent, reaching a plateau after 12 hours [154].

2.1.7 Conjugated linoleic acid supplementation and safety

Based on numerous clinical data, consumption of CLA appears to be safe. The studies by Larsen et al. and Whigham et al. 2004 report the safe use of CLA up to 6 g/day for a duration of up to one year [48, 155] and studies by Gaullier et al. report a safe use of 3.4 g/day for a duration of two years [36, 42, 156]. The basis of safety included no detrimental effects on cardiovascular parameters (lipid metabolism, markers of inflammation, and markers of oxidative stress), insulin sensitivity and glucose metabolism, and maternal milk fat production. Bioavailability studies concluded that there were no adverse effects following a single oral dose of 15 g of CLA-rich oil representing approximately 9 g of CLA isomers and preclinical data have demonstrated an absence of significant toxicological, mutagenic, or reproductive and developmental effects [157]. Commercially available CLA supplements including Tonalin® and Clarinol® are exempted from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because they have been

determined to be Generally Recognized As Safe (GRAS), based on scientific procedures [136].

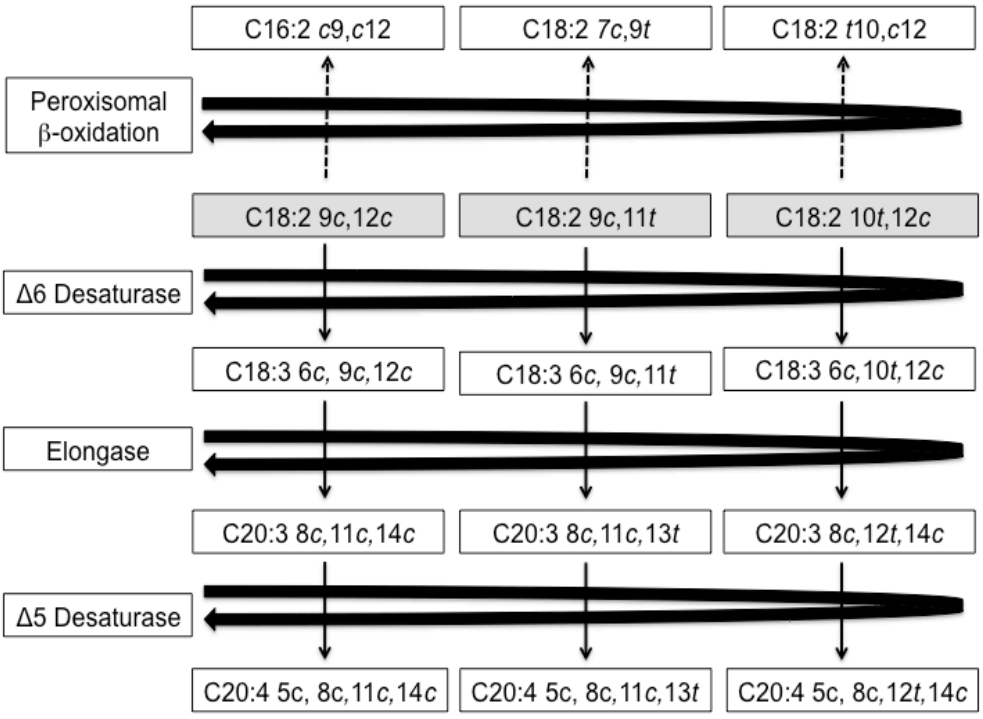

Figure 2.4 Diagram of conjugated linoleic acid metabolism. Both c9, t11 and t10, c12 CLA isomers have been shown to be metabolized and can undergo Δ6 desaturation, elongation and further Δ5 desaturation. Fatty acids of 16-carbons (C16) derived from peroxisomal β-oxidation of CLA have been measured (depicted by dashed arrows).

2.2 Conjugated linoleic acid and the aging musculoskeletal system

There is emerging evidence showing that CLA is beneficial for the maintenance of a healthy musculoskeletal system. Studies, primarily using young and female mice, suggest that CLA increases bone mineralization and protein fractional synthesis rate (FSR) in muscles and prevents bone loss and elevated protein breakdown. However, studies investigating the effects of CLA on bone and muscle using human and other animal models are inconclusive. Moreover, whether CLA can prevent bone and muscle loss in aging males has not been thoroughly addressed. The mechanisms of CLA's effect on factors leading to positive changes in these tissues are still under investigation. This section will focus on the age-related sex hormone deficiency states and inflammation in muscle and bone. A summary of the primary mechanisms that respond to CLA and result in prevention of bone and muscle losses will be discussed. Specifically CLA's effect on musculoskeletal biomarkers, PTH, the COX pathway including the regulation of prostaglandin (PG) E_2, pro-inflammatory cytokines (notably IL-6), and PPAR known to interact in bone and muscle in animal and human studies will be reviewed. The most recent studies investigating the effects of a dietary CLA on bone and muscle are summarized in **Table 2.5**.

2.2.1 Age-related sex hormone deficiency states and inflammation in muscle

Skeletal muscles are long, cylindrical, multinucleated cells composed of myofibrils, which are made of protein bundles. With aging, a decreased myofibrillar protein synthesis associated with decreased bioavailability of sex hormones and increased proinflammatory cytokines is hypothesized, however, this concept has not been thoroughly investigated. Inflammatory markers affect the musculoskeletal system primarily through the catabolism of muscle protein, resulting from increased rate of protein breakdown and decreased rate of protein synthesis [158]. The effects of inflammatory markers on protein degradation are well documented and it is argued that proinflammatory cytokines activate nuclear factor kappa B (NF-κB), which in turn upregulate the adenosine triphosphate dependent ubiquitin-proteasome pathway, which increases myofibrilar protein degradation [159]. Animal models of sarcopenia have suggested that increased activity of NF-κB contributes to age-associated muscle loss [160]. Furthermore, administration of IL-6 or TNF-α in rats causes muscle breakdown [161, 162] and increased TNF-α mRNA and its transcript protein are observed in aged human skeletal muscle signaling for greater protein degradation [163]. On the other hand, protein synthesis occurs through the binding of bioavailable sex hormones to estrogen receptors (ER) and androgen receptors (AR) located on the cell membrane, in the cytoplasm, and on the

nuclear membrane of muscle fibers [164]. Both hormones exert their effects on skeletal muscle through their respective receptor by stimulating the phosphoinositide 3-kinase (PI3K) - protein kinase B (Akt) cascade that stimulates mammalian target of rapamycin (mTOR) resulting in increased protein synthesis [165].

With aging, the aforementioned protein synthesis pathway is thought to be down regulated in part due to estrogen and androgen deficiency. These hormones enhance the cell response to IL-6 by up-regulating the number of IL-6 receptors and cofactors required for cytokine signal transduction and cellular action [166]. Moreover, estrogen-mediated down-regulation of IL-6 expression has been shown to involve the inhibitory effects on NF-kB pathways [167]. Lang et al. demonstrated that TNF-α, impairs skeletal muscle myofibrillar and sarcoplasmic synthesis by decreasing the stimulation of the mTOR signaling pathways in young rats [168]. In a study by Toth et al. comparing young (29±2 y) and old (72±1 y) volunteers, IL-6 and TNF-α receptor II were negatively correlated with skeletal muscle protein synthesis rate [169]. Nevertheless, few studies have assessed declines in protein synthesis rate explained by changes in the production of proinflammatory cytokines modulated by sex hormones using an appropriate aging model [170-172].

2.2.2 Age-related sex hormone deficiency states and inflammation in bone

In addition to age and inflammation-related declines in muscle mass, bone mass may also be reduced due to muscle-bone interactions as well as direct effects on bone tissue. In bone, estrogens and androgens decrease the number of remodeling cycles by attenuating the biogenesis rate of osteoclasts and osteoblasts from their progenitor cells [173] and, also influence the life span of mature bone cells through pro-apoptotic effects in osteoclasts and anti-apoptotic effects in osteoblasts [174]. TNF-α, interleukin 1-beta (IL-1β), and IL-6 can directly stimulate osteoclastogenesis and bone resorption and are up-regulated with decreasing estrogen levels [56]. These cytokines also have an important effect in stimulating receptor activator of nuclear factor kappa-B ligand (RANKL) production by osteoblastic cells and stimulate osteoclast differentiation and activation [175]. Moreover, TNF-α and IL-1β, are known to promote osteocyte apoptosis, where osteocytes are targeted for death and degradation by osteoclasts [176].

In aging men, estrogen is the dominant sex steroid in regulating bone turnover, where it accounts for more than 70% of the total effects on bone formation markers including osteocalcin and type 1 procollagen (P1NP) and bone resorption markers including urinary deoxypyridinoline and collagen Type 1 cross-linked N-telopeptide (NTX) [177]. Despite this physiological dominance of estrogen over testosterone, the latter also

promotes proliferation and differentiation of osteoblasts, inhibits osteoclast recruitment and affects osteoblast-to-osteoclast signaling [178] by regulating cytokines including TGF-β, insulin-like growth factors (IGF) and IL-6 [179]. It also contributes to bone strength by increasing bone size in males during puberty and, most importantly, testosterone is the substrate for the aromatase enzyme, which converts it into estradiol [177]. This suggests that with age, a decrease in these hormones potentially has a direct and indirect catabolic effect on bone and muscle.

2.2.3 Effects of conjugated linoleic acid on the musculoskeletal biomarkers

Recently, CLA was shown to increase and/or prevent muscle and bone loss in various animal and human models. Increases in whole body ash and protein were observed in mice fed 0.5% CLA versus corn oil [180, 181]. It has also been demonstrated that CLA positively affects bone modeling as determined by histomorphometric measurements in male broiler chicks including trabecular bone volume percentage, trabecular thickness, separation, and number [61]. A study by Banu et al. found, in young male mice fed a diet of 0.5% CLA for 14 wk, a significant increase in cancellous bone area (24%), cancellous BMD (46%), cortical BMC (24%), cortical BMD (17%), periosteal perimeter (18%) and endocortical perimeter (21%) at the proximal tibial diaphysis when compared to mice fed safflower oil. At the tibia fibular junction, CLA increased cortical BMC, BMD, thickness, periosteal and endocortical perimeter significantly by

29%, 17%, 4%, 25% and 19% ($p<0.05$) when compared the safflower treated mice [182].

Another study by Rahman et al. found that 12 month old female mice fed a 0.5% CLA diet for 10 wk had increased BMD (64% increase at L4 bone region) and muscle mass (35% increase in quadriceps wet weight), which was accompanied by an ~20% decrease in the proinflammatory cytokines IL-6, TNF-α, and decreased osteoclast differentiation and activation as demonstrated by a decrease in RANKL and TRAP5b [73]. A subsequent study by the same group using a similar design in 12 mo old female mice investigated the differences in muscle mass by using 3 diets of varying CLA isomer composition (c9, t11-CLA 0.5%; t10, c12-CLA 0.5%; 0.25% c9, t11-CLA + 0.25% t10, c12-CLA). After 6 months, all diets lead to significantly higher (8 to 15%) change in total lean mass when compared to the control group [16]. Notably, the control group maintained lean mass over the study and therefore CLA has the capacity to elevate lean mass even at older ages. Male and female mice with induced colon cancer and fed 0.5% CLA had preserved gastrocnemius muscle [183]. In young adult male rats (4 month old, Wistar), a CLA mixture (1% total c9, t11 + t10 c12 CLA) elevates protein FSR in gastrocnemius and soleus muscles with no change to total muscle mass [184].

In humans (18-45 y), CLA supplementation (isomeric mixture, 5 g/d) for 7 wk during resistance training reduced the catabolic effects on muscle as indicated by no change in 3-methylhistadine excretion while the

control group's values were elevated by 92% [72]. Additionally, CLA is known to affect hormones involved in musculoskeletal regulation including IGF-1 and PTH (discussed in next section). CLA has been shown to increase concentrations of IGF-1 and affect IGF-binding proteins, which can lead to bone accretion [60, 61] and muscle anabolism [185, 186], explaining the cross-species positive response to CLA. **Figure 2.5** illustrates the potential antagonistic effects of CLA on muscle loss leading to sarcopenia whereas **Figure 2.6** shows the beneficial effects of CLA on minimizing bone loss.

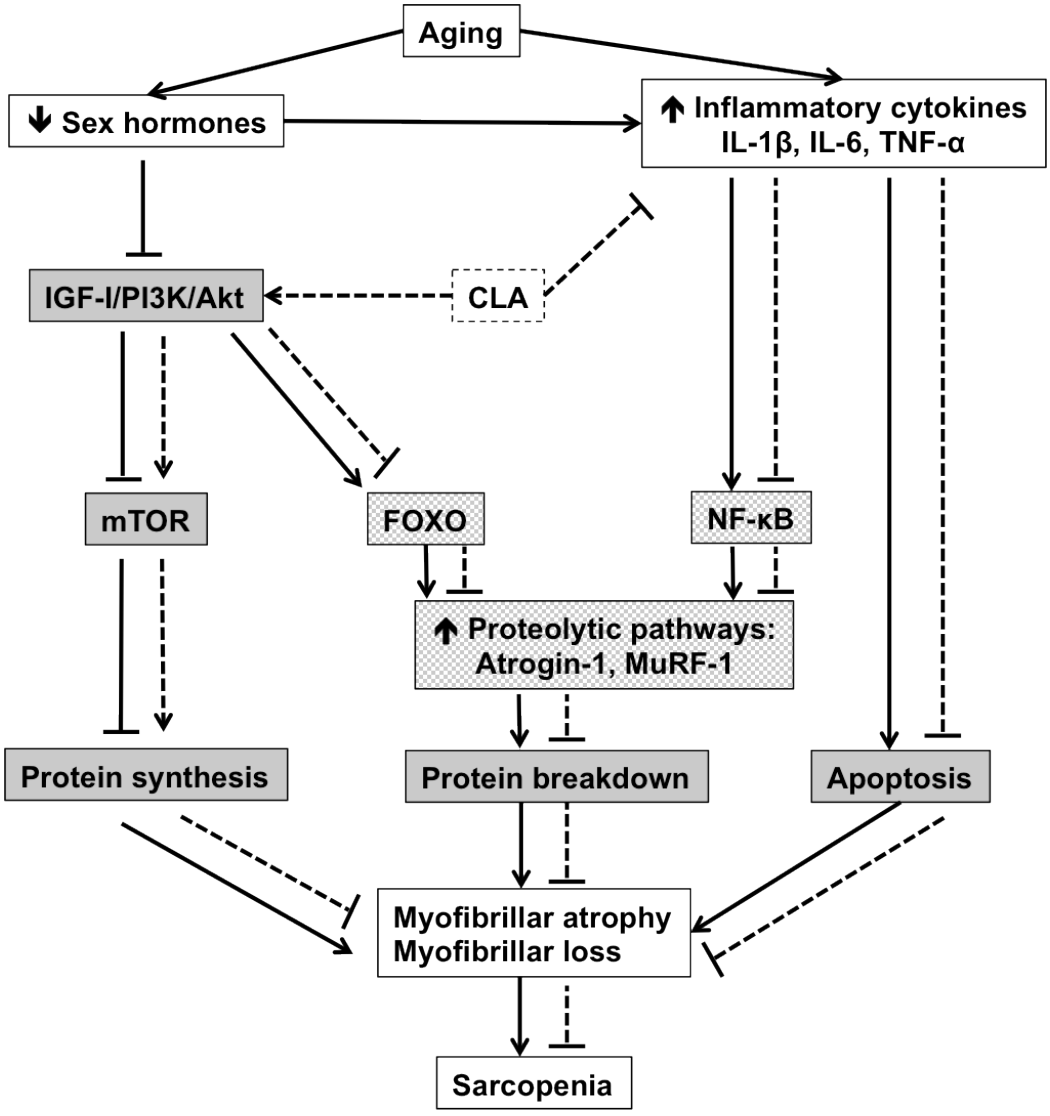

Figure 2.5. A summary of proposed mechanisms by which sex hormones and proinflammatory cytokines can lead to sarcopenia with aging and how CLA can prevent muscle loss. A decrease in sex hormone availability in aging is known to 1) inhibit the modulation of mTOR via the IGF-I/PI3K/Akt pathway leading to reduced protein synthesis and 2) increase the production of proinflammatory cytokines. CLA can inhibit the increase in proinflammatory cytokines observed in aging, which are known to cause apoptosis and modulate NF- κB leading to increased proteolysis. It is also hypothesized that CLA can increase IGF-I leading to increased muscle mass. The pathways are related to an increase in protein turnover favoring protein degradation over synthesis, leading to muscular atrophy and muscle fiber loss, and eventually to sarcopenia. Solid lines (−) represent effects of aging on sarcopenia; dashed lines (---) represent potential effects of CLA on sarcopenia. White boxes (□) represent systemic processes; shaded boxes (■) represent inter/intracellular processes; checkered boxes (▣) represent intranuclear processes.

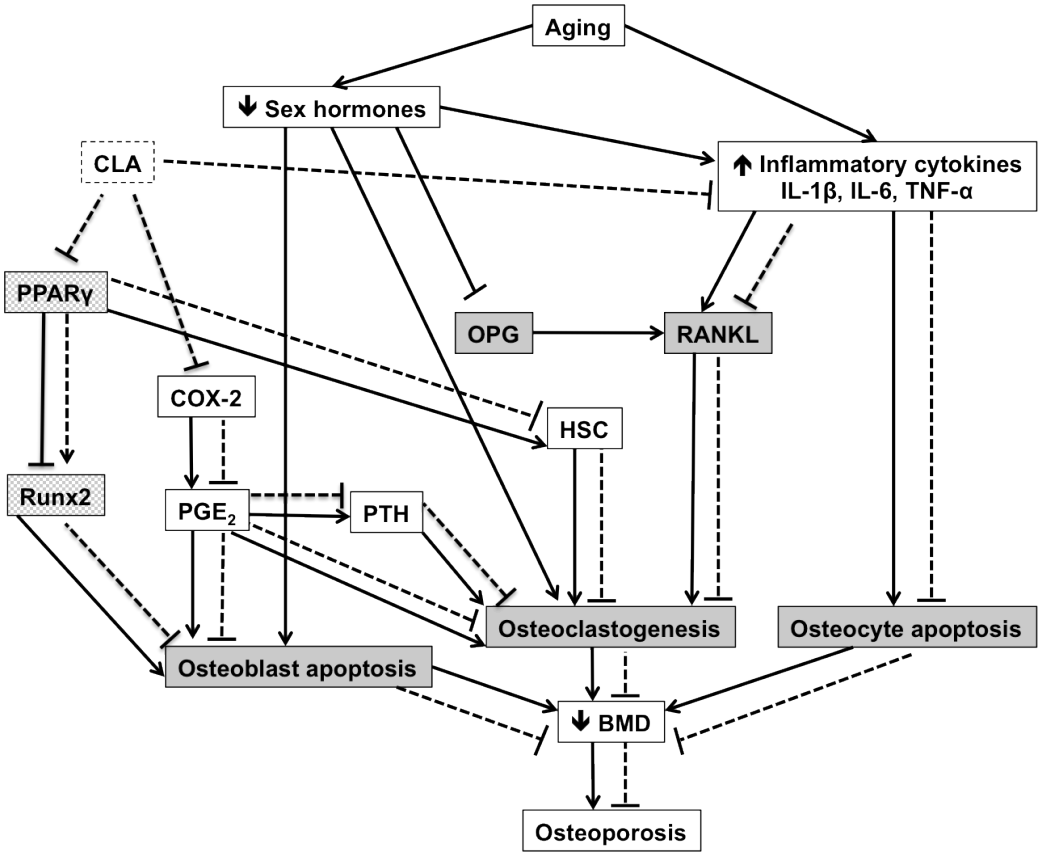

Figure 2.6. A summary of proposed mechanisms by which sex hormones and proinflammatory cytokines can lead to osteoporosis with aging and how CLA can prevent bone loss. A decrease in sex hormone availability in aging is known to 1) stimulate osteoblast apoptosis, 2) osteoclastogenesis via inhibition of osteoprotegerin, amplifying the action of RANKL and 3) increase the production of proinflammatory cytokines. The latter stimulate 1) RANKL, leading to greater osteoclastogenesis and 2) osteocyte apoptosis. CLA inhibits PPARγ, which is known to inhibit Runx2 and stimulate HSC leading to increased osteoblast differentiation and decreased osteoclastogenesis. Moreover, CLA inhibits COX-2 and consequently the production of PGE_2, which can lead to osteoblast apoptosis and increased osteoclastogenesis directly of indirectly by stimulating PTH. The pathways are related to an increase in bone turnover favoring bone breakdown, leading to decreased BMD, and eventually to osteoporosis. Solid lines (−) represent effects of aging on sarcopenia; dashed lines (---) represent potential effects of CLA on sarcopenia. White boxes (□) represent systemic processes; shaded boxes (■) represent inter/intracellular processes; checkered boxes (▣) represent intranuclear processes.

2.2.4 Effects of conjugated linoleic acid on parathyroid hormone

PTH is known to have anabolic properties on bone and muscle tissue [187]. This hormone acts to maintain calcium homeostasis, necessary for nerve signal transduction and muscle contractions and for mineralization of bone. PTH follows a diurnal and circadian rhythm with a nadir existing at 0930 h and peaks in the afternoon [188]. A primary peak is observed at 0314 h with a secondary peak at 1726 h separated by nadirs at 1041 and 2103 h [189] where the lowest concentration is observed at the first nadir. These diurnal patterns do not seem to change with age based on a study in premenopausal women and men [190]. An additional rhythm for PTH exists as ultradian rhythm consisting of seven pulsatile secretions per hour, however this only accounts for 30% of PTH secretion and thus is not likely the major contributor to potential changes due to CLA [191].

Paradoxically, constant high PTH concentrations lead to bone loss while intermittent elevations PTH concentrations induce bone formation [191]. Various mechanisms have been proposed to explain the anabolic and catabolic effects of PTH, however, it appears that effects of PTH are dependent on cellular circumstances such as cell type, stage of cell differentiation, dosage and exposure time [192]. To exert an anabolic action, the PTH ligand binding to the 7-transmembrane G protein-coupled cell surface receptor, initiates multiple intracellular pathways including the cAMP/ protein kinase A and protein kinase C pathways, which promote

the expression of runt-related transcription factor 2 (Runx2) in osteoblasts [192]. However, in cases of elevated PTH concentrations, there is repression of Runx2 and osterix expression in osteoblasts by inhibiting differentiation downstream of early bone morphogenetic protein signaling and by downregulating components of the hedgehog and epidermal growth factor signaling cascade [193]. Pharmacological calcimimetics and 1,25$(OH)_2D_3$ analogs are used to treat hyperparathyroidism and prevent the associated risk of bone loss [193].

CLA was also shown to reduce PTH in rodents. In one study, 52 Han:SPRD-cy rats (a rat model for polycystic kidney disease (PKD)) were fed a diet supplemented with 1% CLA by weight as mixed CLA isomers (1:1 ratio of c9, t11 and t12, c10) for 8 wk. CLA feeding resulted in a 60% decrease in PTH concentrations in both PKD-affected and healthy rats [194]. A subsequent study by Weiler et al. supplemented 48 (24 male and 24 female) Sprague-Dawley rats with CLA (4:1 ratio of c9, t11 and t12, c10) for 16 wk. Again, CLA reduced PTH by over 30% within 4 wk in male rats without adversely affecting bone density [63]. Hence, regardless of the model and health state, PTH was reduced 30 to 40% by CLA. Moreover, these studies demonstrated that the c9, t11 isomer was primarily responsible for the changes seen in PTH. A randomized, double-blind, placebo-controlled trial that included 48 healthy primigravidas with a family history of preeclampsia were supplemented with daily oral doses of calcium carbonate (1484 mg) and 450 mg of CLA (1:1 c9, t11 to t10, c12) or a lactose-starch placebo from wk 18 to wk 22 of gestation until delivery.

PTH was decreased by 8.0% in the calcium-CLA group from baseline to post-supplementation although these results did not reach significance [195]. To date, the effects of dietary or supplemental CLA on PTH in healthy men have not been reported.

2.2.5 Effects of conjugated linoleic acid on cell derived mediators of inflammation

CLA may influence bone and muscle metabolism by modulating anti-inflammatory effects and attenuating the production of pro-inflammatory cytokines [61, 196], and eicosanoids including prostaglandin E_2 [61, 197]. Eicosanoids are physiologically active compounds that originate from polyunsaturated fatty acids composed of 20 carbon atoms. Eicosanoids include compounds such as PG, thromboxanes, leukotrienes, and lipoxins. These compounds can be derived indirectly via dietary sources of linoleic acid, α-linolenic acid, and/or eicosapentaenoic acid (EPA) and directly from arachidonic acid (AA) from the diet or more commonly from AA located in plasma membrane of cells. PGE_2 is involved in bone physiology where higher levels of PGE_2 (10^{-6} M) inhibit bone formation whereas lower levels (10^{-10} - 10^{-8} M) promote bone formation [54]. PGE_2 is synthesized from AA via the cyclooxygenase pathway. Briefly, AA is converted to PGG_2 (very unstable) by the consumption of two O_2 molecules catalyzed by the enzyme COX, (also known as

prostaglandin H synthase). This is followed by a glutathione-dependent oxidation of PGG_2 to PGE_2 via the hydroperoxidase enzyme. The activity of COX is induced by factors such as $1,25(OH)_2D_3$, PTH, IL-1β, and IL-6 [56], which are argued to indirectly increase PGE_2 and consequently promote osteoclastogenesis [57]. Furthermore, COX is present as two isoforms known as COX-1 and COX-2. Hence, PGG_2 is converted to PGH_2 via this peroxidase enzyme. PGH_2 can be converted to PGE_2 via an isomerase enzyme.

CLA decreases bone PGE_2 and may prevent bone loss as previously observed in rat bone organ culture [58]. PGE_2 is produced in bone primarily by osteoblasts and acts as a stimulator for bone resorption [59]. It is argued that CLA may compete with AA in the phospholipid fraction, resulting in diminished prostaglandin production and/or CLA may directly inhibit COX [60] particularly COX-2 production [61], thus decreasing the production of PGE_2. PGE_2 is involved in the release of PTH [62]. Elevated levels of PTH have been reported to decrease bone mass by increasing osteoclastic activity [56]. Hence, due to CLA's inhibitory effects on PGE_2 synthesis, CLA could potentially decrease osteoclastogenesis through a decrease in PTH expression, which may be explained by an upstream decrease in PGE_2 levels, which ultimately would yield positive bone turnover [63]. By inhibiting COX-2 with CLA treatment, this could influence PTH (endocrine) and as well as prostaglandin-dependent osteoclastic bone resorption and PGE_2 (paracrine) receptor-mediated action on bone cells by osteoblasts [64-67].

2.2.6 Conjugated linoleic acid and peroxisome proliferator-activated receptors

PPAR are a member of nuclear receptor protein super family, which includes estrogen, vitamin D, thyroid, glucocorticoid, and retinoic acid receptors, that function as transcription factors regulating the expression of genes [198, 199]. Four isomers have been described including PPAR-α, β/δ, γ1 and γ2. Human osteoblasts express PPAR-γ and PPAR- β/δ [200] that heterodimerize with retinoid X receptor, which further interacts with specific coactivators and corepressors to bind to nuclear PPAR response elements [201].

PPAR-γ is an important transcription factor in adipogenesis and is also expressed in bone marrow mesenchymal stromal cells (MSC) where it regulates osteoblastogenic differentiation [202]. After lineage commitment by MSCs, transcription factors including PPAR-γ and CCAAT/enhancer-binding proteins (C/EBP) manage adipogenesis. PPAR-γ2 promotes adipocytic differentiation [203, 204] in combination with C/EBPβ, which maintains PPAR-γ expression, and the upregulation of C/EBPα expression, which stimulates adipogenesis [205]. PPAR-γ2 also inhibits the Wnt, TGF- β/BMP and IGF-I signaling pathways as well as downregulates the expression of transcriptional regulators Runx2, and osterix [206-208]. PPAR-γ1 enhances osteoclastogenesis by stimulating c-fos expression in osteoclasts precursor cells [209]. Secretory inflammatory factors such as leptin, adipsin, adiponectin, and resistin produced by bone

marrow adipocytes potentially act in a paracrine manner on osteoblasts and suppress osteoblast function and/or differentiation [210].

PPAR-γ ligands include AA derivatives, PG, and polyunsaturated fatty acids. Specifically ligands such as 15-deoxy-D [211, 212], PGJ_2 [213], 9(2)-HODE [214], 15-hydroxy-eicosatetraenoic acid and 8 hydroxyeicosatetraenoic acid [215]. PUFAs and eicosanoids can bind to multiple isomers of PPAR. Both CLA isomers c9, t11 and t10, c12 have a high affinity for PPAR-α [52] and a moderate affinity to PPAR-γ [53]. Thus, CLA acts as an antagonist to PPAR-γ, and can promote osteoblastic differentiation and repress osteoclastic differentiation by inhibiting hematopoietic stem cell differentiation and potentially resulting in positive bone turnover.

Table 2.5. Summary of studies investigating the effects of CLA on bone and muscle biomarkers

Model		Findings[1]	Ref.
Mice			
Species:	12 mo old female C57BL/6 mice (n=80)	6% ↑ %Δ lean mass (c9, t11)	[16]
Diet:	0.5% CLA (c9, t11; t10, c12; mix) (5 g/kg diet)	8% ↑ %Δ lean mass (t10, c12)	
		13% ↑ %Δ lean mass (mix)	
Duration:	6 mo	5% ↑ %Δ hind leg lean (c9, t11)	
		23% ↑ %Δ hind leg lean (t10, c12)	
		17% ↑ %Δ hind leg lean (mix)	
		↔ quad wet weight (c9, t11)	
		23% ↑ quad wet weight (t10, c12)	
		38% ↑ quad wet weight (mix)	
Species:	8 wk old Balb/c mice (n=40)	12% ↑ dist. fem. met. BMD	[182]
Diet:	0.5% CLA (mix) (6.8g CLA/kg diet)	24% ↑ prox. fem. met. BMD	
Duration:	14 wk	24% ↑ fem. Diaphysis BMD	
		11% ↑ tibial diaphysis BMD	
		26% ↑ spine (L3) BMD	
		9% ↑ spine (L4) BMD	
		29% ↑ spine (L5) BMD	
		24% ↑ trab. bone area	
		15% ↑ trab. BMC	
		46% ↑ trab. BMD	
		19% ↑ cort. bone area	
		26% ↑ cort. BMC	
		17% ↑ cort. BMD	
		7% ↑ cort. thickness	
		18% ↑ periosteal perimeter	
		21% ↑ endocort perimeter	
Species:	12 mo old female C57BL/6 mice (n=N/R^2)	43% ↓ fat mass	[73]
Diet:	0.5% CLA (mix) (3.5g CLA/kg diet)	13% ↑ lean mass	
Duration:	10 wk	17% ↑ cortical BMD	
		19% ↑ trabecular BMD	
		20% ↑ gastroc w/w	
		29% ↑ gastroc d/w	
		35% ↑ quad w/w	

		30% ↑ quad d/w 20% ↓ IL-6 22% ↓ TNF-α 15% ↓ RANKL 16% ↓ TRAP5b	
Species: *Diet:* *Duration:*	8 wk old Balb/c male mice (n=40) 0.5% CLA (mix) (6.8g CLA/kg diet) 14 wk	54% ↓ fat mass 4% ↑ lean mass 57% ↓ TNF-α 39% ↓ IL-6	[217]
Species: *Diet:* *Duration:*	5 wk old male Balb/c mice (n=45) 50 g CLA (mix TG & FFA forms)/ kg diet 6 wk	51% ↓ fat mass (TAG) 50% ↓ fat mass (FFA) 2% ↑ protein (TAG) 2% ↑ protein (FFA) 1% ↑ ash (TAG) 4% ↓ ash (FFA)	[45]
Species: *Diet:* *Duration:*	5 wk old male Balb/c mice (n=60) 15 g CLA (mix)/ kg diet 39 d	60% ↓ fat mass ↔ protein ↔ ash	[221]
Species: *Diet:* *Duration:*	4 wk old CD-1 male/fem. mice (n=126) 0.3% CLA (t10, c12) (2.7g CLA/kg diet) 6 wk	81% ↓ epididymal adipose 53% ↓ inguinal adipose ↔ muscle weight (male) ↔ muscle weight (female) 5% ↑ femur weight (male) 7% ↑ femur weight (female)	[218]
Rats			
Species: *Diet:* *Duration:*	4 wk old Sprague-Dawley rats male & female (n=48) 0.5% CLA (c9, t11; t10, c12; mix) (4.2g CLA/kg diet) 16 wk	↔ BMD (whole body) 8% ↓ BMD spine (t10, c12) ↔ BMD (tibia) ↔ BMD (femur) ↔ ionized calcium ↔ serum osteocalcin ↔ urinary ratlaps 31% ↓ PTH males (c9, t11)	[63]

Species:	23 d old male albino Wistar rats (n=30)	9% ↑ water	[216]
Diet:	2.0% CLA (mix) (14g CLA/kg diet)	7% ↑ protein	
Duration:	6 wk	15% ↑ ash	
		16% ↓ fat mass	
Species:	6 wk old fa/fa male Zucker rats (n=120)	↔ lumbar spine BMD	[219]
Diet:	0.4 – 0.8% CLA (c9, t11; t10, c12; mix)	↔ femur BMD	
	(4.15 – 11.0g/kg diet)	↔ femur calcium conc.	
Duration:	8 wk	↔ femur phosphorus conc.	
		↔ bone mass	
		↔ plasma osteocalcin	
Species	3 wk old Han:SPRD-cy male rats (n=52)	60% ↓ PTH (control+PKD)	[194]
Diet:	1% CLA (mix) (16.8 g/kg diet)	↔ plasma osteocalcin	
Duration:	8 wk	↔ ratlaps	
		↔ femur PGE_2	
		↔ femur weight	
		↔ femur length	
		↔ femur area	
		↔ femur BMC	
		↔ femur BMD	
Species:	12 mo old OVX Fisher fem. rats (n=43)	↔ femur length	[220]
Diet:	2.5, 5, 10 g CLA (mix)/ kg diet	↔ femur ash	
Duration:	9 wk	↔ femur BMD	
		↔ femur BMC	
		↔ femur calcium conc.	
		↔ femur magnesium conc.	
		↔ femur phosphorus conc.	
		↔ calcium absorption	
		40% ↓ PGE_2	
		55% ↓ pyridinoline	
		53% ↓ deoxypyridinoline	
Species:	28 d old male Wistar rats (n=40)	↔ femur length	[58]
Diet:	10 g CLA (mix)/kg diet	↔ femur dry weight	
Duration:	8 wk	↔ femur ash weight	
		↔ femur BMD	

		↔ femur BMC ↔ femur calcium conc. ↔ femur magnesium conc. ↔ femur phosphorus conc. 40% ↓ PGE_2 ↔ pyridinoline ↔ deoxypyridinoline ↔ serum osteocalcin ↔ IGF-I 25% ↑ calcium absorption ↔ magnesium absorption ↔ phosphorus absorption	
Species: *Diet:* *Duration:*	21 d old Sprague-Dawley rats (n=40) 1.0% CLA (mix) (10 g/kg diet) 6 wk	*Right Tibia* ↔ trab. thickness ↔ trab. separation ↔ trab. bone volume ↔ trab. number 34% ↓ MAR 45% ↓ BFR *Right Humerus* ↔ bone length ↔ bone dry weight ↔ ash weight ↔ BMD ↔ calcium conc. ↔ magnesium conc. ↔ phosphorus conc. 55% ↓ PGE_2 (tibia) 31% ↓ PGE_2 (femur)	[60]
Pigs			
Species: *Diet:* *Duration:*	Crossbred pigs (weight 26.3 kg) (n=40) 0.12, 0.25, 0.5, 1.0% CLA (mix) When mean weight reached 116 kg	↔ loin weight ↔ intermuscular fat ↔ subcutaneous fat 10% ↑ bone (0.5 &1.0%)	[222]

Chicks			
Species:	160 d old male broiler chicks (n=N/R^2)	*Tibia*	[223]
Diet:	Butter (52 g/kg diet)	↔ bone length	
Duration:	6 wk	13% ↑ bone area	
		7% ↑ medullary cavity area	
		19% ↑ cortical bone area	
		↔ cortical bone width	
		↔ periosteal MAR	
		57% ↑ periosteal BFR	
		68% ↑ total new BFR	
		74% ↑ intracortical porosity	
Humans			
Species:	Healthy college men & women (n=76)	↔ lean tissue mass	[72]
Diet:	5 g/d CLA (mix)	↔ elbow flex muscle thickness	
Duration:	7 wk	↔ body mass	
		↔ % fat	
		↑ Δ lean tissue mass	
		↑ Δ fat mass	
		38% ↓ Urinary 3-methylhistidine	
		↔ urinary NTx	
Species:	Overweight middle-aged men and women (n=134)	↔ body fat mass	[42]
		↔ body lean mass	
Diet:	3.4 g/d CLA (mix) (TG mo 0-12; FFA mo 13-24)	↔ body BMC	
Duration:	24 mo		
Species:	Healthy middle-aged men (n=60)	↔ serum osteocalcin	[70]
Diet:	3 g/d CLA (mix)	↔ bone alkaline phosphatase	
Duration:	8 wk	↔ serum CTx	
		↔ urinary NTx	
		↔ urinary calcium conc.	
Species:	Healthy Caucasian postmenopausal women (n=136)	Dietary CLA is positively associated with BMD	[69]
Diet:	Dietary CLA		
Duration:	Cross-sectional		

Species:	Resistance-trained men & women (n=23)	↔ markers of bone turnover ↔ body mass	[71]
Diet:	3 g/d CLA (mix)	↔ fat mass	
Duration:	4 wk	↔ lean mass ↔ bone mass	

[1] Magnitude of change relative to study control group unless otherwise stipulated.
[2] Not reported

2.3 Summary

While the understanding of the health benefits of certain foods and nutrients is growing exponentially, much remains to be elucidated. Recently, CLA was shown to have beneficial effects on bone and muscle in growing and older animals. However, these results are limited mainly to young and/or female mice. Thus, further research regarding the effects of CLA in the aging musculoskeletal system is warranted.

The pathogenesis of age-related bone and muscle loss is a multifactorial process requiring further investigation to establish mechanisms of action and prescriptive methods to alleviate these chronic diseases. The effects of sex hormones on musculoskeletal health are prevalent and complex and are known to modulate the inflammatory process. Musculoskeletal losses occurring in late life may be modified by a variety of factors including biological processes involving cytokines and hormones. This review has demonstrated that CLA is known to: 1) reduce PTH, 2) modulate cell-derived mediators of inflammation and 3) act as an antagonist when binding with PPAR. Combined, these processes can potentially lead to beneficial effects on the musculoskeletal system.

Chapter 3

Manuscript 1

Free fatty acid and triacylglycerol forms of CLA isomers are not incorporated equally in the liver but do not lead to differences in bone density and biomarkers of bone metabolism

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In Press: Prostaglandins, Leukotrienes and Essential Fatty Acids

12 February 2013 (10.1016/j.plefa.2013.01.006)

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3.1 Abstract

As a dietary supplement, conjugated linoleic acid (CLA) is commercially available in triacylglycerol (TG) and free fatty acid (FFA) forms. The objectives of this study were to assess if FFA or TG CLA diets would: 1) yield similar results regarding tissue incorporation in the liver, 2) produce no differences in assessments of bone density and biomarkers of bone metabolism and 3) not affect calcium, phosphorus and magnesium mass balance. Sprague-Dawley rats (18 male and 18 female) were randomized to 1 of 3 diets: 1) control AIN-93 diet; 2) 1% CLA (1:1 mixture c9, t11: t10, c12) in FFA form and 3) 1% CLA (1:1 mixture c9, t11: t10, c12) in TG form. Assessment of growth, bone and biomarkers of bone metabolism were performed at 8, 12 and 20 wk of age. Differences between groups were tested using a GLIMMIX model repeated for time with diet and sex as fixed effects and individual animals included as a random factor. There were no differences among groups for growth, bone biomarkers or mass nor mineral balance. Liver enrichment of c9, t11 CLA in FFA form was greater than TG form and AIN-93 (FFA: 0.05±0.01 vs. TG: 0.02±0.01 vs. AIN-93 0.001±0.001 % total fatty acids, $P<0.0001$). However, t10, c12 CLA liver enrichment did not differ among groups ($P=0.11$). These findings indicate that c9, t11 (but not t10, c12) CLA in FFA form is incorporated to a greater extent than in TG form. However, the fatty acid forms of CLA do not appear to affect bone or mineral outcomes.

3.2 Introduction

CLA is commercially produced in free fatty acid (FFA) and triacylglycerol (TG) forms, which are branded as having similar health claims. TG CLA is marketed as being better tasting and palatable compared to FFA CLA, which can have a strong astringent taste limiting its application in certain foods [47]. Commonly, FFA CLA is generated from the isomerization of linoleic acid primarily from food grade safflower oil TG, which are saponified to FFA and isomerized to form the CLA isomers under conditions of high pH and temperature. TG CLA is concurrently produced with the addition of a concentration step and a random stereospecific re-esterification of c9, t11 and t10, c12 isomers (in a 1:1 ratio) with glycerol to reform TG, which is further followed by extensive purification processes [224, 225]. The majority of studies investigating physiological effects of CLA are performed using the FFA form of CLA, which could yield different results due to differences in fatty acid absorption and metabolism.

The digestion of CLA varies depending on the form ingested. Synthetically made TG CLA have approximately equal amounts of CLA fatty acids acylated in the *sn*-1,2, and 3 positions, whereas dietary CLA triacylglycerol from dairy [141, 142] and meat sources [143] typically have greater amounts of CLA fatty acids esterified in both the *sn*-1 and 3 positions (~65-75% of esterified fatty acids). This has implications concerning absorption since intestinal gastric (*sn*-3 specific) and

pancreatic lipases (*sn*-1 and 3 specific) hydrolyze TG resulting in two FFA and a monoacylglycerol at the *sn*-2 position (MG). Typically, in fatty acids, MG are more readily absorbed in the enterocytes [226], however, c9, t11 CLA fatty acids in the *sn*-1 and 3 positions are shown to be more bioavailable [141]. In addition to the stereospecificity of fatty acids, the degree of fatty acid absorption varies depending on whether it is ingested as a TG, an ethyl ester or a FFA. Long chain polyunsaturated fatty acids consumed as TG are absorbed more completely than fatty acid ethyl esters, but not as completely as FFAs [138-140]. However, Yamasaki et al. report no significant differences between FFA CLA and TG CLA in the efficiency of lymphatic transport [47].

Beyond absorption, there is no evidence of physiological function differences in animal and human between FFA and TG forms of CLA for body composition, energy balance, cardiovascular, hepatic, hormonal, immune and cancer assessments [36, 42-47]. To date, studies comparing the differences between FFA and TG CLA in bone outcomes are scarce and have only assessed bone mineral content (BMC) [36, 42]. Hence, further assessments including bone density, biomarkers of bone metabolism and mineral balance are warranted. Furthermore, the accumulation of various CLA isomers in animal and human tissues, notably the liver, has been well documented [144, 145]. Nevertheless, isomer-specific tissue enrichment differences between FFA and TG CLA have not been reported. Thus, diets supplemented with either FFA or TG CLA are hypothesized to: 1) produce no differences in assessments of

bone density and biomarkers of bone metabolism; 2) yield similar results regarding tissue incorporation in the liver, and 3) not affect calcium (Ca), phosphorus (P) and magnesium (Mg) mass balance.

3.3 Materials and methods

3.3.1 Study protocol and diets

All procedures and measurements were approved by the University of Manitoba Committee on Animal Care and conformed to Canadian Council on Animal Care guidelines [227]. This study was part of a larger study investigating the effect of CLA on bone and mineral metabolism [63]. Sprague-Dawley rats, (n=36, 18 male and 18 female), were randomized at 3 wk of age to receive 1 of 3 diets from 4 to 20 wk of age. From wk 3 and 4 of life, the rats were acclimatized to the housing conditions and fed the control (CTRL) diet. The rats were housed (12 h light, 12 h dark cycle) in same-sex pairs, fed ad libitum and food disappearance monitored 3 times wkly over the 16-wk feeding trial. The diets all contained 84 g total fat/kg diet to ensure that essential fatty acids were not compromised at the expense of adding CLA. The diets were: 1) CTRL AIN-93G diets [228] made with soybean oil (n-6: n-3 ratio ~ 7:1); 2) CTRL diet combined with 0.5% c9,t11 CLA + 0.5% t10,c12 CLA in free fatty acid form and 3) CTRL diet combined with 0.5% c9,t11 CLA + 0.5% t10,c12 CLA in triacylglycerol form. These CLA mixtures were adjusted so that a single isomer

represented 0.5% of the diet by weight. The CLA isomers were provided in kind from Lipid Nutrition, a division of Loders Croklaan (Channahon, IL, USA). The pre-mixed CLA was 74.5% c9, t11 and t10,c12 CLA with total CLA at 80.6% (Loders Croklaan Inc, certificate of analyses). Total Ca/kg diet was 5.1 g/kg, total P/kg diet was 46.08 g/kg and total Mg/kg was 14.48 g/kg based on mineral content of casein and mineral mix (Harland Teklad certificate of analyses).

3.3.2 Assessment of growth and bone

Body weight was measured weekly throughout the study using a weigh scale with a dynamic weighing application specifically for animal weighing (SB8001, Mettler-Toledo, Columbus, OH). At 8, 12 and 20 wk of age, the rats were anaesthetized using isoflurane gas (AErrane®, Baxter, Mississauga, ON) for measurement of bone mass including whole body, lumbar spine, femur and tibia using a small animal program and dual-energy X-ray absorptiometry (DXA; 4500A Elite Series, Hologic, Bedford, MA, USA). Length was also measured at from nose to base of tail in the anesthetized state. The DXA measurements have been validated using similar hardware (fan beam) and software (specific for small animal) for rats [229, 230].

3.3.3 Biomarkers of bone metabolism

At each of the three time points, a blood sample of no more than 10% blood volume was taken from the tail vein, between 0800 and 1000h to control for diurnal variation. Blood was separated at 2000 g to obtain serum for determination serum PTH, osteocalcin and C-telopeptide of Type 1 collagen (CTx). Both bioactive and intact PTH was measured using an ELISA (Alpco Diagnostics, Windham, NH, USA), osteocalcin using an ELISA (Osteometer, Nordic Bioscience, Herlev, Denmark), in addition to urinary CTx using an ELISA (Ratlaps, Osteometer, Nordic Bioscience). All of these immunoassays are specific to rodents. Regarding serum ionized Ca, samples were measured within 4 h of collection using a Nova analyzer (Model 11, Nova Biomedical) and a CV <1.6 over the study period. In the last 5 days of each study phase, rats were housed in metabolic cages and mass balance studies conducted by measuring disappearance of food and excretion of nutrients over the last 3 days; the first 2 days are adaptation to the new housing. Minerals (Ca, P, Mg) and urinary CTx were measured from the 72-h pooled samples of urine and feces following digestion in nitric acid and using inductively coupled plasma optical emission spectroscopy (Varian Liberty 200, Varian Canada). However, since many male rats were too large for the metabolic cages at 20 wk of age, the dataset was not complete and thus not analyzed. Results are therefore reflective of only 8 and 12 wk of age.

3.3.4 Gas chromatography

Lipid extraction was performed using a modified Folch procedure, as previously described [231]. Liver tissue was examined as previous studies have shown this tissue to be a more sensitive and consistent marker to confirm absorption and incorporation of the ingested PUFA [231, 232]. Samples were dissolved in 1 ml of dry toluene, mixed with 2.0 ml of 0.5 M sodium methoxide and heated to 50 °C for 10 min, then mixed with 0.1 ml of glacial acetic acid, 5 ml of distilled H2O, and 5 ml of hexane. This method was selected as being reliable for the methylation of the CLA isomers, which were primary interest in tissue analysis in this study [233]. After vortexing, samples were centrifuged at 2500Xg for 10 min and the hexane fraction removed. Fresh hexane was added to the remaining solution and the previous steps repeated. The hexane fractions were dried under anhydrous sodium sulfate, evaporated under nitrogen and the lipid esters redissolved in 1 ml hexane. Gas chromatography was performed on a Varian Chrompack 3800 instrument, using a Varian CP-Sil 88 100 meter column (Varian, Walnut Creek, CA).

3.3.5 Statistical analyses

Data were analyzed using SAS statistical package software version 9.2.0 (SAS Institute Inc., Cary, North Carolina, USA). A GLIMMIX model included diet and sex repeated for time, which was used combined with the individual animals included as a random factor. This relatively new model was used to accommodate the Gaussian and non-Gaussian distribution and unequal variances of the data [234]; all pair wise differences in diet means were tested using Tukey-Kramer approximation and a P-value of ≤0.05 was accepted as significant. Data are presented as mean ± SEM unless otherwise stated.

3.4 Results

3.4.1 Dietary intake

There were no differences in food intake between male and female rats, however, a main effect for diet was observed where the CTRL group had an average wkly food intake greater than the FFA CLA group but not TG CLA. Feed efficiency ratios (gain in body mass/food intake) were higher in the FFA CLA group but did not reach significance (CTRL: 0.19±0.01 vs. FFA CLA 0.22±0.01 vs. TG CLA 0.20±0.01, P=0.29). There was also a main effect for time, which is expected to be greater with increased growth by wk 8 and 16. No interaction effects were found for food intake after post hoc testing (**Table 3.1**).

3.4.2 Growth and body composition

There was no main effect for diet on weight, length and tail length (data not shown for tail length). Main effects for sex and time were detected as anticipated with male rats being heavier than female rats and growth resulting in increased weight over the course of the study. No interaction effects were detected for weight, length and tail length. There was no main effect of diet, and a main effect of time for all the body composition variables including fat mass, WBF%, lean mass, WBL%. Main

effects for sex were observed in fat mass and lean mass measurements but not in WBF% and WBL% (**Table 3.1**).

3.4.3 Bone assessment

There were no main effects of diet on BMC and BMD. Time was significantly related to all of the bone assessment variables as a reflection of bone growth over the 16 wk study. Also, there was main effect of sex for WB BMC, tibia BMC, femur BMC, spine BMC, tibia BMD, femur BMD, and spine BMD, however there was a main effect of sex for whole body BMD. No significant interaction effects were found for these body composition variables (**Table 3.2**).

3.4.4 Biochemistry

No main effects of diet were found for any of the biochemistry measurements. There was a main effect of sex for serum osteocalcin and urinary ratlaps, and a main effect of time for all the variables except INT PTH. No significant interaction effects were observed (**Table 3.3**).

3.3.5 Mass balance

Mass balance was conducted at 8, 12 and 20 wk of age. There were no main effects of diet or sex for Ca, P, and Mg mass balance. A main effect for time was observed in Ca and P, but not in mass balance of Mg (**Table 3.3**).

3.3.6 Liver incorporation of CLA

Liver c9, t11 CLA and t10, c12 CLA enrichment was compared between AIN-93, FFA and TG CLA diets. FFA CLA fed rats had the highest enrichment of c9, t11 CLA in the liver followed by TG CLA and AIN-93 all of which were significantly different (**Figure 3.1**). However, t10, c12 CLA did not have significantly different enrichment among groups (**Figure 3.2**). There were no sex differences for liver c9, t11 CLA and t10, c12 CLA enrichment among group.

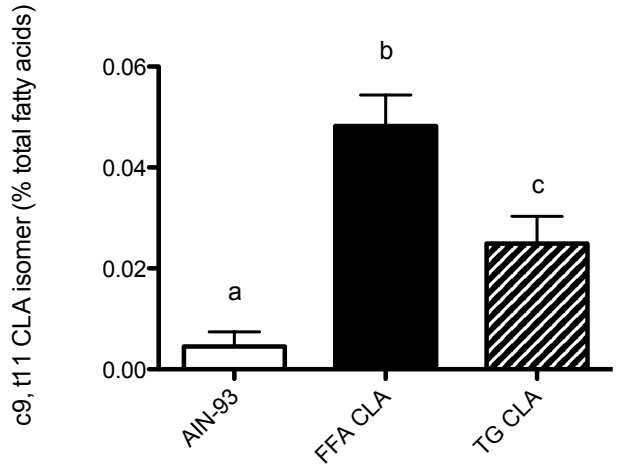

Figure 3.1. Liver c9, t11 CLA proportion as percent total of measured fatty acids. Differences detected using GLIMMIX model with post hoc testing using Tukey's HSD test. Different letters indicate significant differences ($P<0.05$). Values are means ± SEM; n = 12 per diet group.

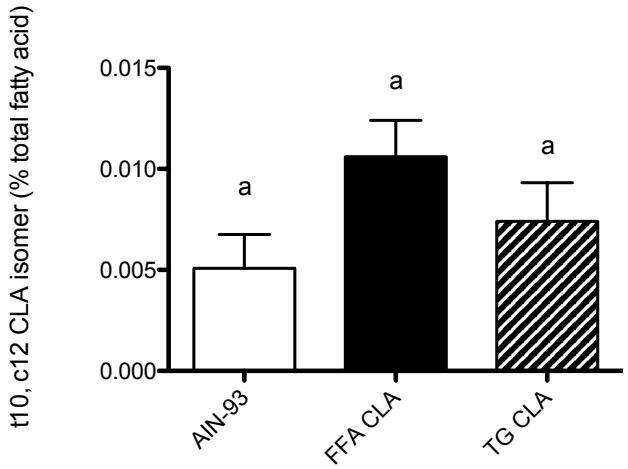

Figure 3.2. Liver t10, c12 CLA proportion as percent total of measured fatty acids. Differences detected using GLIMMIX model with post hoc testing using Tukey's HSD test. Different letters indicate significant differences ($P<0.05$). Values are means ± SEM; n = 12 per diet group.

Table 3.1. Main effects of CTRL, FFA CLA or TG CLA diet at 4, 8 and 16 wk of study on food intake, growth and body composition of male and female rats

	Diet			Sex		Time (wk)		
	CTRL	FFA CLA	TG CLA	Male	Female	4	8	16
Diet Characteristics								
Food Intake (average g/wk)	166.3±5.3^{z}	137.1±5.1^{y}	154.3±4.9^{z}	158.1±4.8	147.0±3.9	118.6±2.8^{A}	158.9±3.4^{B}	180.2±3.7^{C}
Growth								
Weight (g)	370.8±24.5	352.9±22.3	364.8±23.5	460.9±17.7^{a}	264.7±7.3^{b}	257.4±9.1^{A}	365.3±17.4^{B}	465.7±25.5^{C}
Length (cm)	42.3±0.8	41.4±0.7	41.4±0.7	44.3±0.6^{a}	39.1±0.4^{b}	37.6±0.4^{A}	42.3±0.5^{B}	45.1±0.6^{C}
Body Composition								
Fat mass (g)	59.6±6.0	52.3±5.7	63.3±7.8	74.6±6.0^{a}	42.2±3.5^{b}	31.9±1.9^{A}	50.5±3.7^{B}	92.7±7.6^{C}
WBF (%)	15.2±0.7	13.7±1.0	15.6±1.0	15±0.7	14.6±0.8	11.9±0.5^{A}	13.4±0.7^{B}	19.2±1.0^{C}
Lean mass (g)	308.7±19.3	299.3±18.0	300.8±16.9	384.6±12.7^{a}	221.2±4.6^{b}	226.2±7.8^{A}	314.5±15.4^{B}	368.1±19.8^{C}
WBL (%)	82.1±0.8	83.2±1.3	81.6±1.0	82.2±0.8	82.5±0.8	85.1±0.9^{A}	84±0.8^{B}	77.9±1.0^{C}

Data are mean±SEM; n = 36 in total. Main effects determined using GLIMMIX model with Tukey-Kramer approximation. Only main effects presented herein. Different letters indicate significant differences (P<0.05).

Table 3.2. Main effects of CTRL, FFA CLA or TG CLA diet at 4, 8 and 16 wk of study on whole body and regional bone mineral content and density of male and female rats

	Diet			Sex		Time (wk)		
	CTRL	FFA CLA	TG CLA	Male	Female	4	8	16
Whole body								
WB BMC (g)	10.3±0.6	9.8±0.6	10.2±0.6	11.9±0.5^{a}	8.2±0.3^{b}	6.6±0.2^{A}	10.1±0.3^{B}	13.5±0.5^{C}
WB BMD (g/cm^2)	0.147±0.003	0.144±0.003	0.146±0.003	0.147±0.003	0.144±0.002	0.121±0.001^{A}	0.147±0.001^{B}	0.169±0.001^{C}
Appendicular bone								
Tibia BMC (g)	0.37±0.02	0.37±0.02	0.37±0.02	0.43±0.02^{a}	0.31±0.01^{b}	0.26±0.01^{A}	0.39±0.01^{B}	0.47±0.02^{C}
Tibia BMD (g/cm^2)	0.201±0.007	0.204±0.006	0.209±0.007	0.213±0.006^{a}	0.196±0.005^{b}	0.165±0.004^{A}	0.211±0.004^{B}	0.238±0.005^{C}
Femur BMC (g)	0.52±0.03	0.50±0.03	0.52±0.03	0.59±0.03^{a}	0.43±0.01^{b}	0.34±0.01^{A}	0.54±0.01^{B}	0.66±0.03^{C}
Femur BMD (g/cm^2)	0.366±0.014	0.363±0.012	0.370±0.014	0.391±0.012^{a}	0.341±0.008^{b}	0.280±0.005^{A}	0.383±0.005^{B}	0.435±0.010^{C}
Axial bone								
Spine BMC (g)	0.57±0.03	0.53±0.03	0.53±0.03	0.59±0.03^{a}	0.50±0.01^{b}	0.36±0.01^{A}	0.57±0.01^{B}	0.70±0.02^{C}
Spine BMD (g/cm^2)	0.269±0.007	0.255±0.008	0.262±0.008	0.249±0.007^{a}	0.275±0.005^{b}	0.213±0.004^{A}	0.264±0.004^{B}	0.308±0.003^{C}

Data are mean±SEM; n = 36 in total. Main effects determined using GLIMMIX model with Tukey-Kramer approximation. Only main effects presented herein. Different letters indicate significant differences ($P<0.05$).

Table 3.3. Main effects CTRL, FFA CLA or TG CLA diet at 4, 8 and 16 wk of study on biomarkers of bone metabolism and mass balance of male and female rats

	Diet			Sex		Time (wk)		
	CTRL	FFA CLA	TG CLA	Male	Female	4	8	16
Biochemistry								
Ionized Ca (mmol/L)	1.43±0.01	1.42±0.01	1.43±0.01	1.42±0.01	1.43±0.01	1.45±0.01^{A}	1.40±0.01^{B}	1.43±0.01^{C}
Bio PTH (pmol/L)	11.1±1.1	11.8±1.1	10.1±1.0	10.9±0.8	11.1±0.9	11.7±1.2^{A}	12.7±1.0^{B}	8.5±0.8^{C}
Intact PTH (pmol/L)	22.4±1.3	22.1±1.1	20.2±1.2	22.2±1.0	20.9±1.0	20.7±1.1	23.6±1.2	20.4±1.2
Serum Osteocalcin (nmol/L)	53.3±5.0	52.4±4.6	50±4.6	58.4±3.9^{a}	45.4±3.6^{b}	82±3.1^{A}	49.3±2.9^{B}	24.4±1.3^{C}
Urinary ratlaps (nmol/d)	2.13±0.46	2.0±0.35	1.97±0.33	2.95±0.38^{a}	1.13±0.15^{b}	4.08±0.44^{A}	1.54±0.20^{B}	0.44±0.07^{C}
Mass balance								
Calcium (mg/balance)	151.8±15.3	134.1±11.5	158.3±11.4	165.1±12.3	129.8±6.5	184.7±10.0^{A}	109±5.4^{B}	N/A
Phosphorus (mg/balance)	68.5±8.0	62.7±5.8	73.2±6.6	78±6.1	57.6±4.1	86.1±5.5^{A}	49.1±3.2^{B}	N/A
Magnesium (mg/balance)	8.9±1.1	8.0±1.2	9.5±1.2	9.9±1.1	7.6±0.8	9.3±1.1	8.3±0.7	N/A

Data are mean±SEM; n = 36 in total. Main effects determined using GLIMMIX model with Tukey-Kramer approximation. Only main effects presented herein. Different letters indicate significant differences (P<0.05).

3.5 Discussion

This study has demonstrated for the first time an isomer-specific liver enrichment difference between TG and FFA forms of CLA. The observed pattern of CLA isomer incorporation in the liver suggests that it occurs via a selective process, where c9, t11 CLA fatty acids from FFA sources are preferentially incorporated in liver over similar fatty acids of TG origin. However, the hepatic enrichment of t10, c12 CLA isomer did not differ significantly between forms and from the CTRL group. This suggests that incorporation of t10, c12 CLA isomer was minimal and could have been insufficient to detect differences between forms. This is of significance for studies investigating potential effects on liver metabolism specifically gene regulation via c9, t11 and t10, c12 CLA isomers interaction with peroxisome proliferator-activated receptors [235].

These findings differ from Porsgaard et al. who found no differences in c9, t11 and t10, c12 CLA isomer incorporation in liver TG, phospholipids or cholesterol esters in hamsters fed 0.5% TG CLA or FFA CLA [236]. However, Kramer et al., examined the relative FFA CLA (but not TG CLA) isomeric distribution in liver lipids, using a similar commercial mixture of CLA as in the present study, found similar proportions of c9, t11 and t10, c12 CLA isomer incorporation in the liver [237]. Furthermore, the distribution of c9, t11 CLA isomer was the major isomer in most liver lipids including: phospholipids, sphingomyelin, cholesteryl ester, TG and FFA, whereas t10, c12 CLA isomer showed significantly lower levels in the

aforementioned liver lipids compared to the distribution found in the diet [237].

The differences in relative accumulation of the CLA isomers could be explained by the structural bioassembly of TG CLA, which could allow for a variation in the distribution in liver lipids, and differences in tissue fatty acid metabolism, oxidation, and incorporation. The enrichment of c9, t11 CLA could be due to slower metabolism and/or preferential incorporation. In contrast, low content of t10, c12 CLA could be a result of faster metabolism and/or selective discrimination. Moreover, the TG form could potentially favor oxidation versus incorporation [237], which would explain the lower values of c9, t11 and t10, c12 CLA observed in **Figure 3.1** and **3.2**.

This study also showed that supplemental CLA form did not lead to differences in body composition among groups despite the FFA CLA group having significantly lower wkly average food intake. Feed efficiency ratios did not differ significantly among diet groups, which further explains the lack of change in body composition due to diet. Mineral metabolism was also not affected by the form of CLA, this is in agreement with Kelly et al. who supplemented weanling male rats with 1% CLA (1:1 c9, t11: t10, c12 isomer mixture) for 8 wk and found no differences in Ca, P, and Mg balance and absorption in a typical soybean oil based diet [58].

Regarding bone and biomarkers of bone metabolism, no significant differences in the measured bone outcomes were observed when comparing TG and FFA forms of CLA. Our whole body BMC results are in

agreement with Gaullier and al. who did not detect differences in BMC in subjects supplemented with FFA and TG CLA for up to 24 months [36, 42]. Moreover, the majority of studies comparing both forms of CLA found no differences [43-47]. However, Yamasaki et al. demonstrated in 4 wk old male mice fed 1% TG CLA but not in 1% FFA CLA, a >200% downregulation of certain cytokines, including interleukin-6 (IL-6), a proinflammatory cytokine associated with bone loss with aging [47]. A potential difference in structural reorganization between TG CLA and FFA CLA can occur after intestinal absorption where products of lipase-digested TG-CLA, typically 2-monoglyceride containing CLA at the *sn*-2 position, can be reshuffled in the endoplasmic reticulum of the enterocytes to the *sn*-1 and/or 3 position and affect lipid metabolic function [238, 239]. Hence, despite similar lymphatic absorption efficiency [47], the resynthesized TG in epithelial cells may have discrepancies, which can affect inflammatory function. Nevertheless, the animals used in this study were young healthy rats and despite all having reached peak bone mass at 12 wk of age [240] and having final bone measurements at 20 wk of age, the proinflammatory effects of IL-6 are most likely negligible, which supports the lack of differences observed for the bone measurements.

In summary, c9, t11 CLA isomer in FFA form is preferentially incorporated in the liver compared to the TG form whereas hepatic enrichment of the t10, c12 isomer did not differ. This suggests that studies investigating tissue enrichment, and lipid fraction properties of CLA should take into consideration the form of the ingested fatty acid. Furthermore, no

significant differences were observed in body composition, biomarkers of bone metabolism and mineral mass balance among diet groups suggesting that one supplemental form of CLA does not appear to have any added value over the other regarding potential bone density and metabolism benefits.

3.6 Acknowledgements

This study was funded jointly by Dairy Farmers of Canada and NSERC and the CLA provided in kind by Lipid Nutrition. The work was conducted at the University of Manitoba facilities and the associated Manitoba Institute of Child Health. The authors acknowledge the assistance with feeding and some sample collection by S. Fitzpatrick, M. Kaur, and R. Mollard and A. Dick. The authors have no conflict of interest.

Bridge statement I

The pre-clinical results obtained in chapter 3 are significant for improving the development and design of future clinical trials investigating the mechanisms of CLA on bone and biomarkers of bone metabolism. As shown, the FFA form of c9, t11 CLA isomer is preferentially incorporated in the liver and no significant differences were observed for bone biomarkers of bone metabolism between FFA and TG forms of CLA supplement were observed. This suggests that a CLA supplement formulation composed of primarily of the c9, t11 isomer in FFA form could be favored over a supplement comprised of an equal mixture of c9, t11 and t10, c12 CLA isomers. Moreover, a study by Weiler et al. established that the c9, t11 isomer in FFA form, was responsible for a reduction in PTH of up to 36% over 16 wk in male rats, which could have positive bone health implications in the long term [63]. In order to proceed with a randomized clinical trial in attempt to verify these changes in PTH, further research regarding the effects of fasting, gender, and generation of PTH assay on PTH measurements must be elucidated to ensure the optimization and standardization of the data collected.

Chapter 4

Manuscript 2

Feeding reduces serum concentrations of endogenous bioactive (1-84), but not total intact parathyroid hormone in female Sprague-Dawley rats

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Published in: Open Journal of Nephrology 2013, 3: 61-65

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4.1 Abstract

Interpretation of parathyroid hormone (PTH) measurements requires an understanding of its structural conformation. PTH is commonly measured in the fasted state using 2nd generation assays, known to cross-react with PTH 7-84 fragments, which is not observed with 3rd generation assays. The objective was to determine if plasma PTH in the fed and non-fed state differ while controlling for diurnal rhythm and the generation of PTH assay. Blood was sampled, 2 d apart, from 60 Sprague-Dawley rats (30 male and 30 female) in both fed and non-fed states at 20 wk of age (weight: 470.2±23.2 g) for measurement of ionized calcium (iCa), total intact (INT) and bioactive 1-84 (BIO) PTH. Differences between groups were tested using a GLIMMIX model with sex and feeding state as fixed effects and individual rats as a random effect. Females had a lower iCa compared to males (F: 1.43±0.01 vs. M: 1.46±0.01 mmol/L, P=0.03). In males and females, there was no difference between fed and non-fed groups when PTH was assessed using the INT PTH assay (M Fed: 21.58±1.34 vs. M Non-fed: 22.64±2.27 pmol/L, P=0.59; F Fed: 19.33±1.49 vs. F Non-fed: 22.80±2.92 pmol/L, P=0.39). However, in females only, PTH measured using the BIO PTH, was significantly lower in the fed group versus the non-fed (Fed: 8.44±0.77 vs. Non-fed: 16.66±3.37 pmol/L, P=0.05). These results suggest that fasting elevates BIO PTH. Studies related to PTH should consider standardization of fast duration, sex differences, and preferentially use BIO PTH assays.

4.2 Introduction

Parathyroid hormone (PTH) is the primary determinant of the extent of bone remodeling activity in the skeleton by regulating bone metabolism and mineralization in addition to maintaining calcium homeostasis. PTH follows a diurnal and circadian rhythm [63, 194, 241, 242] and is altered acutely by certain nutrients such as calcium [241, 242] and chronically by conjugated linoleic acid (CLA) [63, 194]. Thus when assessing PTH, both timing of sampling and standardized food intake patterns prior to sampling are required in trials designed to assess the physiological response of PTH to diet or supplements. Furthermore, PTH assays have evolved based on their clinical performance in differentiating bone diseases associated with renal failure [243].

First generation assays allowed for the description of circulating PTH immunoreactivity, however, these mainly reacted with PTH fragments without bioactivity. Second generation assays, known as intact PTH (INT), were initially thought to react only with the bioactive form PTH (1-84) [243]. It was later discovered that these assays cross-reacted with the PTH fragments, notably PTH (7-84), which are suggested to antagonize the calcemic and bone resorptive effects of PTH (1-84). Third generation assays (BIO), with no cross-reactivity with the PTH fragments were thus developed to address the issue of the detection of fragments with biologically opposite effects [243]. Recently, in male and female cynomolgus monkeys, feeding reduced INT PTH suggesting that feeding

state should be considered when interpreting PTH values [244]. The objective of this study was to confirm these results in another species and to assess possible differences in feeding state, sex and type of PTH assay used. It was hypothesized that: 1) feeding would reduce both INT and BIO PTH; 2) there would be sex differences for both INT and BIO PTH; and 3) INT and BIO PTH assays will yield different values for PTH.

4.3 Materials and methods

4.3.1 Study protocol and diets

All procedures and measurements were approved by the University of Manitoba Committee on Animal Care and conformed to Canadian Council on Animal Care guidelines [227]. This study was part of a larger study investigating the effect of CLA on bone and mineral metabolism [63]. Sprague-Dawley rats, (30 male and 30 female), were fed AIN-93G diets [228] from 3 to 20 wk of age with the exception of slightly higher fat content (84 g/kg) in all groups with the control group containing only soybean oil and the other dietary groups receiving 0.5 to 1% CLA. The rats were acclimatized to the housing conditions and housed (12 h light, 12 h dark cycle) in same-sex pairs, fed ad libitum and food disappearance monitored 3 times wkly over the study.

4.3.2 Assessment of growth, PTH and ionized calcium

Body weight was measured wkly throughout the study using a weigh scale with a dynamic weighing program specifically for animal weighing (SB8001, Mettler-Toledo, Columbus, USA). Length was measured at wk 16 from nose to base of tail in the anesthetized state using isoflurane gas (AErrane®, Baxter, Mississauga, Canada). At the wk 16 time-point, a blood sample of no more than 10% blood volume was taken from the tail vein, between 0800 and 1000h to control for diurnal variation and separated to obtain serum for determination of PTH. The fed state was a reflection of ad libitum intake during normal nocturnal feeding. Food was removed at 2000 h the night before the non-fed blood sampling 2 days after the fed blood sampling. Blood was separated at 2000 g to obtain serum for determination of PTH using both second generation INT PTH and third generation BIO PTH using separate ELISA kits specific to rodent PTH (Alpco Diagnositics, Windham, USA). To assess intra- and inter-assay precision of both assays, the coefficient of variation (CV%) was calculated from 20 triplicate determinations of two samples each performed in a single assay. For the BIO assay, intra-assay CV ranged from 2.5-3.9% and inter-assay CV ranged from 7.8-8.9%. For the INT assay, intra-assay CV ranged from 2.1-2.4% and inter assay CV ranged from 5.1-6.0%. Regarding serum ionized calcium (iCa), samples were measured within 4 h of collection using a Nova analyzer (Model 11, Nova Biomedical, Waltham, USA) with a CV <1.6%.

4.3.3 Statistical analyses

Data was analyzed using SAS statistical package software version 9.2.0 (SAS Institute Inc., Cary, USA). A GLIMMIX model included feeding state, sex and type of assay used combined with the individual animals included as a random factor. This relatively new model was used to accommodate the Gaussian and non-Gaussian distribution and unequal variances of the data; all pair wise differences in diet means were tested using Tukey-Kramer approximation and a P-value of ≤0.05 was accepted as significant. Data are presented as mean ± SEM unless otherwise stated.

4.4 Results

At 20 wk of age, females had 47.5% lower weight than males (F: 323.1±32.6 vs. M: 615.2±51.6 g, P<0.0001), 18.6% lower length (F: 21.5±0.5 vs. M: 26.4±0.8 cm, P<0.0001), and 8.8% less food intake (F: 179.3±22.9 vs. M: 196.6±18.0 g, P=0.003). In males, iCa was 2.0% lower in the non-fed group versus fed (M non-fed: 1.43±0.01 vs. M fed: 1.46±0.01 mmol/L, P=0.003). A similar trend was observed in females (1.4% lower), however, it did not reach significance (F fed: 1.44±0.01 vs. F non-fed: 1.42±0.01 mmol/L, P=0.08). There was a significant difference between sexes where females had a 2.1% lower iCa compared to males (F: 1.43±0.01 vs. M: 1.46±0.01 mmol/L, P=0.03). Also fed rats had

significantly higher (1.4%) iCa versus non-fed (fed: 1.45±0.01 vs. non-fed 1.43±0.01 mmol/L, P=0.0002).

BIO PTH values were 48.6% lower (P<0.0001) than INT PTH. In females, there was no difference between fed and non-fed groups when PTH was assessed using the INT assay however, PTH measured using the BIO was lower in the fed group versus the non-fed. In males, there was no difference between fed and non-fed groups when measured with either BIO or INT assays (**Figure 4.1**).

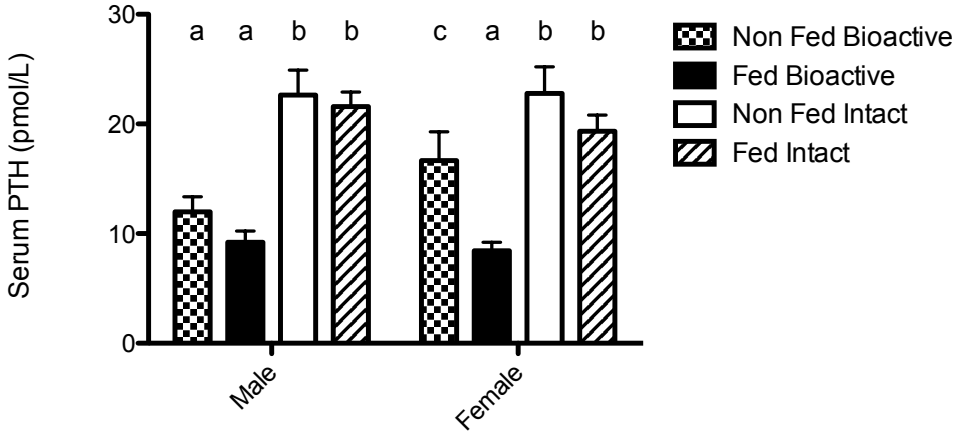

Figure 4.1. Effect of feeding in endogenous serum PTH concentration assessed using INT and BIO PTH assays in male and female rats. Differences detected using GLIMMIX model with post hoc testing using Tukey-Kramer approximation. Different letters indicate significant differences (P<.05). Values are mean ± SEM; n = 30 per sex.

4.5 Discussion

The results of the present study indicate that for the biological assessment of PTH, one should take into consideration sex, feeding state, and generation of assay (ELISA) used. The assays used in this study were similar except for the horseradish peroxidase conjugated detection antibody. The detection antibody in the INT assay binds to epitopes within the 13-34 amino acid region of the rat PTH molecule while the detection antibody in the BIO PTH assay binds to an initial amino terminal epitope of amino acids 1-3. Generally, the second-generation INT PTH assay reacts with both the PTH (1–84) and the shorter PTH fragments notably PTH (7-84), which, in a group of renal transplant patients, was reported to account for 44.1% of total plasma PTH [245].

Evidence suggests that PTH (7-84) fragments can lead to skeletal resistance to PTH and inhibit bone resorption and thus display an antagonistic action to PTH (1–84) [245, 246]. Moreover, PTH (7-84) fragments have a half-life of ~10 minutes versus ~4 minutes for PTH (1-84) [247]. This could explain in part the higher PTH values measured by the INT PTH assay compared to the BIO PTH assay could reflect the sum of agonist and slow degrading antagonist fragments of PTH. These results are in agreement with Inaba et al. where the mean concentrations measured by the BIO PTH and INT PTH assays in healthy Japanese men and women were 62% higher in the INT assay, indicating that the PTH fragments account for ~40% of circulating PTH immunoreactivity [248].

Thus, the results obtained with the second-generation INT assay may not accurately reflect parathyroid function or serum PTH concentration.

Nutritional intake and extended fasting periods (33 to 96 hours) can modulate the circadian rhythm of PTH [249, 250]. Additionally, plasma phosphate (PO_4) and iCa are known to be correlated with PTH [250-255], which emphasizes the importance of meal times, type of food ingested and the resulting flux of PO_4 and iCa on PTH. Serum iCa, PO_4 and $1,25(OH)_2D_3$ are known to regulate serum PTH, and in turn are regulated by changes in PTH [256, 257]. An increase in iCa mediates a decrease in PTH secretion within seconds of being detected by calcium receptors and this is accompanied by the breakdown of circulating PTH over the following hours [258]. PO_4 independently increases PTH synthesis and secretion via a post-transcriptional mechanism occurring over a span of hours [259, 260], while $1,25(OH)_2D_3$ decreases PTH via inhibition of PTH gene expression [261]. This study has demonstrated that being in a non-fed state for 8 to 10 h is sufficient to decrease iCa, whereas the increase in PTH values true for the BIO PTH assay only in females. It is possible that the already lower values for BIO PTH in some of the dietary groups of males but not females from the original study [63], precluded detection of differences in INT and BIO PTH between feeding states in males. Nonetheless, BIO PTH values for rats in the control diet were not different between feeding states (Fed 9.13±1.715 vs. non-fed 9.82 ± 2.34 pmol/L, P=0.81); the same was true for INT PTH (Fed 22.12±2.28 vs. non-fed

21.71±4.55 pmol/L, P=0.97). In addition the iCa values for males were higher than females whereas BIO PTH was lower.

Overall our work contrasts that of Ruh et al. who found that in male and female monkeys, withdrawing food increases INT PTH [244]. The animals' food was removed for 7 hours and collected the blood samples in the late afternoon, which would also reflect circadian rhythm near the end of the expected feeding period. One of the reasons for the discrepancy might relate to the use of isoflurane anesthesia in rats, which is known to decrease iCa and increase INT PTH after induction of anesthesia and peaking after 30 minutes of exposure [262]. However, sampling in the rats was rapid within 10 to 15 min total. In summary, the present study suggests that the INT assay could mask the changes in PTH seen with sex and feeding states, at least in rats.

The assessment of PTH is difficult due to its complex metabolic and structural properties. Mallette first proposed that PTH could be considered as a "polyhormone" based on the multiple biological actions of single peptide regions of the PTH molecule [263]. This theory continues to be verified with ongoing discoveries of different forms of circulating PTH [264-266]. This reinforces the importance of careful selection of PTH assays depending on the purpose of the study. As demonstrated in this study, 2nd generation assays yield higher values than 3rd generation assays and importantly, these appear to be unable to differentiate differences incurred based on sex. Future studies related to PTH should consider standardization of fast duration, sex effects, and measure BIO PTH.

4.6 Acknowledgements

The work was conducted at the University of Manitoba facilities and the associated Manitoba Institute of Child Health. The authors acknowledge the assistance with feeding and some sample collection and measurement by S. Fitzpatrick, M. Kaur, R. Mollard and A. Dick. The authors have no conflict of interest.

Bridge statement II

The assessment of PTH is complex since it is affected by many behavioral (eating and sleeping patterns), environmental (ultraviolet light exposure via vitamin D production) and physiological (ionized calcium) factors. Additionally, there are different types of PTH assays to consider, which can yield different results. The pre-clinical results obtained in chapter 4 add additional evidence of methodological considerations for future clinical trials seeking to investigate the mechanisms of CLA on bone and biomarkers of bone metabolism. First, fasting leads to significant increases in BIO PTH in female, but not male rats, which was not observed when PTH was assessed using INT PTH. This suggests that future studies designed to assess dietary effects on PTH should consider standardization of fast duration, sex effects, and preferentially measure BIO PTH. As such, the results described in the next chapter are based on both BIO and INT PTH following dietary supplementation with CLA in an RCT framework in healthy men.

Chapter 5

Manuscript 3

Conjugated linoleic acid is related to bone mineral density, but does not affect parathyroid hormone in men

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Published in: Nutrition Research 2012, 32: 911-920

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5.1 Abstract

The relationship between CLA status, bone, body composition, and the effect of CLA on calciotropic hormones are unclear. A cross-sectional study was designed to examine the association between c9, t11 CLA status in erythrocyte membranes (RBC) and body composition. This preceded a dose-response trial investigating if c9, t11 CLA affected parathyroid hormone (PTH). Fifty-four men (19 – 53 y) were included in the cross-sectional analysis of which, 31 were studied in the dose response trial and randomized to 1 of 3 groups: placebo (n=10) or 1.5 g/d (n=11) or 3.0 g/d (n=10) of c9, t11 CLA for 16 wk. Men with RBC c9, t11 CLA status above the median had higher whole body bone mineral density (BMD) (1.359±0.024 vs. 1.287±0.023 g/cm^2; P=0.04) and whole body lean mass % (WBL) (78.8±0.9 vs. 75.3±1.0 %; P=0.01), whereas body mass index (BMI) (24.8±0.5 vs. 27.3±0.9 kg/m^2; P=0.01) and whole body fat mass % (WBF) (17.3±0.9 vs. 21.3±1.1 %; P=0.007) were lower. In regression analysis, RBC c9, t11 CLA status accounted for a significant proportion (r^2=0.10) of the variation in whole body BMD (P=0.03). There were no time or treatment differences among any bone or biomarkers of bone metabolism including PTH. These findings indicate that RBC c9, t11 CLA status, a reflection of long-term (~ 4 mo) dietary CLA intake, positively relates to BMD. However, c9, t11 CLA supplementation does not appear to affect PTH in healthy men.

5.2 Introduction

Conjugated linoleic acid (CLA), specifically the *cis*-9, *trans*-11 (c9, t11) and *trans*-10, *cis*-12 (t10, c12) CLA isomers, is a commercially available nutraceutical marketed to promote weight loss or improve body composition. In addition to its positive effects on adipose [36] and lean mass in humans [267], CLA supplementation increases whole body ash in mice [180, 181]. In growing mice and chicks, CLA results in greater amounts of trabecular and cortical bone [182, 223, 268]. There are few studies that have examined the relationship between dietary CLA and bone in humans. A cross-sectional analysis in postmenopausal women suggested that dietary CLA was positively related to bone mineral density (BMD) of the hip, specifically Ward's triangle [69]. However, two RCT in adults and children show mixed results. In middle-aged overweight (body mass index (BMI): 25-30 kg/m^2) men and women supplemented for 24 mo with either 1:1 mix (c9, t11 and t10, c12) CLA (3.4 g/d) in free fatty acid (FFA) form or triacylglycerol (TG) form, a significant positive change in whole body bone mineral content (BMC) from 12 to 24 mo was found in the FFA group compared to TG and placebo groups [42]. However, no differences in BMC and BMD were observed in children (6-10 y), after a 7 mo trial of 1:1 mix (c9, t11 and t10, c12) CLA (3.0 g/d) in TG form or a placebo [269]. These studies suggest that dietary and supplemental CLA in FFA form may be associated to bone measurements in humans.

There is evidence that PTH is responsive to CLA in humans and rats and this might explain the various responses in bone. In primigravidas with a family history of preeclampsia supplemented with calcium (600 mg/d) and 1:1 mix (c9, t11 and t10, c12) CLA (450 mg/d) from wk 18 to wk 22 of pregnancy until delivery, PTH was reduced by 8.0% but this change was not significant [195]. Furthermore, the c9, t11 CLA isomer decreased PTH by 40% in both healthy [63] and polycystic kidney diseased [194] male rats. In line with average values for PTH being in the normal range for each rat model, no changes in BMC or BMD were observed [16-17]. Chronic high PTH concentrations are known to promote bone loss whereas intermittent or pulsatile PTH administration increases bone formation, BMD and reduce osteoporosis-related fractures [191]. Enhanced BMD in human trials of calcium supplementation are ascribed to reduced PTH levels [241, 242]. Therefore, in order to clarify the relationship between dietary CLA and BMD, it is essential to assess PTH response following CLA supplementation. The study was conducted in men since c9, t11 CLA reduced PTH in male but not female rats [63]. The objectives of this study were to 1) investigate if basal c9, t11 CLA status in RBC, as assessed by gas chromatography analysis, is associated with enhanced body composition and bone mass (assessed by dual-energy X-ray absorptiometry (DXA)); and 2) to determine the response of bioactive and intact PTH concentrations, measured by chemiluminescence, to supplemental c9, t11 CLA. It was hypothesized that higher c9, t11 CLA status in RBC at baseline, which reflects long term (~4 mo) CLA intake,

will be associated with a lower fat and higher bone mass and that following a dose-response trial, PTH will be reduced by 30% in the highest c9, t11 CLA supplemental dose versus the placebo group

5.3 Materials and methods

5.3.1 Participants

Fifty-four community dwelling adult men from the greater Montreal area were studied for the cross-sectional analysis and screened for entrance criteria for the dose-response study. All study visits took place at the Mary Emily Clinical Nutrition Research Unit, School of Dietetics and Human Nutrition. Participants gave written consent prior to inclusion into the study. Participants were excluded from the dose response trial (but not for the cross-sectional analysis) if they were taking prescribed medication, had allergies to nuts or gelatin, consumed more than 14 alcoholic drinks per wk, or if they were diagnosed with diabetes, Crohn's, celiac, liver, or kidney disease. Inclusion criteria were BMI between 18.5 and 27 kg/m^2 and/or a percentage body fat less than 23 (based on predictions of percentage body fat by BMI, age, sex, and ethnicity [270]), plasma 25(OH)D concentration greater than 60 nmol/L, lumbar spine vertebrae 1-4 BMD Z-score greater than -1.0, and general health clearance by the study physician. Of the 54 men screened, 31 met the criteria and were included in the dose-response study (**Figure 5.1**). This study was

approved by the Research Ethics Boards of McGill University (IRB A01-M11-09B; ClinicalTrials.gov identifier: NCT00608400) and the supplements used for the purpose of this study received a letter of no objection by Health Canada's Natural Health Products Directorate.

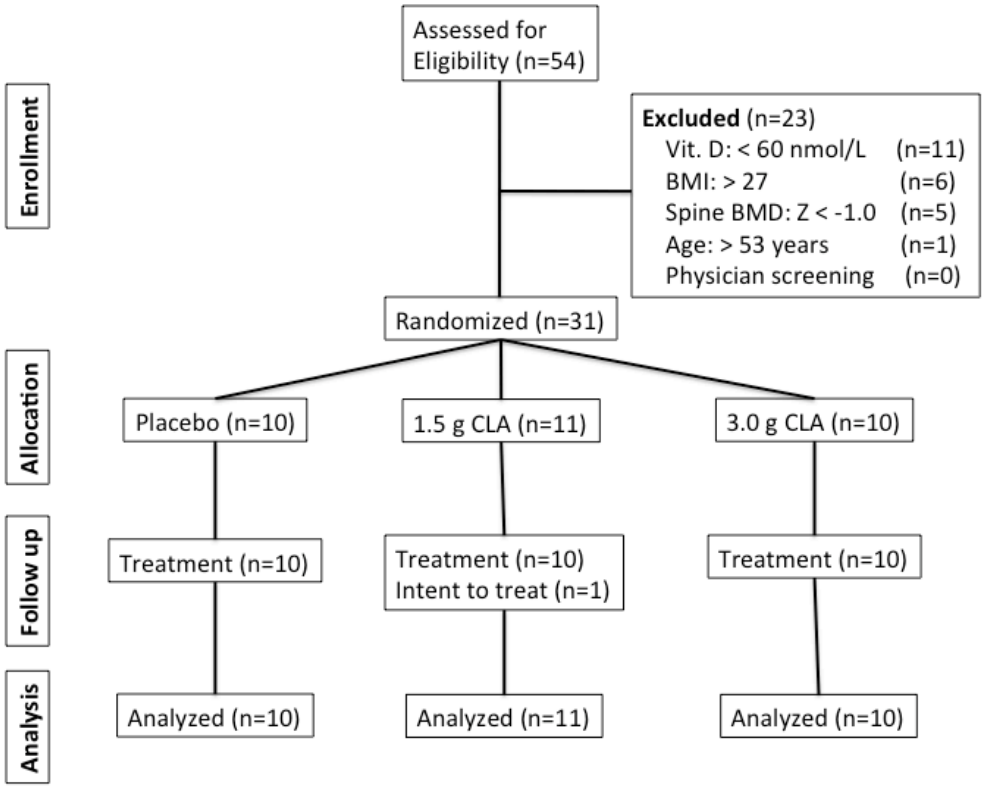

Figure 5.1. Consort diagram. One participant dropped out and was classified as intent to treat. Duration of intervention was 16 weeks.

5.3.2 Dose-response trial design

This study was a double-blind, placebo-controlled randomized clinical trial phase II. The participants were stratified by age (< 37 or > 38 y) and then randomized to 1 of 3 groups: 1) 4.2 g olive oil (placebo), n=10), 2) 2.1 g olive oil and 2.1 g (1.5 g c9, t11 CLA (1.5CLA), n=11) or 3) 4.2 g (3.0 g c9, t11 CLA (3.0CLA), n=10) as shown in **Figure 5.1**. The incomplete randomization block was started prior to the decision to stop recruiting, resulting in n=11 in the 1.5CLA group only. Olive oil was selected as the placebo since the small amount used has no significant biological activity on PTH, bone, or body composition [36, 42, 271]. The fatty acid composition of the CLA (in kind, Lipid Nutrition, Loders Croklaan B.V.) and the extra virgin olive oil (Cibaria International Inc) are shown in **Table 5.1**. Both were encapsulated in amber opaque soft gel capsules identical in taste, smell and appearance. Each participant took a total of 6 capsules per day (placebo and/or CLA capsules) with lunch (3) and dinner (3) meals to arrive at their respective dosage of CLA (0, 1.5, or 3 g/d). During the 8 wk and 16 wk visits all unused capsules were recovered for compliance assessment and a new supply was provided. Participants were defined as compliant if they took ≥ 80% of the supplements provided. Participants were asked to maintain their usual dietary and physical activity habits throughout the study.

Table 5.1 Fatty acid composition of CLA[1] and olive oil[2] capsules

	CLA		Olive Oil	
	g / 100 g	%	g / 100 g	%
C14:0	0.2	0.2	0.0	0.0
C16:0	5.4	5.6	11.9	12.2
C16:1	0.1	0.1	1.1	1.2
C18:0	1.2	1.3	2.8	2.9
C18:1	13.7	14.1	71.1	72.8
C18:2	2.5	2.6	9.2	9.5
C18:3	0.1	0.1	0.7	0.8
C18:2 (c9,t11 CLA)	60.7	60.2	0.0	0.0
C18:2 (t10,c12 CLA)	14.6	15.1	0.0	0.0
C20:0	0.1	0.1	0.3	0.3
C20:1	0.6	0.6	0.3	0.3
Total	99.3	100.0	97.6	100.0
Saturated	7.0	7.2	15.0	15.4
Monounsaturated	14.4	14.8	72.5	74.3
Polyunsaturated	77.8	78	10.0	10.3
n-3	0.1	0.1	0.7	0.8
n-6	2.5	2.6	9.2	9.5

[1] CLA supplied by Lipid Nutrition, Loders Croklaan B.V.
[2] Olive oil supplied by Cibaria International Inc.

5.3.3 Clinical assessments

Anthropometry data (height, weight, BMI, waist circumference) as well as systolic and diastolic blood pressure (SBP; DBP) and heart rate (HR) were recorded at baseline and wk 4, 8, 12, and 16. Body composition was assessed at baseline and at wk 16. All 5 visits (baseline, 4, 8, 12, and 16 wk) took place following a 12 h fast at 7:00-10:00 h during which, a blood sample (15 mL) was collected in lithium heparin and plasma separated and stored at -80 °C. RBC were washed twice with saline then stored at -80 C in an equal weight of water:methanol:BHT solution to prevent polyunsaturated fatty acid (PUFA) oxidation as described by Magnusardottir et al. [272]. Plasma was used to analyze intact (LIAISON® N-TACT® PTH assay 310660), bioactive (LIAISON® 1-84 PTH assay 310630) PTH, 25(OH)D (LIAISON® 25 OH Vitamin D Total 310600), OC (LIAISON® Osteocalcin 310950), and BAP (LIAISON® BAP Ostase® 310970) at McGill University using chemiluminescence assays (Liaison, DiaSorin, Stillwater, MN). In addition, blood was analyzed for ionized calcium (iCa) (ABL800 FLEX analyzer, Radiometer, Copenhagen, Denmark), phosphate (PO_4), and lipid profile including serum HDL cholesterol (HDL), LDL cholesterol (LDL), triacylglycerol (TG), and total cholesterol (TC) (SYNCHRON LX®20 PRO Clinical System, Beckman Coulter, Brea, CA) at the McGill University Health Centre (Montreal General Hospital, QC). The coefficient of variation (CV%) for all parameters (inter- and intra-assay) was less than 10%.

5.3.4 *Diet and physical activity*

Usual dietary intake was assessed during the screening visit by a nutritionist using an interviewer administered modified semi-quantitative food frequency questionnaire (FFQ) (Harvard/Willett [131]). The modification captured supplement use and frequency. Prior to use, the FFQ was translated to French and back translated to ensure consistency of data collection among the English and French speaking participants. The FFQ was a 131 item semi-quantitative FFQ designed to classify individuals according to their level of daily intake of multiple nutrients [131]. Questions that originally combined beef and lamb were separated to improve estimation of CLA. Furthermore, dietary assessment was performed using a 24-hour food recall at each visit plus every 2 wk by phone for a total of 9 recalls for the duration of the study. Nutrient analysis of listed food items was determined using Nutritionist Pro™ version 2.2 (Nutritionist Pro, Axxya Systems, Stafford, TX) and the 2010 Canadian Nutrient File. Estimates for CLA intake for both the FFQ and food recalls were calculated by multiplying the fat content of a food item by the concentration of CLA [127, 129, 273]. Physical activity was assessed during the screening visit using the Paffenbarger Physical Activity Questionnaire that estimates physical activity over a 12-month period and is valid in several population groups including healthy men [274-280].

5.3.5 Anthropometry and body composition

Blood pressure was measured using a standard radial cuff and sphygmomanometer and HR ((beats/15 s)X4) was assessed manually via palpation of the radial artery in a seated position after 5 min of rest. Height and weight were measured and standardized by having participants wear thin cotton pants and t-shirt without any metal. Weight was measured using a standard balance platform beam scale (Detecto, Webb City, MO), height was measured using a stadiometer (Seca 213, Birmingham, U.K.) and BMI was calculated according the Health Canada guidelines [31]. Waist circumference was measured between the lower costal margin and the iliac crest according to Health Canada guidelines [281]. In addition, even though the 16 wk study would likely not enable sufficient time to detect minimal changes in BMC and BMD we conducted the measures at both baseline and wk 16 for the purpose of safety monitoring. Whole body composition WBL, WBF and measurement of bone area, BMC and BMD of the whole body, spine vertebrae lumbar 1-4, and hip (total hip plus femoral neck) were assessed using DXA with Hologic Apex v3.2 analysis software (QDR 12.3:5, 4500A Discovery Series, Hologic Inc., Bedford, MA). The CV% for BMD, BMC and area (assessed using a spine phantom over the course of the study) was 0.325, 0.498 and 0.383% respectively.

5.3.6 *Fatty acid analyses of lipids in plasma and erythrocytes*

CLA was measured from fasting samples using a modified Bondia-Pons method [282] to determine short (plasma c9,t11 CLA) and long-term (RBC c9, t11 CLA) supplement compliance in all groups, as well as basal c9, t11 CLA status. Modifications included the use of 100 µL of plasma or 200 µL of RBC combined with 10 µL of heptadecanoic acid (internal standard) and the addition of 300 µL of hexane (600 µL for RBC) after methylation. Fatty acid methyl esters (FAME) were separated using a 100 m CP-Sil-88 capillary column (Varian-Chrompack, CP7489), installed in a Varian CP-3800 Gas Chromatograph, (Varian, Inc., Walnut Creek, CA, USA) with a flame-ionization detector. For identification and quantification of the FAME peaks, authentic standard 461 (cat # GLC-461, Nu-Chek Prep, Inc. Elysian, MN) was used. In addition, c9, t11 octadecadienoic acid and t10, c12 octadecadienoic acid (cat # UC60M & UC61M, Nu-Chek Prep, Inc. Elysian, MN) and standards of known concentration were used to calculate recovery (>90%). The fatty acids were expressed as a percentage of the total fatty acids identified from dodecanoic acid (C12:0) to docosahexaenoic acid (C22:6).

5.3.7 Adverse effects and safety monitoring

At each visit, participants were asked using a questionnaire if they experienced any illness or been treated for acute illnesses. Frequency of

headaches, dizziness, constipation, cramping, nausea, vomiting, diarrhea or abdominal pain was documented. The study physician provided safety monitoring throughout this study including objective assessment of iCa, PO_4 or PTH concentrations.

5.3.8 Statistical analyses

A sample size of 10 was calculated for an ANOVA design with 3 groups based on a standard deviation of 10.43 pg/mL [283] combined with an alpha value set at 0.05 and power at 0.80 and a detectable difference of 10.34 pg/mL from the means. Data were analyzed using SAS statistical package software version 9.2.0 (SAS Institute Inc., Cary, North Carolina, USA) and was tested for normality and homogeneity of variances prior to using a mixed model with Tukey post-hoc comparisons. A mixed model repeated measures design included, time, CLA dosage and plasma 25(OH)D concentration as a fixed effect and individual participants nested for treatment and block randomization as random effects. Pearson's correlation coefficients (r) were calculated as preliminary evaluation to determine the degree of association between plasma and RBC CLA versus body composition and bone related assessments for the cross-sectional analysis. Multiple regression models controlled for energy (kcal) and dietary protein intake (g), WBL (%), BMI (kg/m^2), and plasma 25(OH)D concentration (nmol/L); all of which are known to affect bone. Data are presented as means ± standard error of the means (SEM).

5.4 Results

5.4.1 Cross-sectional analysis of the baseline assessment

5.4.1.1 Conjugated linoleic acid status, bone and body composition

Anthropometric, body composition, and dietary measurements for all men were within healthy ranges Table 5.2. When participants were stratified on RBC CLA level above and below median RBC CLA, whole body BMD (1.359±0.024 vs. 1.287±0.023 g/cm^2; P=0.04) and WBL (78.8±0.9 vs. 75.3±1.0 %; P=0.01) were higher in the above median group, whereas BMI (24.8±0.5 vs. 27.3±0.9 kg/m^2; P=0.01) and WBF (17.3±0.9 vs. 21.3±1.1 %; P=0.007) were lower. Waist circumference and specific regions examined for BMD such as hip and spine were not different above and below the median RBC CLA.

Body composition measurements that correlated with RBC CLA included BMI (r = -0.29, P = 0.04), whole body BMD (r = 0.30, P = 0.03), WBF (r = -0.34, P = 0.01), and WBL (r = 0.32, P = 0.02). Plasma CLA was not associated with body composition measures but was associated with dietary intake of CLA (r = 0.40, P=0.05). In a regression model containing energy and dietary protein intakes, BMI, plasma 25(OH)D concentration, and RBC CLA status, the latter 2 were significant predictors of whole body BMD. The model accounted for 22.0% of the total variance in BMD where RBC CLA alone accounted for 10.0% of the total variance in whole body

BMD based on the partial correlation coefficient (**Table 5.3**). The model did not reach significance when age, waist circumference, and exercise were included (P=0.21).

5.4.2 Dose response trial

5.4.2.1 Participants and lifestyle characteristics

Of the participants enrolled, 30 (96.8%) completed the study and 1 withdrew due to new employment. Compliance with the supplements was greater than 80% for all groups. There were no significant differences among groups at baseline (**Table 5.4**) except for exercise, which was higher in the 3.0CLA compared to the 1.5CLA and placebo groups (P=0.04). Throughout the study, 5 participants reported mild gastrointestinal discomfort as related to the supplement and placebo.

5.4.2.2 Conjugated linoleic acid levels in plasma and erythrocytes

There were no differences among the plasma and RBC CLA baseline values. In plasma, CLA proportion was significantly increased in both the 1.5CLA and 3.0CLA compared to baseline for all time points except wk 16 in the 1.5CLA group (P=0.61). Furthermore, CLA proportion at wk 12 in 3.0CLA was significantly greater than all time points in the 1.5CLA and placebo groups (**Figure 5.2**). For RBC CLA, the proportion

was significantly increased from baseline at all time points in both 1.5CLA and 3.0CLA. Additionally, CLA proportion in the 3.0CLA group was significantly greater at wk 8 than all other time points of all groups except for wk 16 (**Figure 5.3**). There were no time or treatment differences for other fatty acids including linoleic acid, alpha linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid.

5.4.2.3 Vital signs and blood lipid profile

There were no differences among groups for HR (44 – 82 bpm), DBP (60 – 94 mmHg), HDL (0.77 – 2.25 mmol/L), iCa (1.03 – 1.53 mmol/L), PO_4 (0.62 – 1.56 mmol/L), TG (0.2 – 3.45 mmol/L), and TC (2.99 – 6.49 mmol/L) throughout the study. Across the study, higher values were observed in the 3.0 CLA vs. placebo groups for SBP (117.9±1.2 vs.112.5±1.0 mmHg, $P<0.0001$) and LDL (2.96±0.13 vs. 2.76±0.10 mmol/L, $P<0.0001$). Nevertheless, there was no significant change from baseline to wk 16 in any of the parameters and they were all within normal ranges throughout the study.

5.4.2.4 Anthropometry and body composition

Higher values were observed in the 3.0CLA group at all time points for weight ($P<0.0001$), waist circumference ($P<0.0001$) and BMI ($P=0.0019$) (**Table 5.4**). There were no group or time differences for WBF,

WBL, whole body BMC, whole body BMD, hip BMC, hip BMD. Spine BMD was significantly higher in the 3.0CLA group versus the 1.5CLA (P=0.05) group across the duration of the study, whereas placebo was not different from any group (**Table 5.4**). There was no significant change in any variable from baseline to wk 16.

5.4.2.5 Calciotropic hormones and biomarkers of bone metabolism

Plasma intact PTH concentration was lower (P=0.006) in the 3.0CLA group versus 1.5CLA and placebo across the study and did not change significantly over time. At baseline and wk 12, plasma bioactive PTH concentration was significantly (P=0.0002) lower in 3.0CLA versus 1.5CLA and placebo. Additionally, there was no significant group or time differences in plasma 25(OH)D, bioactive PTH, intact PTH, OC or BAP concentrations when expressed as percent change relative to baseline values (**Figure 5.4**). Plasma BAP concentration was significantly higher in the 1.5CLA group compared to 3.0CLA and placebo across the study but did not change significantly over time. There were no treatment or time differences for plasma 25(OH)D, iCa, OC and PO_4 concentrations.

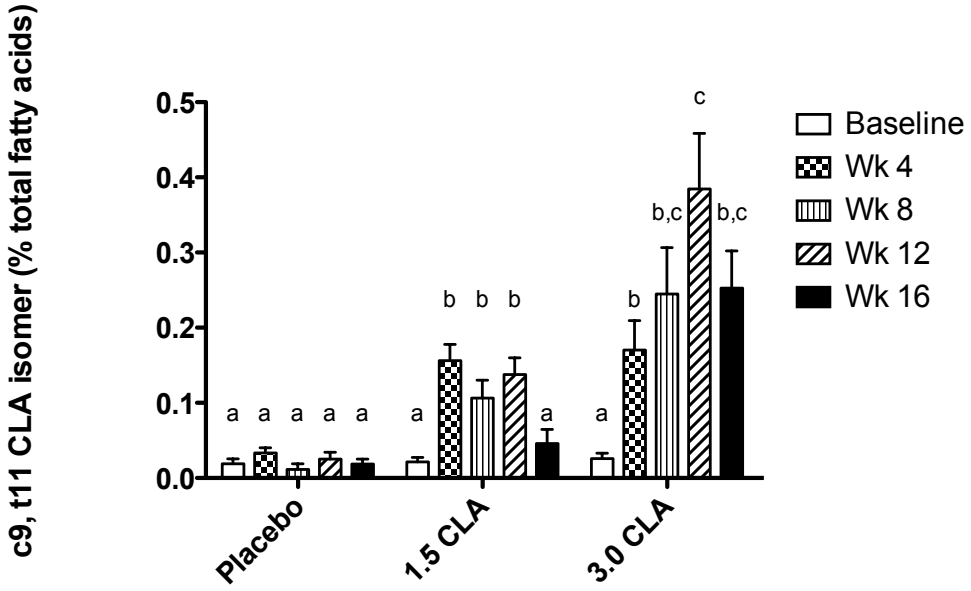

Figure 5.2. Plasma c9, t11 CLA content in dose response study. Differences detected using MIXED model with post hoc testing using Tukey's HSD test. Different letters indicate significant differences (P<0.05). Values are means ± SEM; n = 10 per diet.

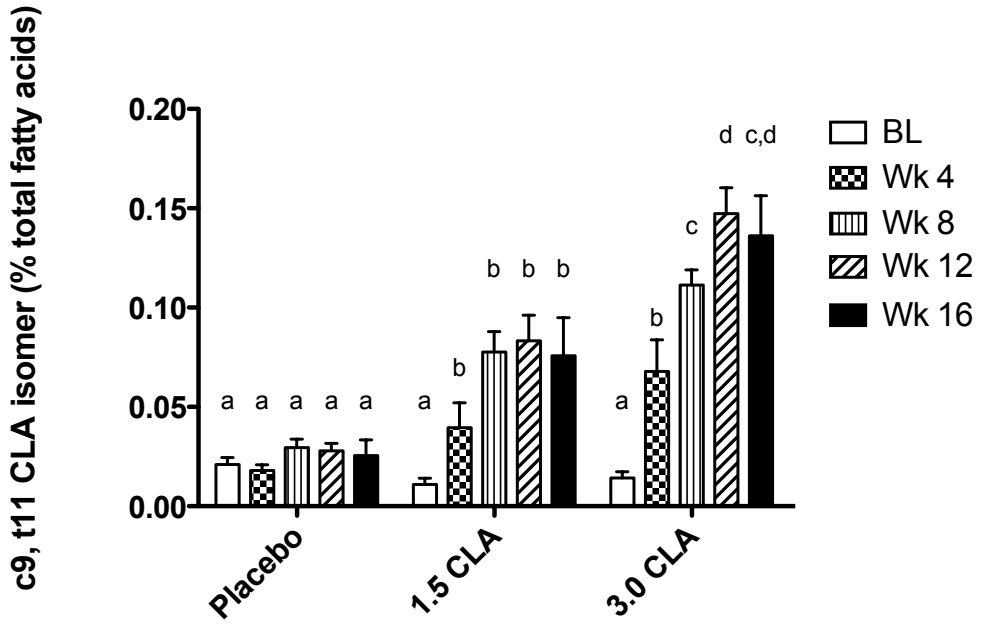

Figure 5.3. RBC c9, t11 CLA content in dose response study. Differences detected using MIXED model with post hoc testing using Tukey's HSD test. Different letters indicate significant differences ($P<0.05$). Values are means ± SEM; n = 10 per diet.

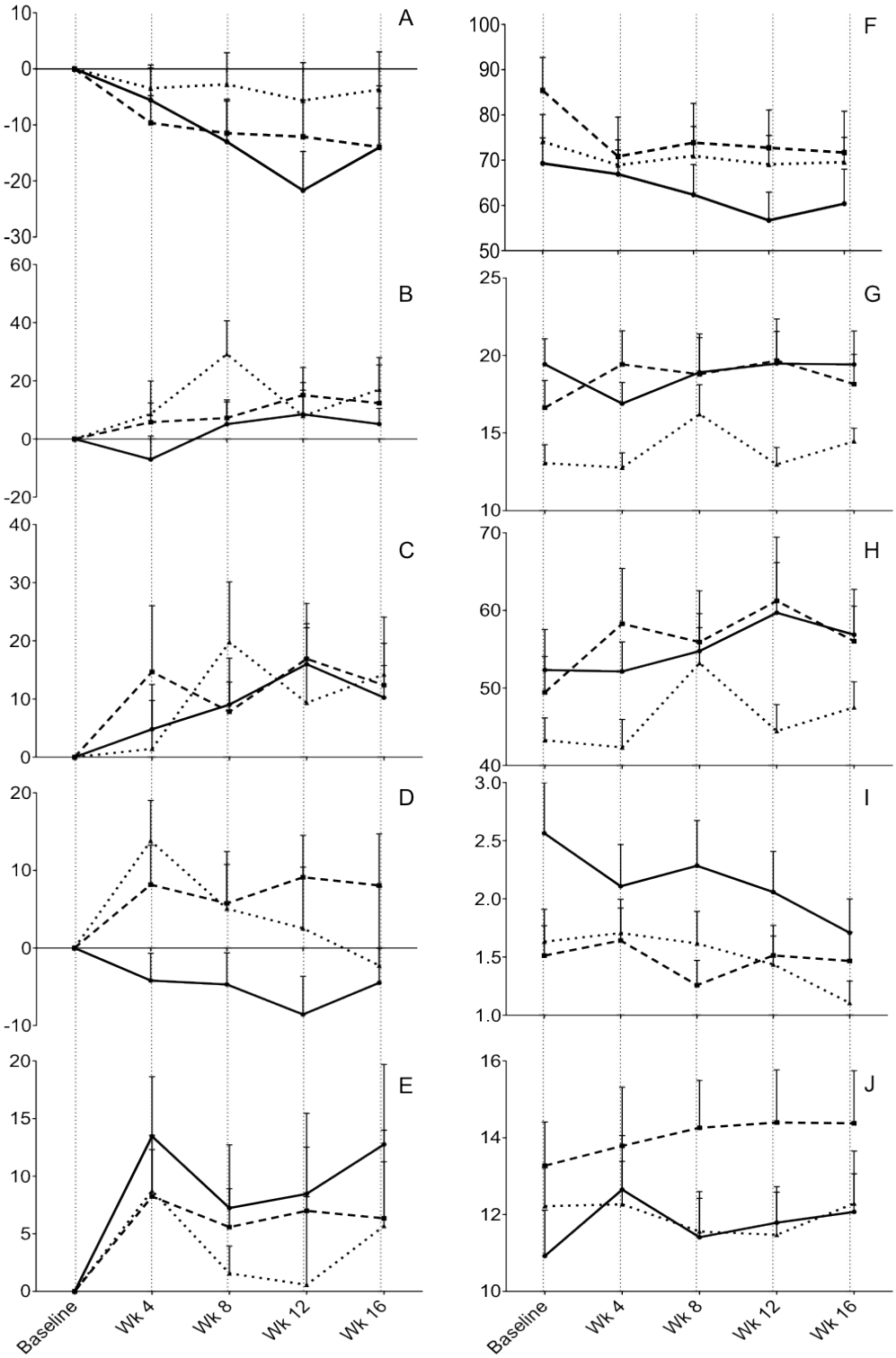

Figure 5.4. Percent change in plasma 25(OH)D (A), bioactive PTH (B), intact PTH (C), OC (D) and BAP (E) concentrations relative to baseline. Change in 25(OH)D (nmol/L) (F), bioactive PTH (ng/L) (G), intact PTH (ng/L) (H), OC (nmol/L) (I) and BAP (ug/L) (J) concentrations over time. Placebo (−), 1.5CLA (- -), 3.0CLA (···). Differences detected using MIXED model with post hoc testing using Tukey's HSD test. Values are means ± SEM; n = 10 per diet.

Table 5.2 Cross-sectional analysis baseline characteristics

Age (y)	39±10
Weight (kg)	81.7±12.3
Height (m)	1.77±0.07
BMI (kg/m2)	26.1±3.8
Waist Circumference (cm)	86.3±6.5
WB Fat (%)	19.2±5.5
WB Lean (%)	77.1±5.1
Whole Body BMD (g/cm^2)	1.323±0.017
Hip BMD (g/cm^2)	1.062±0.021
Spine BMD (g/cm^2)	1.062±0.017
Plasma 25(OH)D (nmol/L)	69.8±24.3
Exercise (kcal/wk)	2236±231
Dietary CLA (mg/d)	149.9±77.5
Dietary Calcium (mg/d)	1176±415
Total Vitamin D[1] (µg)	6.00±2.22
Alcohol (g/d)	9.0±4.3

Data are presented as means ± SEM, n = 54.
[1]Total vitamin D includes both dietary and supplemental vitamin D intake.

Table 5.3 Multiple regression model for whole body BMD

Dependent Variable	Explanatory Variables	t Ratio	P-Value	Partial r^2	Model r^2 adjusted
Whole Body BMD (g/cm^2)	Energy (kcal)	1.47	0.15	0.05	0.22
	Dietary Protein (g)	-1.32	0.19	0.04	P=0.03
	BMI (kg/m^2)	1.10	0.27	0.03	
	Plasma 25(OH)D (nmol/L)	2.31	0.03	0.10	
	RBC c9, t11 CLA (% total FA)	2.23	0.03	0.10	

Table 5.4 Dose response characteristics

	Time (wk)	Placebo (n=10)	1.5 g CLA (n=11)	3.0 g CLA (n=10)
Anthropometry				
Age (y)	0	36±3	39±3	35±4
	16	36±3	39±3	35±4
Weight (kg)	0	79.6±2.6[b]	77.4±2.0[b]	83.8±1.5[a]
	16	79.5±2.5[b]	76.5±2.1[b]	84.3±1.4[a]
Height (m)	0	1.81±0.03	1.77±0.02	1.80±0.02
	16	1.81±0.03	1.77±0.02	1.80±0.02
BMI (kg/m^2)	0	24.3±0.8	24.7±0.6	25.8±0.7
	16	24.3±0.8	24.4±0.7	26.0±0.8
Waist Circumference (cm)	0	84.9±1.8[b]	85.9±2.1[b]	88.1±2.1[a]
	16	84.7±1.8[b]	86.4±1.7[b]	89.0±1.6[a]
Body Composition				
WBF (%)	0	17.0±2.0	18.9±1.4	16.6±1.2
	16	17.7±2.0	18.7±1.6	17.1±1.1
WBL (%)	0	79.0±1.9	77.2±1.3	79±5±1.1
	16	77.9±2.0	77.3±1.4	79.0±1.0
Whole Body BMD (g/cm^2)	0	1.346±0.040	1.326±0.033	1.409±0.032
	16	1.349±0.039	1.331±0.032	1.407±0.028
Hip BMD (g/cm^2)	0	1.072±0.140	1.074±0.080	1.148±0.140
	16	1.081±0.143	1.080±0.087	1.143±0.140
Spine BMD (g/cm^2)	0	1.094±0.112[a,b]	1.063±0.107[b]	1.143±0.090[a]
	16	1.086±0.123[a,b]	1.062±0.101[b]	1.143±0.079[a]
Biochemistry				
Plasma 25(OH)D (nmol/L)	0	69.3±6.0	85.4±7.3	74.1±5.7
	16	60.4±7.6	71.7±9.1	70.0±5.5
Dietary Intake[1]				
CLA (mg/d)	0	130.0±14.4	124.0±15.4	155.0±22.6
	16	N/A	N/A	N/A
Calcium (mg/d)	0	1318±122.5	924.2±104.6	1108±139.6
	16	N/A	N/A	N/A
Total Vitamin D[2] (µg)	0	11.26±1.87	11.25±3.35	5.47±1.49
	16	N/A	N/A	N/A
Alcohol (g/d)	0	10.3±3.9	14.2±5.5	15.4±4.4
	16	N/A	N/A	N/A
Exercise				
Energy expenditure (kcal/wk)	0	1543±295[a]	2163±315[a]	2940±466[b]
	16	N/A	N/A	N/A

Data are represented as means ± SEM, total n = 31. Differences detected using MIXED model with post hoc testing using Tukey's HSD test. Different letters indicate significant differences ($P<0.05$).

[1] Dietary intake assessed using FFQ.

[2] Total vitamin D includes both dietary and supplemental vitamin D intake.

5.5 Discussion

This is the first study to demonstrate a relationship between long-term CLA status (based on diet) and body composition assessments (WBF, WBL, BMI) in adult men. Rosell et al. also found an inverse association between BMI and dairy intake of CLA in 19,352 Swedish women aged 40-55 y studied over 9 y. The authors argued that dietary CLA could be partly responsible for this relationship, however, when adjusted for CLA intake, dairy intake still remained a significant covariate in the model [284]. In the present cross-sectional analysis, RBC CLA was also positively associated with whole body BMD. In a multiple regression model accounting for important confounders known to affect BMD including plasma 25(OH)D concentration, RBC CLA accounted for 10.0% of the total variance in whole body BMD. This is in agreement with a cross-sectional analysis in postmenopausal women (n=136; 68.6±7.1 y) by Brownbill et al. who documented an association between dietary CLA (63.1±46.8 mg/d) and Ward's triangle BMD (r = 0.04) [69]. Thus, we accept our first hypothesis where dietary CLA status appears to be associated with enhanced body composition and BMD.

This study is also the first to assess the effects of CLA on PTH in healthy men. c9, t11 CLA did not appear to significantly affect intact or bioactive PTH over 16 wk of supplementation, therefore we reject our second hypothesis. All men had low normal healthy concentrations of bioactive PTH ranging from 7.2 to 34.0 pg/mL throughout the study, which

may have diminished our ability to document further reduction following CLA supplementation. These results do not agree with observations in rats where a 36.1% reduction in bioactive PTH was observed in males fed a diet of 0.5% c9, t11 CLA after 16 wk of supplementation [63]. A study in rats with PKD demonstrated that a 1% CLA supplementation resulted in a 60% attenuation in PTH in PKD and non-PKD rats [194]. Notably, in both these rat studies, CLA intake expressed as a percentage of total fat intake ranged from 6.0 and 14.3% whereas in this study, CLA intake represented 0.17% of total fat intake. Moreover, bioactive PTH was greater (>100 pg/mL) than in the healthy men. Hence, greater CLA intake and physiological values could allow for a greater decrease as seen with the CLA supplementation in the rats. Furthermore, chronic high PTH concentrations are known to promote bone loss whereas intermittent or pulsatile PTH secretion or administration increases BMD [191]. Reductions in PTH have been observed in humans studies where the participants were fed or supplemented with calcium and it was concluded that greater calcium absorption combined with lower PTH could support higher BMD [241, 242]. A randomized, double-blind, placebo-controlled trial that included 48 healthy primigravidas with a family history of preeclampsia and with diastolic notch were supplemented with daily oral doses of calcium carbonate (1484 mg) and 450 mg of CLA (1:1 c9, t11 to t10, c12) or a lactose-starch placebo from wk 18 to wk 22 of gestation until delivery. Bioactive PTH (3.7 – 54.7 pg/mL) was decreased by 8.0% percent in the calcium-CLA group from baseline to post-supplementation

although these results did not reach significance [195]. Hence, CLA combined with calcium supplementation could potentially decrease PTH given elevated physiological concentrations.

This dose response trial did not observe any differences in measurements of bone or biomarkers of bone metabolism. This is in agreement with a study by Gaullier et al. who supplemented obese (BMI: 32-35 kg/m^2) men (n=21) and women (n=84) with 4.5 g CLA (3.4 g CLA, 37.5% c9, t11, 38% t10, c12) for 6 months and found no changes in BMC between or within groups [156]. Also, in a study with overweight and obese (BMI percentile: 94.6±3.2 – 96.1±2.8) children (8.1±0.6 – 9.3±0.8 y) supplemented with 3.0 g of CLA (1:1 c9, t11 to t10, c12) for 7 months resulted in no change in whole body BMC accretion in the CLA group [269]. Moreover, a double-blind, placebo-controlled trial by Doyle et al. in which 60 healthy adult males (39-64 y) were randomly assigned to receive 3.0 g/d CLA (1:1 c9, t11 to t10, c12) for 8 wk found no significant differences in OC, BAP, and several biomarkers of bone resorption or on serum or urinary calcium levels, which is consistent with our findings [70]. This study assessed BMC and BMD in healthy men over a relatively short period of time (16 wk) therefore significant changes were not expected.

We also did not observe any significant differences in body composition over time and among groups. This is in agreement with Zambell et al. who supplemented 17 healthy women (BMI < 25) with 3.0 g/d of CLA (22.6% t10, c12, 17.6% c9, t11 isomer) for 64 days and reported no change in weight and percent body fat [285] and Petridou et

al. who gave 16 young healthy sedentary women 2.1 g/d of CLA (mixed isomers) for 45 d and found no differences in body fat (measured by skinfold thickness at 10 sites) [286]. However, Gaullier et al. showed that BW, WBF and BMI were reduced in middle-aged men and women (BMI 25-30 kg/m^2) after 24 mo of CLA supplementation. It is possible that the longer duration of 24 mo, higher dosage of CLA (4.5 g) and higher baseline BMI were needed to see changes in body composition. Two other studies using higher dosages (3.6 – 4.2 g/d) of CLA for shorter durations (6 -12 wk) in healthy men and women resulted in decreased WBF [37, 287]. Thus, it is likely a dosage of 3.0 g/d CLA was lower than required to observe changes in body composition within a 16 wk timeframe.

The effect of CLA on lipid metabolism has been studied in many clinical trials, often as safety parameters, the majority of which do not report any significant differences in blood total, HDL- and LDL-cholesterol, and triacylglycerol which is congruent with the present results [286-289]. However, Smedman et al. reported a significant increase in LDL-cholesterol compared to baseline but not to placebo after 12 wk of CLA supplementation (4.2 g/day) in 53 healthy subjects [37]. HDL-cholesterol decreased relative to baseline in some studies [35, 290, 291], nevertheless, the changes in HDL-cholesterol were all within normal healthy population range and did not have any clinical significance. Triacylglycerol levels were increased in a study by Whigham et al., however, this change was also considered within the normal healthy range [155].

The different outcomes for body composition, bone and lipid profile among the CLA intake studies may be explained by differences in gender [292], study duration [212] isomeric specificity [293], and intake source (dietary vs. supplemental) of CLA [288, 293]. The study most similar to ours in terms gender and CLA supplementation showed similar body composition and blood lipid outcomes [294]. Animal studies have demonstrated that CLA isomers incorporate in multiple tissues including bone (cortical, marrow and periosteum), brain, heart, liver, skeletal muscle, serum and spleen leading to their involvement in the metabolism of these tissues [60, 295]. Despite no differences in the quantified bone biomarkers and an observed relationship between RBC CLA and BMD, this suggests CLA affects other factors, most likely cell derived mediators of inflammation such as eicosanoids and cytokines [56, 58, 59]. Limitations of our study include the small number of participants and issues with outcome variables (weight, BMI, intact and bioactive PTH, exercise) being significantly different among groups at baseline and across the study. However, this study demonstrated an adequate method for estimating dietary CLA intake based on its positive association with plasma CLA content.

In summary, we demonstrated an association between RBC CLA and body composition and bone-related outcomes but did not show an effect of CLA supplementation on PTH. Based on other studies where PTH was reduced by CLA in humans (trend) [195] and rodents [63, 194], future studies should consider a larger scale study of the effects of CLA in

older individuals with elevated PTH or in patients with secondary hyperparathyroidism or polycystic kidney disease (PKD).

5.6 Acknowledgements

This study was funded by Dairy Farmers of Canada and the CLA provided *in kind* by Lipid Nutrition. The work was conducted at McGill University facilities and the associated Mary Emily Clinical Nutrition Research Unit. The authors acknowledge the financial assistance of the Elizabeth and Andre Rossinger Fellowship in Canadian Rural Sustainability (JD), the Canada Research Chairs (CRC) program (HW), the Canada Foundation for Innovation (CFI) for funded infrastructure and the assistance of D. Blank, W. Parsons, S. Agellon, I. Mak, and S. Dell'Elce. Dr. Gustavo Duque holds a grant from the National Health and Medical Research Council of Australia (Grant # 632767). The authors have no conflict of interest.

Bridge statement III

The clinical results obtained in chapter 5 demonstrated that dietary CLA is positively associated with lean mass percentage and bone density in a cross-sectional design, but supplemental c9, t11 CLA in an dose-response RCT did not affect PTH in men suggesting that CLA affects the musculoskeletal system via other physiological pathway, which potentially include the inhibition of proinflammatory cytokine release, which are known to negatively affect bone and muscle. Proinflammatory cytokine production increases with age, which is in part caused by a decrease in estrogen and androgen hormone concentrations observed in aging. The following chapter described research designed to test whether aging is associated with declines in muscle as a function of inflammatory cytokines using a model of advanced aging. The potential for CLA to reduce inflammatory cytokines and thus help preserve muscle mass was tested.

Chapter 6

Manuscript 4

Conjugated linoleic acid prevents bone loss but does not affect muscle protein synthesis in an orchidectomized middle-aged guinea pig model of advanced aging

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6.1 Abstract

Age-related musculoskeletal diseases display decreased bone and muscle mass in part due to reduced anabolic hormonal stimulus and development of catabolic inflammatory responses. The objective of this study is to determine if conjugated linoleic acid (CLA) can attenuate the catabolic effects of orchidectomy-induced aging in bone and muscle. Male guinea pigs (n=40; 70 – 72 wk; pigmented), were block randomized on weight into 4 groups: 1) SHAM+Control diet, 2) SHAM+ CLA diet, 3) Orchidectomy (ORX)+Control diet, 4) ORX+CLA diet. Body composition and blood sample measurements were performed at wk 0, 2, 4, 8 and 16. At wk 16 only, protein fractional synthesis rate, bone microarchitecture and strength were assessed. The metaphysis femur and tibia had significant increases in Tb.Sp in the ORX CTRL but not ORX CLA diet group and significant decreases in vBMD, Tb.N and Conn.D in the ORX groups compared to SHAM CTRL group. The mid-diaphysis of the femur had decreased porosity in the ORX CLA but not in the ORX CTRL diet group. ORX decreased free testosterone (wk 0: 0.14±0.02 vs. wk 16: 0.06±0.01 nmol/L, $P<0.0001$) whereas interleukin-6 increased (wk 0: 0.014±0.001 vs. wk 16: 0.089±0.006 pmol/L, $P<0.0001$). ORX decreased trabecular bone in the metaphysis and increased intracortical porosity in the bone; however, no differences in bone strength, sarcoplasmic and myofibrillar protein fractional synthesis rate were detected. This model achieved the desired effect of creating a catabolic environment similar to aging, and positively responded to CLA in terms of ameliorating bone loss.

6.2 Introduction

Osteoporosis and sarcopenia are age-related musculoskeletal diseases of decreased bone and muscle mass and strength leading to impaired physical function, increased fracture risk, decreased quality of life and consequently in some cases death [15]. Epidemiological studies demonstrate that the typical age-related increase in fractures seen in women was also prevalent in men where 13 to 20% of men over age 50 will experience an osteoporotic fracture in their lifetime [10, 296] and approximately one third of all hip fractures occur in men [297]. Men also have higher rates of one-year mortality following a hip fracture compared to women (31–38% for men versus 12–28% for women) [298, 299].

Prevalence of sarcopenia ranges from 13 to 24% in persons aged 65 to 70 y and was over 50% for those older than 80 y [7]. Moreover, the prevalence is higher for men over 75 y (58%) than for women (45%) [7]. It is estimated that in men over the age of 50 decreased whole body muscle mass and bone mineral content (BMC) progress at a rate ranging between 1 to 2% per year [11-14]. These age-related declines in bone and muscle are partly due to a withdrawal of anabolic hormonal stimulus (testosterone and estrogen) and a development of a catabolic inflammatory state [25].

Androgens are recognized as playing major roles in the growth and the maintenance of muscle and as well as in trabecular and cortical bone mass in men [179, 300]. Aging is linked with a decline of up to 50% in free testosterone [26, 28] and estradiol [301] and an increase of 124% of sex

hormone binding globulin, inhibiting the biological activity of testosterone and estradiol [301]. Moreover, estrogen and testosterone are shown to inhibit the production of the inflammatory interleukin 6 (IL-6) which is linked to decreases in muscle mass and BMD [170-172]. Hence, with age, a decrease in these hormones potentially has a direct and indirect catabolic effect on the musculoskeletal system. To counter these consequences of aging, pharmaceutical approaches are still predominant, however, benefits from certain foods such as CLA are emerging.

CLA has been shown to positively affect lean mass [74], muscle mass [73], bone mineral content (BMC), bone mineral density (BMD) [73], and bone microarchitecture in young male [182] and middle-aged female mice [73]. Furthermore, CLA decreased IL-6 by up to 20% in the middle-aged females [73]. Moreover, male and female mice with induced colon cancer supplemented with CLA had preserved gastrocnemius muscle [302]. In young adult male rats, a CLA mixture elevated protein fractional synthesis rate (FSR) in gastrocnemius and soleus muscles with no change to total lean body mass [303]. In humans (18-45 y), CLA supplementation (CLA mixture, 5 g/d) for 7 wk during resistance training reduced the catabolic effects of exercise on muscle as indicated by no change in 3-methylhistadine excretion while in the control group values it increased by 92% [72]. Whether CLA could blunt muscle and bone loss of aging in males has not been thoroughly addressed and warrants further investigation.

Despite the current evidence showing the benefits of CLA and its isomers on muscle and bone, the studies used primarily female murine models. Since these animals are constantly growing, this may not reflect the hormonal changes seen with aging in the human. Hence, to account for these limitations, it is proposed to use a guinea pig model of accelerated aging. Specifically, mature males beyond age of peak bone mass would undergo an orchidectomy. The objective of this study is to determine if 16 wk of dietary c9, c11 CLA following orchidectomy-induced aging will prevent muscle and bone catabolism by counteracting an androgen withdrawal and proinflammatory response. A guinea pig model was chosen since it is a good model for studies involving lipid research due to the fact that their blood lipid profiles and responses to dietary lipid interventions are similar to those of humans [304].

The hypothesis is that CLA will prevent age-related losses in bone density and muscle mass by reducing the effects of the withdrawal of anabolic stimuli (testosterone) and by reducing the catabolic effect of increased systemic inflammation (IL-6). Furthermore, the changes in bone and muscle will be related as a function of musculoskeletal interactions.

6.3 Methods and materials

6.3.1 Study protocol

Male, retired breeder guinea pigs (n=40; 70 – 72 wk; pigmented) were obtained from Elm Hill Laboratories (Boston, USA) and housed singly. The room temperature was maintained at 21-22 °C and a relative humidity of 50% with a lighting cycle from 0600 to 1800 h. Seven days prior to the introduction to the experimental diets, the guinea pigs underwent DXA measurements and a blood sample was collected (baseline). The animals were block randomized by weight into 4 groups: 1) SHAM+Control diet, 2) SHAM+ CLA diet, 3) Orchidectomy (ORX)+Control diet, 4) ORX+CLA diet and underwent their respective surgical intervention and were allowed 3 d of recovery prior to the start of the diet intervention. Subsequent DXA measurements and blood sampling were performed at wk 2, 4, 8 and 16. At wk 16 protein fractional synthesis rate was studied by administration of a flooding dose of L-[ring-$^{2}H_{5}$]-phenylalanine with serial blood sampling and tissue sampling. Fifty minutes following the administration of the flooding dose, and while anaesthetized with AErrane isoflurane gas (Baxter Inc., Mississauga, ON, Canada) blood and left quadriceps were sampled and the muscle was snap frozen in liquid nitrogen (**Figure 6.1**). The guinea pigs were terminated by exsanguination. Ethical approval was obtained from the McGill University Animal Care Committee, and all practices were in

accordance with the guidelines of the Canadian Council on Animal Care [227].

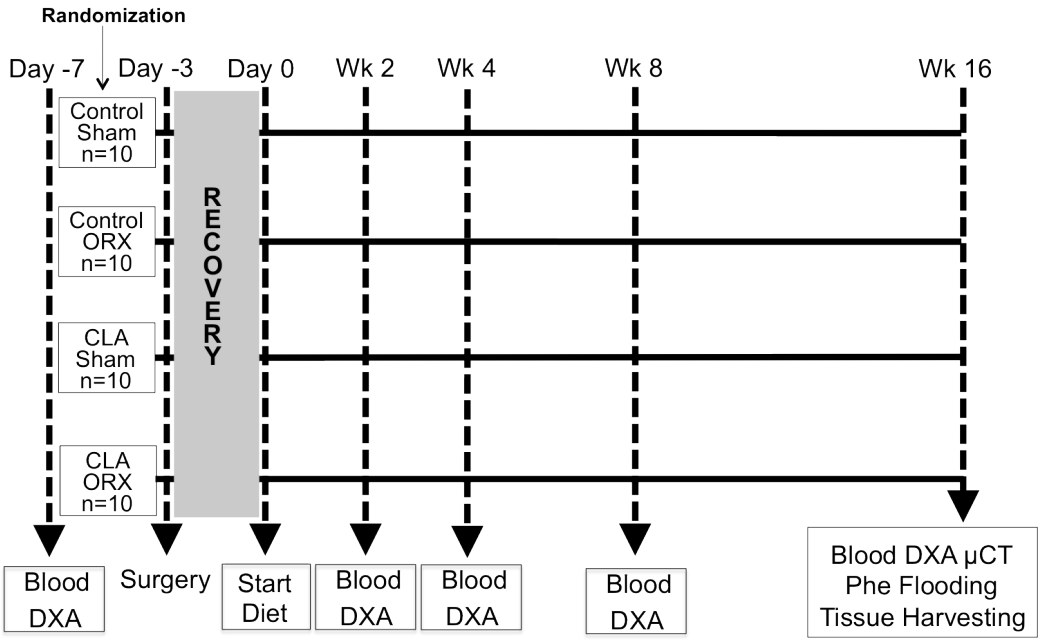

Figure 6.1. Diagram of study protocol. Control, control diet; sham, sham surgery, ORX, orchidectomy surgery; CLA, 1% conjugated linoleic acid diet; blood, blood sample; DXA, dual-energy x-ray absorptiometry; µCT, micro computed tomography; Phe flooding, flooding dose of L-[ring-$^{2}H_{5}$]-phenylalanine stable isotope tracer.

6.3.2 Experimental diets

Prior to randomization into their respective groups and at the baseline measurement, the guinea pigs were fed ad libitum a high fiber diet, since there is a requirement for additional fiber (cellulose) for better condition and performance of these animals when in stressful situations [305] (product 2041, Teklad Diets, Harlan, Madison, WI, USA). Three days following surgery, the guinea pigs received ad libitum either the control diet (product 5SUS, TestDiet, Richmond, IN, USA) or the control diet where 1% of soybean oil was substituted with 1% CLA (product 5ALC, TestDiet, Richmond, IN, USA). The isomeric composition of the CLA oil (Lipid Nutrition, Loders Croklaan, Channahon, IL, USA) was 4:1 c9, t11:t10, c12 with negligible quantities of other fatty acids. Both diets were in pellet form, isoenergetic (**Table 6.1**) and stored at 4°C. Fresh deionized water was provided ad libitum daily. The control and CLA diets were considered representative for the adult guinea pig based on requirements from the National Research Council [306]. The guinea pigs were adapted to their study diets over the course of 2 days by mixing (1:1) the in-house diet with the control or experimental diet. They continued to consume the study diets for the duration of the study. Diet consumption was monitored daily for the first 14 days post surgery. Subsequently, food intake was monitored every 3 days by weighing the remaining food from the previous feeding day.

6.3.3 *Surgical intervention*

Prior to orchidectomy, all guinea pigs had their abdomens and scrotums shaved. Anesthesia was induced in a chamber with 5% isoflurane and followed by 2% maintenance using a cone mask. The guinea pigs were positioned in dorsal recumbency and a prepubic skin incision to show the linea alba. The guinea pigs in the sham groups had the same skin incision made, but then closed with tissue adhesive and returned to their cages for recovery. The guinea pigs in the orchidectomy groups had a further incision performed through the linea alba and the testes were expressed from the scrotal sac through the abdominal incision. The testes, epididymis, fat and gubernaculum were exteriorized and excised including ligation of the blood vessels and vas deferens. The peritoneal cavity was closed and the skin incision was closed with tissue adhesive. All guinea pigs were returned to their cages and were monitored for 14 consecutive days, weight gain, eating and drinking. One orchidetomized guinea pig developed a pustule/cyst ~30 days post surgery, which was successfully removed with no decrease in weight or food intake in the following wk. No guinea pigs were excluded from the study.

6.3.4 Blood sampling and DXA measurements

Blood was drawn, prior to DXA measurements to control for effects of the anesthetic, from the lateral saphenous vein at baseline (wk -1), wk 2, 4, 8 and 16, between 0730 and 0930 h into microtainers containing lithium heparin. All blood samples were centrifuged at 2200 x g for 15 min in order to obtain plasma, serum and RBC. RBC were washed with saline then stored in an equal weight of water:methanol:BHT solution to prevent polyunsaturated fatty acid (PUFA) oxidation as described by Magnusardottir et al. [272]. All blood samples were stored at -80°C. Bone and body composition including whole body mass, lean mass, fat mass, BMC (WB BMC), areal bone mineral density (WB aBMD), Left (L) femur BMC, L femur aBMD, L tibia BMC, L tibia aBMD, and lumbar spine vertebrae 1 to 4 (LS 1-4) were determined using DXA (Discovery Series 4500A densitometer, Hologic Inc., Bedford, MA) under isoflurane anesthesia. Anesthesia was induced in a chamber with 5% isoflurane and followed by 2% maintenance using a cone mask. The guinea pigs were scanned in the prone position with limbs extended in whole body and regional (spine and left and right tibia and femur) modes. Analysis was performed using the small animal software (QDR 4500A small animal software version 12.5). Guinea pigs were anesthetized for approximately 20 minutes, returned to their respective cage and monitored for 48 hr for fluid and food intake.

6.3.5 Blood and tissue fatty acid profiling

CLA was measured from RBC samples using a modified Bondia-Pons method [282] to determine the fatty acid profile (including c9, t11 and t10, c12 CLA isomers). Modifications included the use of 200 µL of RBC combined with 10 µL of heptadecanoic acid (internal standard) and the addition of 600 µL of hexane after methylation. CLA in experimental diets, heart, liver, spleen, brain, right kidney, right quadriceps muscle and left and right adipose tissue were assessed using the O'Fallon method [307].

For all samples, fatty acid methyl esters were separated using a 100 m CP-Sil-88 capillary column (Varian-Chrompack, CP7489), installed in a Varian CP-3800 Gas Chromatograph, (Varian, Inc., Walnut Creek, CA, USA) with a flame-ionization detector. For identification and quantification of the FAME peaks, authentic standard 461 (cat # GLC-461, Nu-Chek Prep, Inc. Elysian, MN) was used. In addition, *c*9, *t*11 octadecadienoic acid and t10, c12 octadecadienoic acid (cat # UC60M & UC61M, Nu-Chek Prep, Inc. Elysian, MN) and standards of known concentration were used to calculate recovery (>90%). The fatty acids were expressed as a percentage of the total fatty acids identified from dodecanoic acid (C12:0) to docosahexaenoic acid (C22:6).

6.3.6 Biochemistry

Blood was separated at 2000 g to obtain plasma for determination of testosterone (Cat. # E05T0123 Shanghai BlueGene Biotech Co. Ltd., Shanghai, China) and estrogen (Cat. # CSB-E12956Gp Cusabio Biotech Co. Ltd., Wuhan, China) and serum for IL-6 (Cat. # CSB-E06757Gu Cusabio Biotech Co. Ltd., Wuhan, China) using ELISA kits specific to guinea pig. Regarding serum ionized calcium (iCa), samples were collected in heparinized plastic capillary tubes and measured within 4 h of collection (ABL800 FLEX analyzer, Radiometer, Copenhagen, Denmark). The coefficient of variation (CV%) for all parameters (inter- and intra-assay) was less than 10%.

6.3.7 Bone microarchitecture

Ex vivo bone microarchitecture was performed by μCT on excised left femurs using a Skyscan 1174 scanner (Skyscan, Belgium). The X-ray tube voltage was 50 kV and a 1 mm aluminum filter was used to reduce beam hardening artifacts [308]. Images were obtained using a scanning angular rotation of 180° with an increment of 0.7° per image, exposure time was 7500 ms, and a frame averaging of 2. Acquired images were reconstructed with the manufacturer's software (NRecon v1.6.4.1; Skyscan) and subsequently segmented into binary images, which separate bone and marrow regions (CT Analyser, v1.11.8.0; Skyscan). An

adaptive threshold of 40-255 and a fixed global threshold of 128-255 were applied for trabecular and cortical bone respectively. The threshold was optimized by visually comparing the binarized images with original gray-scale images. Trabecular bone proximal to the epiphysis was segmented by manually drawing the anatomical region of interest a few voxels away from the endocortical surface. The ROI started at a longitudinal distance of 1.5 mm from the epiphysis, and extended a further 3.06 mm in the proximal direction for analysis. To minimize bias and inconsistency, a single operator performed the contouring. The ROI contained 100 slices, each with the same thickness as the pixel size (30.56 μm). In total, the ROI reached a total voxel size of 3.06 mm^3. Trabecular thickness (Tb.Th) and separation (Tb.Sp) were calculated by the sphere-fitting local thickness method. The trabecular number (Tb.N), connectivity density (Conn.D) and structure model index (SMI) were automatically calculated by the software (CT Analyser, v1.11.8.0; Skyscan). Cortical bone at the mid-1/3 proximal and 1/3 distal diaphysis of the femur was analyzed an ROI of 100 slices (1 slice = 30.56μm). The periosteal (outer) and endosteal (inner) boundaries of these slices of cortical bone of the femur were obtained automatically using a 3D “shrink-wrap” function provided by the software tool (CT Analyser, v1.11.8.0; Skyscan) and also allowed for cortical porosity (Ct.Po) and cortical bone fraction (Ct.Ar/Tt.Ar) measurements. Total volume (TV) represented all voxels within the periosteal boundary, while bone volume (BV) signified all the voxels segmented as bone within the volume of interest, which allowed to

determine bone volume fraction (BV/TV). Medullary volume (MV) was calculated as TV-BV. The areal moment of inertia (MOI) in the x (I_x) and y (I_y) direction (x and y representing the rotational axis and quantifying the distribution of material around a given axis) and polar moment of inertia (Ip) (reflecting torsional rigidity) were calculated using 2D analyses (for bone strength testing).

6.3.8 Bone strength testing

Left femurs underwent three-point bending (Instron 5544, Canton, MA). Bones stored at -20 C were thawed to room temperature (20 - 23 °C) in a water bath. The location of the mid-diaphysis was measured from the lateral condyle to the greater trochanter using electronic digital calipers (Fisher Scientific, Ottawa, ON). The bone was positioned horizontally with the anterior surface upward, centered on the fulcrums with a constant span length of 40 mm. A preload was set with a sensitivity of 0.5 N, and a rate of 10 mm/min. When the loading fixture touched the bone, the bone was visually inspected to ensure stability on the fulcrums. The load then continued at a rate of 1 mm/min at the mid-diaphysis. Bones were loaded to failure (until bone fractured and load detected was decreased by 50%). Load versus deformation curve was plotted (Bluehill v.2; Instron, Canton, MA), and maximum flexure load (F_{max}, N), maximum extension at maximum flexure load (d_{max}, mm), and energy at break (W, mJ) were derived from the curve. Extrinsic stiffness (K, N/mm) was calculated as the

slope of the linear portion of the curve. Stress (σ_{max}) and strain (ε_{max}) at maximum flexure load, and Young's modulus (E) were derived using the formulas:

$$\sigma = \frac{F_{MAX} \times L \times c}{4l}, \qquad \varepsilon = \frac{12 \times c \times d_{MAX}}{L^2}, \qquad E = \frac{K \times L^3}{48 \times l}$$

where F_{max} = maximum flexure load; L = distance between fulcrums; c= distance from neutral axis to outer perimeter (measured from μCT cross-sectional images); I = I_x (obtained from μCT 2D analyses); d_{max} = maximum extension at maximum flexure load.

6.3.9 Protein fractional synthesis rates

A flooding dose of L-[ring-$^2H_5$]-phenylalanine (40 mol %, Cambridge Isotope Laboratories, Cambridge, MA), in unlabeled phenylalanine (Ajinomoto U.S.A Inc., Raleigh, N.C.), was prepared in sterile saline for a final concentration of 0.15 mol/L and passed through a 0.22 μm syringe filter. The solution was injected intraperitoneally at a dose of 0.01 L/kg body weight with a 20 G Insyte needle (Becton Dickinson & Co., Franklin Lakes, NJ) over a 10-s period in order to determine protein fractional synthesis rates of mixed muscle quadriceps proteins. At 50 minutes, prior to termination and while under anesthesia, the guinea pigs' left quadriceps were snap frozen in liquid nitrogen and stored at -80ºC.

6.3.9.1 Tissue and protein preparation

Myofibrillar and sarcoplasmic proteins from the left quadriceps muscle were separated according to methods previously described by Welle et al. [309, 310]. To isolate sarcoplasmic and myofibrillar proteins as well as free amino acids of both muscle compartments, approximately 100 mg of tissue was homogenized in 4 mL distilled H_2O and centrifuged at 1500 g at 4°C for 10 min. The supernatant was removed and separated into 2 portions in 4 mL glass vials. The free amino acid supernatant from the first 4 mL glass vial was acidified with 1 mL glacial acetic acid for $^2H_5$-phenylalanine enrichment. The myofibrillar protein pellet was washed 3 times with 2 mL cold distilled H_2O and supernatant was discarded. The pellet was dissolved in 2 mL 0.3N NaOH to precipitate non-soluble proteins and the supernatant containing myofibrillar proteins was transferred to a 4 mL glass vial and acidified with 2 mL 6N HCl. Samples were capped under N_2 and heated at 110°C overnight. In the supernatant of the second glass vial, 2 mL of 10% perchloric acid was added to precipitate sarcoplasmic proteins. Samples were centrifuged at 1500 g at 4°C for 10 min and washed 4 times with 2 mL 5% perchloric acid to remove free amino acids. Samples were washed with 2 mL distilled H_2O, centrifuged at 1500 g at 4°C for 10 min, followed by 2 mL of ethanol to remove perchloric acid and transferred to a 4 mL glass vial. Finally, samples were dried in Speed Vac (Thermo Savant SC 210A, Waltham,

MA, USA). The dried pellet was acidified with 2 mL 6M HCl, capped under N_2 and heated at 110°C overnight.

6.3.9.2 Isolation and derivatization of amino acids

Tissue free and bound-amino acids pools were isolated by ion exchange chromatography (Dowex 50WX8, Bio-Rad, Mississauga, ON, Canada), dried under nitrogen, esterified, and derivatized to their n-propyl ester heptafluorobutyramide derivatives using n-propanol and acetyl chloride followed by heptafluorobutyric anhydride [311, 312].

6.3.9.3 Gas chromatography-mass spectrometry

Phenylalanine enrichment was analyzed by methane negative chemical ionization gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies, Model 5975C, Mississauga, ON, Canada), by monitoring the $[M\text{-}FH]^-$ ions at mass to charge ratio 383 to 388 (free amino acid) and 385 to 388 (sarcoplasmic and myofibrillar), plotted against a standard (STD) curve of corresponding unlabeled and labeled ions. Tracer:tracee ratios were calculated from the raw ion abundances and analysis of the tracer and natural abundance of phenylalanine.

6.3.9.4 Calculations

The protein fractional synthesis rate (FSR) of mixed proteins in each tissue was calculated using:

$$FSR\ (\%/d) = (E_{bound} \times 24 \times 100) / (E_{free})$$

where E_{bound} is the net tracer:tracee ratio of the tissue protein bound phenylalanine at 60 min; and E_{free} is the net tracer:tracee ratio of tissue free phenylalanine at isotopic steady state. The tracer:tracee ratio of free phenylalanine in tissue (E_{free}) was assumed to reflect the steady-state tracer:tracee ratio of the phenylalanine pool from which proteins were synthesized.

6.3.10 Statistical analyses

Data were analyzed using SAS statistical package software version 9.2.0 (SAS Institute Inc, Cary, NC, USA) and was tested for normality and homogeneity of variances before using a mixed model with Tukey post hoc comparisons. A mixed-model repeated-measures design included time, diet, and surgical intervention as a fixed effect and individual animals nested for treatment and block randomization as random effects. A P-value of ≤0.05 was accepted as significant. Data are presented as mean ± SEM unless otherwise stated.

6.4 Results

6.4.1 Dietary intake and tissue incorporation of fatty acids

Guinea pigs in the ORX CTRL group (338.7±5.0 g/wk) ate significantly less than SHAM CTRL (377.9±5.9 g/wk, P=0.001) but not ORX+CLA (353.8±8.3 g/wk) or SHAM CLA (363.3±7.2 g/wk) groups.

Regarding fatty acid incorporation in RBC, c9, t11 CLA isomer proportion was significantly increased in both SHAM CLA and ORX CLA groups compared with baseline for all time points, whereas, in SHAM CTRL and ORX CTRL, no c9, t11 CLA was detected (**Appendix C - Table C.1**). As for the t10, c12 CLA isomer, no differences were observed among groups and over time (data not shown). Omega-6 (n-6) polyunsaturated fatty acids (PUFA) including linoleic acid (LA; C18:2) and arachidonic acid (AA; C20:4) did not significantly differ among groups over time, whereas omega-3 (n-3) PUFA including alpha linolenic acid (ALA; C18:3), eicosapentaenoic acid (EPA; C20:5), and docosahexaenoic acid (DHA, C22:6) did not differ among groups, however, they decreased, compared to baseline, over time reaching significance at wk 8 for ALA in the SHAM CLA group, at wk 8 and 16 for EPA and DHA in the SHAM CLA group and at wk 16 for EPA and DHA for all groups (**Appendix C - Table C.1**).

In liver tissue, both c9 t11 and t10, c12 CLA isomer percent of total fatty acids were significantly higher in the CLA groups compared to the

CTRL groups. LA was significantly lower in the SHAM CLA group compared to SHAM CTRL and ORX CTRL groups but not ORX CLA. There were no significant differences in percent total fatty acids among groups for ALA, AA, EPA and DHA (**Appendix C - Table C.2**).

In mixed muscle tissue (mixed muscle fiber types) from the quadriceps, both c9 t11 and t10, c12 CLA isomer percent of total fatty acids were significantly higher in the CLA groups compared to the CTRL groups. EPA was significantly higher in the ORX CTRL group compared to all other groups. There were no significant differences in percent total fatty acids among groups for LA, ALA, AA, and DHA (**Appendix C - Table C.2**).

6.4.2 Anthropometry and body composition

There was no main effect of diet or time for the body mass measurement; however, there was a main effect for surgery where the ORX group had a lower body mass than the SHAM group. There was no main effect of diet and surgery for fat mass, whole body fat mass percentage (WBF%) and whole body lean mass percentage (WBL%). A main effect for surgery was observed for lean mass where the ORX group had less lean mass than the SHAM group. Furthermore, a main effect for time was detected for fat mass, WBF%, WBL% but not for lean mass. Fat mass and WBF% increased significantly from baseline to wk 16, whereas, WBL% decreased significantly from baseline (**Table 6.2**). No interaction effects were detected for anthropometry and body composition outcomes.

6.4.3 Whole body, axial and appendicular bone composition

No main effects of diet were observed for WB BMC, WB aBMD, L) femur BMC, L femur aBMD, L tibia BMC, L tibia aBMD, LS 1-4 BMC and aBMD. There was a main effect of surgery for WB BMC, L femur BMC and LS 1-4 aBMD, where BMC and aBMD values in ORX groups were significantly lower than SHAM groups. No effect of surgery was observed for WB aBMD, L femur aBMD, L tibia BMC, L tibia aBMD and LS 1-4 BMC. A main effect of time was seen in L tibia BMC and L tibia aBMD measurements only (**Appendix C - Table C.3**). For LS 1-4 BMC only, an interaction effect between diet and surgery was observed where SHAM CTRL (0.797±0.010 g) was significantly higher than ORX CTRL (0.754±0.010 g) but not SHAM CLA (0.774±0.010 g) or ORX CLA (0.780±0.010 g).

6.4.4 Biochemistry

No differences among diet groups were observed for 25(OH)D, however, there was a main effect of surgery where 25(OH)D was significantly higher in the SHAM group compared to the ORX group (**Table 6.3**). No differences among groups and main effects in iCa and estradiol were detected. For free testosterone, no differences among groups were observed at wk 0, however, free testosterone was significantly decreased from wk 0 to 16 in the ORX groups but not the SHAM groups and, at wk

16 only, free testosterone was significantly lower in the ORX groups versus the SHAM groups. Moreover, there were no differences at wk 0 among groups for IL-6 and all groups significantly increased IL-6 concentration from wk 0 to wk 16. Additionally, at wk 16, IL-6 was significantly greater in the ORX groups than the SHAM groups (**Table 6.3**). For free testosterone and IL-6 only, an interaction effect between time and surgery was observed. For free testosterone, ORX at wk 16 (0.005±0.001 pmol/L) was significantly lower than ORX at wk 0 (0.014±0.001 pmol/L) SHAM at wk 0 (0.012±0.001 pmol/L) and SHAM at wk 16 (0.014±0.001 pmol/L). For IL-6, SHAM (0.013±0.003 pmol/L) and ORX (0.014±0.003 pmol/L) at wk 0 did not differ from each other, however both were significantly different from ORX at wk 16 (0.089±0.003 pmol/L) and SHAM at wk 16 (0.046±0.003 pmol/L) which in turn were significantly different from each other.

6.4.5 Bone microarchitecture

At the distal left femur metaphysis region, vBMD, BV/TV, Tb.N and Conn.D were significantly lower in ORX CTRL compared to SHAM CTRL and SHAM CLA but not ORX CLA. SMI was significantly lower in SHAM CTRL compared to ORX CTRL and ORX CLA but not SHAM CLA. A main effect of surgery was detected for all measures except Tb.Th, which was also not significantly different among groups. Values were significantly

lower in the ORX groups versus the SHAM groups (higher for Th.Sp and SMI) (**Table 6.4**).

At the proximal left tibia metaphysis region, vBMD, BV/TV and Tb.N were significantly lower in the ORX CTRL group compared to SHAM CTRL but not SHAM CLA or ORX CLA. A main effect for surgery was found for vBMD, BV/TV, Tb.Sp and Tb.N where values were significantly lower in the ORX groups versus the SHAM groups (higher for Th.Sp) (**Table 6.4**).

At the L3 spine metaphysis, SMI was significantly lower in SHAM CLA compared to ORX CLA but no SHAM CTRL and ORX CTRL. Also, a main effect of surgery was observed for Tb.Sp, Tb.N and SMI where ORX was higher for Tb.Sp and SMI compared to SHAM and ORX was lower for Tb.N compared to SHAM (**Table 6.4**).

At the left femur mid-diaphysis, Ct.Po was lower in ORX CLA versus ORX CTRL but not SHAM CTRL or SHAM CLA (**Table 6.5**). A main effect of diet was found where the CLA groups had lower porosity than the CTRL groups (CTRL: 1.40±0.11 vs. CLA 1.01±0.12 %, P=0.02). There were no significant differences among groups for vBMD or Ct.Ar/Tt.Ar and no main effects of surgery. At 1/3 distal, vBMD was significantly lower in SHAM CTRL compared to SHAM CLA but not ORX CTRL or ORX CLA. Moreover, Ct.Po was significantly higher in ORX CTRL compared to SHAM CTRL but not SHAM CLA or ORX CLA. A main effect of surgery was also found for Ct.Po. Ct.Ar/Tt.Ar did not differ among groups. At the 1/3 proximal, Ct.Po was significantly lower in SHAM CLA

versus SHAM CTRL but not ORX CRTL of ORX CLA. A main effect of surgery was found for Ct.Ar/Tt.Ar where ORX lead to lower cortical bone fraction than SHAM. There were no differences among groups for vBMD at this region (See Appendix B for bone microarchitecture images).

6.4.6 Bone strength testing

No significant differences were observed among groups for F_{max}, d_{max}, W_{break}, σ_{max}, ε_{max} and E. No main effect of diet and surgery were observed (**Table 6.6**).

6.4.7 Skeletal muscle protein fractional synthesis rate

There were no significant differences among groups for sarcoplasmic and myofibrillar FSR (**Figures 6.2**). No main or interaction effects were detected.

Table 6.1. Diet composition

Nutritional profile (g/kg)	CTRL	CLA[1]
Carbohydrates		
Starch	154.1	154.1
Glucose	2.7	2.7
Fructose	7.3	7.3
Sucrose	22.3	22.3
Lactose	5.6	5.6
Fiber	144.0	144.0
Fats		
Cholesterol (ppm)	0.043	0.043
Linoleic acid	17.1	12.3
Conjugated linoleic acid	0.0	10.0[2]
Linolenic acid	2.9	2.1
Arachidonic acid	0.0	0.0
Omega-3 fatty acids	3.1	2.3
Saturated fatty acids	17.6	16.9
Monounsaturated fatty acids	19.4	18.8
Polyunsaturated fatty acids	17.6	19.5
Protein		
Arginine	11.3	11.3
Histidine	4.7	4.7
Isoleucine	10.7	10.7
Leucine	14.8	14.8
Lysine	10.8	10.8
Methionine	4.2	4.2
Cystine	2.6	2.6
Phenylalanine	9.7	9.7
Tyrosine	6.5	6.5
Threonine	7.3	7.3
Tryptophan	2.6	2.6
Valine	10.4	10.4
Alanine	10.1	10.1
Aspartic acid	24.9	24.9
Glutamic acid	37.8	37.8
Glycine	8.6	8.6
Proline	13.1	13.1
Serine	10.7	10.7
Vitamin[3] and mineral[4] premix	174.0	174.0
Energy (%)		
Carbohydrates	56.7	56.7
Fat	17.8	17.8
Protein	25.5	25.5

1. CLA supplied by Lipid Nutrition, Loders Croklaan BV. 2. 1% CLA (10 g/kg) composed of 61% c9, t11 isomer and 15% t10,c12 isomer. 3. Vitamin mix contained the following: carotene, 12.7 mg/kg; vitamin A, 8.5 mg/kg (25 IU/g); DL-α-tocopheryl, 36.1 mg/kg (54 IU/kg); menadione, 5.0 mg/kg; thiamin, 9.0 mg/kg; riboflavin, 6.1 mg/kg; niacin, 62 mg/kg; pantothenic acid, 21 mg/kg; folic acid, 4 mg/kg; pyridoxine, 4 mg/kg; biotin, 0.2 mg/kg; vitamin B-12, 0.01 mg/kg; choline chloride, 1850 mg/kg; and ascorbic acid, 500 mg/kg.

4. Mineral mix contained the following: calcium, 11 g/kg; phosphorous, 6.5 g/kg; phosphorous (available), 4.3 g/kg; potassium, 15.5 g/kg; magnesium, 3.6 g/kg; sodium, sulfur, 2.8 g/kg; 3.5 g/kg; chlorine, 7.1 g/kg; fluorine, 26.6 mg/kg; iron, 353 mg/kg; zinc, 78 mg/kg; manganese, 81mg/kg; copper, 14.0 mg/kg; cobalt, 3.4 mg/kg; iodine, 0.88 mg/kg; chromium, 2.0 mg/kg; and selenium, 0.47 mg/kg.

Table 6.2. Main effects of diet, surgery and time on anthropometry and body composition of male guinea pigs

	Diet		Surgery		Time (wk)				
	CTRL	CLA	SHAM	ORX	Baseline	2	4	8	16
Anthropo-metry									
Body mass (g)	1198.5±11.6	1201.8±13.0	1224.9±12.7[a]	1175.3±11.4[b]	1207.1±21.3	1173.1±18.5	1189.0±18.3	1194.9±18.3	1236.5±20.1
Body comp.									
Fat mass (g)	230.3±8.6	230.9±8.5	235.1±9.4	226.1±7.6	215.1±16.0[a,b]	212.3±12.2[a]	226.2±12.8[a,b]	233.1±12.3[a,b]	266.3±12.7[b]
WBF (%)	18.9±0.6	18.8±0.5	18.7±0.6	19.0±0.5	17.3±1.0[a]	17.8±0.8[a]	18.7±0.8[a,b]	19.2±0.8[a,b]	21.2±0.7[b]
Lean mass (g)	930.7±6.4	932.9±7.3	951.6±5.6[a]	911.9±7.3[b]	954.4±10.1	923.1±11.5	925.2±10.5	924.10.0	931.9±11.4
WBL (%)	78.0±0.5	78.0±0.5	78.2±0.5	77.8±0.5	79.6±0.9[a]	79.0±0.8[a]	78.1±0.8[a,b]	77.7±0.7[a,b]	75.6±0.7[b]

Data are mean±SEM. Main effects determined using MIXED model with Tukey post hoc comparisons. Only main effects presented herein. Different letters indicate significant differences ($P<0.05$).

Table 6.3. Differences in biochemical measurements among dietary and surgery groups

	Time (wk)	SHAM CTRL (n=10)	SHAM CLA (n=10)	ORX CTRL (n=10)	ORX CLA (n=10)	Surgery	
Biochemistry						SHAM	ORX
Plasma 25(OH)D (nmol/L)	16	219.2±15.6	192.6±11.3	175.2±13.1	170.1±11.0	206.0±9.9^{A}	172.7±8.3^{B}
Ionized Calcium (mmol/L)	16	1.57±0.02	1.58±0.01	1.57±0.02	1.61±0.02	1.58±0.02	1.59±0.02
Plasma Estradiol (pmol/L)	0	8.44±0.26	7.59±0.46	8.03±0.25	8.19±0.22	8.04±0.27	8.11±0.16
	16	8.16±0.31	8.00±0.32	8.10±0.42	8.44±0.27	8.09±0.22	8.26±0.25
	%Δ(16-0)	-3.0±3.2	9.0±10.2	1.2±5.2	3.9±4.9	3.0±5.1	2.5±3.5
Plasma Free Testosterone (nmol/L)	0	0.13±0.01^{a}	0.12±0.02^{a}	0.12±0.01^{a}	0.16±0.01^{a}	0.13±0.02^{A}	0.14±0.02^{A}
	16	0.15±0.01^{a}	0.13±0.01^{a}	0.05±0.02^{b}	0.06±0.02^{b}	0.14±0.01^{A}	0.06±0.01^{B}
	%Δ(16-0)	26.5±17.4^{a}	25.6±22.3^{a}	-52.3±23.^{b}	-59.9±11.8^{b}	26.0±13.3	-56.1±12.5
Interleukin-6 (pmol/L)	0	0.013±0.002^{a}	0.013±0.001^{a}	0.016±0.001^{a}	0.011±0.001^{a}	0.013±0.002^{A}	0.014±0.001^{A}
	16	0.043±0.005^{c}	0.049±0.006^{c}	0.087±0.005^{b}	0.088±0.006^{b}	0.047±0.006^{B}	0.089±0.006^{C}
	%Δ(16-0)	146.4±15.5^{a}	306.0±43.2^{b}	630.1±93.6^{c}	650.5±74.8^{c}	237.6±33.1^{A}	640.3±58.2^{B}

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences (P<0.05). Uppercase letters designate main effect differences.

Table 6.4. Differences in bone microarchitecture in femur, tibia and spine metaphyses among dietary and surgery groups

	SHAM CTRL	SHAM CLA	ORX CTRL	ORX CLA	Main Effect	
					SHAM	ORX
Distal left femur metaphysis						
vBMD (mg/mm^3)	0.253±0.023^{a}	0.237±0.025^{a,b}	0.139±0.019^{c}	0.165±0.021^{b,c}	0.244±0.016^{A}	0.152±0.014^{B}
BV/TV (%)	14.7±0.8^{a}	14.1±0.9^{a,b}	10.8±0.7^{c}	11.7±0.7b^{b,c}	14.4±0.57^{A}	11.2±0.49^{B}
Tb.Th (mm)	0.155±0.002	0.156±0.002	0.156±0.002	0.160±0.002	0.156±0.001	0.158±0.001
Tb.Sp (mm)	0.488±0.024^{a}	0.509±0.024^{a,b}	0.613±0.036^{b}	0.585±0.037^{a,b}	0.499±0.025^{A}	0.599±0.016^{B}
Tb.N (1/mm)	0.944±0.048^{a}	0.905±0.049^{a,b}	0.690±0.041^{c}	0.730±0.049^{b,c}	0.924±0.033^{A}	0.710±0.031^{B}
SMI	1.88±0.050^{a}	1.91±0.05^{a,b}	2.12±0.05^{b}	2.11±0.06^{b}	1.90±0.03^{A}	2.11±0.04^{B}
Conn.D (1/mm^3)	12.18±0.93^{a}	10.99±1.03^{a,b}	7.29±0.85^{c}	7.44±0.98^{b,c}	11.61±3.10^{A}	7.42±2.81^{B}
Proximal left tibia metaphysis						
vBMD (mg/mm^3)	0.428±0.017^{a}	0.394±0.031^{a,b}	0.326±0.021^{b}	0.351±0.031^{a,b}	0.411±0.019^{A}	0.338±0.019^{B}
BV/TV (%)	20.9±0.6^{a}	19.6±1.1^{a,b}	17.3±0.7^{b}	18.2±1.1^{a,b}	20.2±0.6^{A}	17.7±0.7^{B}
Tb.Th (mm)	0.122±0.002	0.120±0.002	0.127±0.003	0.124±0.003	0.121±0.002	0.126±0.002
Tb.Sp (mm)	0.289±0.008	0.291±0.011	0.333±0.019	0.334±0.021	0.290±0.007^{A}	0.339±0.014^{B}
Tb.N (1/mm)	1.71±0.06^{a}	1.64±0.08^{a,b}	1.39±0.05^{b}	1.44±0.11^{a,b}	1.68±0.05^{A}	1.42±0.06^{B}
SMI	2.26±0.12	2.43±0.12	2.57±0.11	2.56±0.11	2.34±0.09	2.56±0.07
Conn.D (1/mm^3)	42.3±2.5	48.0±7.1	41.0±7.7	38.5±9.4	45.1±3.7	39.7±5.9
L3 spine metaphysis						
vBMD (mg/mm^3)	0.586±0.045	0.660±0.063	0.575±0.040	0.533±0.053	0.623±0.039	0.554±0.033
BV/TV (%)	26.4±1.6	29.0±2.2	26.0±1.4	24.5±1.9	27.7±1.3	25.3±1.1
Tb.Th (mm)	0.119±0.002	0.123±0.003	0.122±0.002	0.123±0.003	0.121±0.002	0.123±0.002
Tb.Sp (mm)	0.253±0.004	0.254±0.005	0.262±0.005	0.269±0.008	0.254±0.003^{A}	0.265±0.005^{B}
Tb.N (1/mm)	2.199±0.093	2.336±0.124	2.111±0.064	1.984±0.117	2.267±0.077^{A}	2.048±0.067^{B}
SMI	1.479±0.082^{a,b}	1.238±0.127^{a}	1.509±0.071^{a,b}	1.651±0.110^{b}	1.358±0.078^{A}	1.580±0.066^{B}
Conn.D	78.25±5.11	72.15±5.72	71.17±7.13	71.87±6.62	75.20±3.79	71.52±4.74

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences (P<0.05). Uppercase letters designate main effect differences. n=10 per group.

Table 6.5. Differences in bone microarchitecture in femur diaphysis among dietary and surgery groups

	SHAM CTRL	SHAM CLA	ORX CTRL	ORX CLA	Main Effect	
					SHAM	ORX
Left femur mid-diaphysis						
vBMD (mg/mm^3)	1.608±0.005	1.626±0.007	1.611±0.007	1.605±0.008	1.608±0.005	1.617±0.004
Ct.Ar/Tt.Ar (%)	60.3±0.8	60.3±1.0	58.2±1.0	59.2±1.0	60.3±0.6	58.7±0.7
Ct.Po (%)	1.12±0.16^{a,b}	1.11±0.17^{a,b}	1.68±0.11^{a}	0.91±0.18^{b}	1.11±0.11	1.27±0.14
Left femur diaphysis 1/3 distal						
vBMD (mg/mm^3)	1.559±0.008^{a}	1.591±0.010^{b}	1.565±0.008^{a,b}	1.561±0.007^{a,b}	1.575±0.007	1.563±0.005
Ct.Ar/Tt.Ar (%)	44.6±0.8	45.8±1.3	44.2±1.1	45.8±1.0	45.2±0.8	45.0±0.8
Ct.Po (%)	0.84±0.15^{a}	1.09±0.26^{a,b}	1.73±0.34^{b}	1.50±0.32^{a,b}	0.97±0.21^{A}	1.61±0.20^{B}
Left femur diaphysis 1/3 proximal						
vBMD (mg/mm^3)	1.761±0.064	1.779±0.060	1.704±0.010	1.693±0.007	1.770±0.043	1.698±0.005
Ct.Ar/Tt.Ar (%)	74.3±3.1	74.5±2.9	69.4±0.9	68.6±1.2	74.4±2.1^{A}	69.0±0.7^{B}
Ct.Po (%)	1.41±0.23^{a}	0.77±0.19^{b}	0.64±0.18^{b}	1.03±0.19^{a,b}	1.08±0.16	0.85±0.13

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences (P<0.05). Uppercase letters designate main effect differences. n=10 per group.

Table 6.6. Differences in bone strength among diet and surgery groups in guinea pigs

	SHAM CTRL (n=10)	SHAM CLA (n=10)	ORX CTRL (n=10)	ORX CLA (n=9)	Surgery	
					SHAM	ORX
Left femur mid-diaphysis						
F_{max} (N)	174.1±14.6	170.8±13.5	157.9±8.4	175.1±15.7	172.4±9.7	166.0±8.7
d_{max} (mm)	0.81±0.10	0.92±0.06	0.85±0.06	0.80±0.07	0.86±0.06	0.83±0.04
W_{break} (mJ)	80.1±15.5	65.0±10.7	61.8±8.0	65.3±12.7	72.5±9.3	63.5±7.1
σ_{max} (MPa)	98.1±8.2	97.0±8.2	85.1±7.0	100.9±9.5	97.6±5.7	92.6±5.9
ε_{max} (%)	2.95±0.35	3.36±0.27	3.15±0.22	2.86±0.23	3.15±0.22	3.01±0.16
E (MPa)	19402±1296	18529±746	15380±1008	19529±1287	18966±723	17346±925

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences ($P<0.05$).

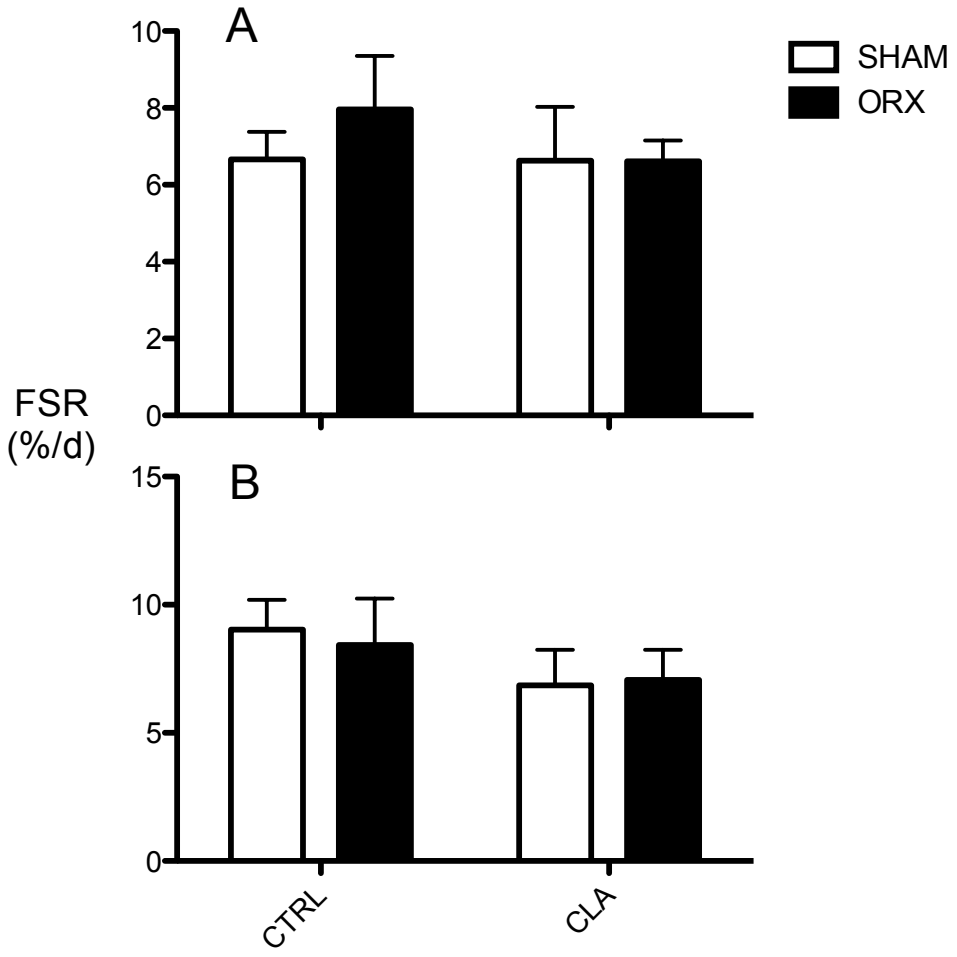

Figure 6.2. Fractional synthesis rate of sarcoplasmic (A) and myofibrillar (B) protein in mixed muscle of the left quadriceps. Differences detected using MIXED model with post hoc testing using Tukey's HSD test. No significant differences were detected ($P<0.05$). Values are mean±SEM; n=8 for SHAM+CTRL and ORX CTRL, n=9 for SHAM+ CLA and n=6 for ORX+CLA.

6.5 Discussion

To date, studies investigating the musculoskeletal effects of CLA have used primarily young and/or female murine models. Since these animals are constantly growing, the typical hormonal changes seen with aging in the human are not well reflected. This is the first study to investigate the effects of CLA on bone and muscle using an accelerated aging model (orchidectomized middle-aged (70 wk) guinea pigs). The orchidectomy intervention resulted in the desired significant increase (640.3%) in proinflammatory cytokine (IL-6) and the significant reduction in total plasma free testosterone (57.1%), body mass (4.0%), lean mass (4.2%), and trabecular bone density (up to 37.7% in distal femur metaphysis), which are typical to aging in men. However CLA did not prevent decreased free testosterone and increased IL-6 as hypothesized. These findings are in line with a study by Erben et al., who assessed the effects of aging and orchidectomy on bone and body composition in adult (~56 wk) male rats over ~39 wk [313]. They observed a decrease in total serum free testosterone (84.7%), body mass (~5.0%) and trabecular bone. This group also found a significant decrease (81.1%) in serum estradiol, which we did not observe.

In the distal femur and proximal tibia metaphysis, vBMD was 58.2 and 27.1% lower in the ORX CTRL compared to SHAM CTRL, however this loss in density was diminished to 42.1 and 19.8% in ORX CLA. Also BV/TV was 30.6 and 18.8% lower in the ORX CTRL compared to SHAM

CTRL whereas these losses were 22.7 and 13.8% in ORX CLA. For Tb.N, 31.1 and 20.6% lower in ORX CTRL compared to SHAM CTRL but losses were reduced in ORX CLA (25.6 and 17.1% respectively). Together, these suggest that CLA has a modest effect in preventing trabecular bone loss. Moreover, ORX led to a 20.0 and 15.6% increase in Tb.Sp as well as a 23.3 and 16.8% decrease in Tb.N in the femur and tibia respectively. No change was observed in Tb.Th. This is also in agreement with Erben et al. who observed at 82 wk, which coincides with the endpoint of our study, an increase in Th.Sp (~200%), a decrease in Tb.N (~33.3), and no change in Tb.Th in the proximal tibia metaphysis [313]. Moreover, Erben et al. found similar effects of ORX in lumbar vertebral trabecular bone (L1) where Tb.Sp was increased by ~40% (4.3% in this study) and Tb.N was decreased by ~20% (10.7% in this study), however, we did not detect any changes in Tb.Th whereas they found a ~9.0% decrease [313]. The differences in magnitude of change for Tb.Sp between studies could be explained by the animal species used (rat vs. guinea pig). For the same time point, the control rats' metaphyseal region had a Tb.Sp ~0.100 mm, which is considerably less than the metaphyseal region of the distal femur and proximal tibia (data not shown) of our guinea pigs (~0.500 mm) and could allow for greater changes in Tb.Sp. In proximal tibia metaphysis specimens of men aged 60 to 94 y, Tb.Th and Tb.N values are consistent with our study and Th.Sp ranged from ~0.450 to 0.950 mm [314], which would suggest that this model of accelerated aging provides a better

representation of human bone microarchitecture at least for trabeculae of the metaphysis.

Regarding cortical bone, left femur diaphysis microarchitecture was assessed at 3 sites (mid-, 1/3 distal and 1/3 proximal). CLA decreased porosity (Ct.Po) by 32.4% at the mid-diaphysis compared to CTRL, however, these changes were not observed at the distal and proximal region. Interestingly, ORX caused a significant 49.6% decrease in Ct.Po at the distal region, which was not observed at mid-diaphysis or proximal 1/3.

Despite no evidence of cortical thinning, bone strength testing was performed at the mid-diaphysis of the femur since that area had the largest change in porosity, which is known to reduce both compressive strength and resistance to bending [315]. However, no significant differences were observed among groups for all variables assessed during 3 point bending test (**Table 6.6**). During the mechanical testing, the femurs would fracture during the elastic portion of the load-deflection curve and thus did not exhibit much plasticity, which could be a consequence of age-related reduced bone stiffness associated with a modification of the content of collagen crosslinks [316].

FSR from sarcoplasmic and myofibrillar protein of mixed muscle of the quadriceps was not affected by diet or surgery despite the ORX guinea pigs having a significantly lower (2.0%) lean mass than SHAM, which suggests that these observations in lean mass in the ORX groups are likely a results of increased protein degradation. This is in contrast to Mirand et al. who measured a 30% increase in FSR in the gastrocnemius

muscle in 4 mo old male Wistar rats fed 1.0% CLA for 6 wk using a flooding dose of C-13-valine isotope [184].

Nevertheless, the adaptive changes in bone morphology and geometry that underlie the increased skeletal fragility in aging including bone mineral loss by trabecular and cortical resorption, which result notably in decreased bone volume and density and increased cortical porosity have been demonstrated using this model. Based on these data, this model of accelerated aging has achieved the desired effect of creating a catabolic environment similar to one observed with aging at least in the bone milieu.

This study is also the first to quantify the tissue and cell incorporation of a 4:1 of c9,t11 to t10,c12 CLA mixture (Appendix C -**Table C.2**). Li et al. reported tissue incorporation values of a 1:1 c9, t11:t10, c12 CLA mixture, which, when proportionally adjusted, are in agreement with our values [295]. The dietary intervention (CLA) did not appear to affect any of the measured outcomes except for Tb.Sp at the distal left femur metaphysis where it was increased significantly by 23.0% in ORX CTRL and but did not reach significance in ORX CLA (18.0%) compared to the SHAM CTRL group, which suggests that CLA could have a modest protective effect on the occurrence of increased distance between trabeculae observed with aging. This is in contrast to Rahman et al. who found an increase in aBMD (9.9%) at the distal femur metaphysis in adult (57 wk old) female mice fed 1% CLA (1:1 isomer mix) for 10 wk compared to corn oil fed animals.

This lack of response could be due in part to a species specific induction of PPAR responsive genes following CLA supplementation, where mice have a greater sensitivity to peroxisome proliferators than rats [317] and guinea pigs, like humans, express PPAR at a reduced level and do not respond to peroxisome proliferators, such as CLA, to the extent observed in mice and rats [318]. Also, a displacement of omega-3 fatty acids, which are known to be beneficial to the regulation of bone cell maturation and activity [319], by CLA in bone, which is exemplified by the decrease in ALA (45.3%), EPA (60.5%) and DHA (60.4%) and no plateauing of CLA over time in RBC (a proxy for bone [295]) of SHAM CLA and ORX CLA groups.

The CLA study diet did not contain a sufficient amount of omega-3 fatty acids (2.3 g/kg of chow) and should be taken into consideration for future guinea pig studies investigating dietary effects on bone. Additional limitations to the study include the scarcity of inflammatory marker bioassays with guinea pig specific cross-reactivity. Also, 9 guinea pigs did not display sufficient free amino acid pool enrichment, necessary for FSR calculations, due to difficulties with the intraperitoneal injections of the isotope.

In summary, ORX decreased trabecular bone in the metaphysis and increased intracortical porosity in the bone; however, no differences in bone strength, sarcoplasmic and myofibrillar protein fractional synthesis rate were detected. This model has achieved the desired effect of creating

a catabolic environment similar to aging, and CLA did prevent some negative bone changes observed with aging.

6.6 Acknowledgements

This study was funded jointly by NSERC and the CLA provided in kind by Lipid Nutrition. The work was conducted at the McGill University facilities and the associated Mary Emily Clinical Nutrition Research Unit. The authors acknowledge the assistance with feeding and some sample collection by A. Babar, N. Tabatabaei, and J. Zhou. The authors have no conflict of interest.

Chapter 7

Study conclusions, limitations and future directions

7.1 Summary

The overall objective of this study was to assess the biological responses underlying the potential effects of conjugated linoleic acid (CLA) on the musculoskeletal system in middle-aged and advanced aging population groups. Both human and animal models were developed to address 5 major outstanding questions. These questions were addressed in 4 manuscripts and are summarized in greater detail in the subsequent sections. These questions were answered using a randomized placebo controlled double blind clinical trial and two different animal models. The selection of the animal model used was based on the appropriateness of this model to answer the specific outstanding question. A rat model was used since it is a useful model of human musculoskeletal metabolism, including the measurement of parathyroid hormone. Also, rat models are used extensively when investigating the effects of CLA (**Figure 2.5**). Subsequently, a guinea pig model was used in order to better mimic human physiologic responses to CLA. Guinea pigs have a healthy longevity and provide a good model for: 1) aging due to similarities in sex hormones and 2) lipid research due to the fact that their blood lipid profiles and responses to dietary lipid interventions are similar to those of humans [304]. Moreover, guinea pigs, like humans, express PPAR at a reduced level and do not respond to peroxisome proliferators, such as CLA, to the extent observed in mice and rats [318].

As a whole, this research has advanced the understanding of tissue enrichment of CLA and the biological responses including hormones (PTH, testosterone, estrogen), and proinflammatory cytokines (IL-6) of CLA on bone and muscle. Moreover it has provided pre-clinical evaluations of CLA as a dietary supplementation strategy to prevent age-related degenerative conditions such as osteoporosis and sarcopenia. The findings in this dissertation provide direction for future research focused on mechanistic approaches regarding age related loss of muscle and bone.

7.1.1 Conjugated linoleic acid in free fatty acid versus triacylglycerol form

> Objective 1: To investigate the tissue incorporation, bone mass and biomarkers of bone metabolism differences between FFA and TG forms of 1.0% 1:1 mixture of c9, t11 to t10, c12 CLA isomer in rats.

CLA is commercially produced in FFA and TG forms, which are branded as having similar health claims. The digestion of CLA varies depending on the stereospecificity [141] of the ingested CLA and the degree of fatty acid absorption varies depending on whether it is ingested as a TG, an ethyl ester or a FFA [138-140]. There is no evidence of physiological differences in animal and human between FFA and TG forms of CLA [36, 42-47], however, differences in tissue incorporation, bone density, biomarkers of bone metabolism and mineral balance have not been investigated comprehensively. In growing rats fed a 1:1 mixture of c9, t11:t10, c12 CLA

isomers in either FFA or TG form, an isomer-specific liver CLA enrichment was found where c9, t11 CLA isomer in FFA form was preferentially incorporated in liver tissue compared to c9, t11 CLA isomer in TG form. Considering these findings, the hypothesis that no differences in tissue incorporation would occur between FFA and TG forms of CLA was rejected. However, no differences in liver enrichment of t10, c12 CLA isomer between both forms were detected. Moreover, in these same rats bone content and density as well as biomarkers of bone metabolism and mineral mass balance did not differ significantly in animals fed FFA CLA versus TG CLA. Thus, the hypothesis that there are no differences in bone and biomarkers of bone metabolism between the FFA and TG form of CLA was accepted. These findings provide an important basis for the future selection of a CLA supplement in the development of a study design investigating the effects of bone related outcomes.

This study did not assess CLA incorporation in bone or bone marrow, which is a limitation in regard to the potential preferential incorporation of c9, t11 CLA isomer in FFA over TG. However, based on tissue enrichment data from Li et al. who demonstrated comparable proportions of c9, t11 and t10, c12 CLA isomer in liver and bone, one could infer that incorporation in bone occurs similarly as in the liver [295], CLA in FFA form could be argued to be preferred over TG in attempt to elicit a greater response. Further work is required to confirm isomer-specific enrichment in bone. Also, studies investigating the effects of synthetically made CLA in TG form with known CLA isomer

stereospecificity could be designed to compare the bioavailability, effects on tissue incorporation and physiological function of naturally occurring CLA to commercially made CLA in TG form.

7.1.2 Standardization of parathyroid hormone assessment

> Objective 2: To determine sex, fasting, and PTH assay generation effects on measurements of PTH in rats.

PTH is the primary determinant of the extent of bone remodeling activity in the skeleton by regulating bone metabolism and mineralization in addition to maintaining calcium homeostasis, which can have implications in skeletal muscles. The assessment of PTH is complicated by its diurnal and circadian rhythm and by the intake of nutrients, such as calcium, alcohol and CLA, which can alter its secretion patterns [63, 194, 241, 242, 320]. Additionally, different generations of PTH assays, with varying cross-reactivity to specific epitopes, exist and are known to yield different results. Hence, for reliable PTH assessment, the timing of blood sampling, the standardized food intake patterns prior to sampling, and the more sensitive PTH assay generation are required in trials designed to assess the physiological response of PTH to diet or supplements. To confirm using a clinical trial the results observed in rats by Weiler et al. [63, 194] and to obtain supporting data for a future study design, it was necessary to first determine the effects of PTH assay of different generations, gender

(sex) and fasting duration on measurements of PTH. In growing rats fed a 1:1 mixture of c9, t11:t10, c12 CLA isomers in FFA form, it was found that the selection of PTH assays was important depending on the purpose of the study since PTH assays (2^{nd} generation (intact) versus 3^{rd} generation (bioactive)) appear to be unable to differentiate differences incurred based on sex and fasting duration. Thus the hypothesis stating that there will be sex, fasting, and PTH assay generation effects on measurements of PTH, was accepted. This strengthened the need to ensure a standardization of fast duration, sex effects, and measurement of PTH using a bioactive PTH assay.

A limitation to this study relates to the use of isoflurane anesthesia in the rats, which is known to decrease iCa and increase INT PTH after induction of anesthesia and peaking after 30 minutes of exposure [262]. Moreover, whether these factors prove to be important in humans remains to be determined. Future studies should attempt to duplicate these findings in humans and investigate the effects of fasting duration, gender and PTH assay generation on PTH measurements. Additionally, dietary factors including calcium intake and alcohol as well as physical activity could be manipulated to determine further effects on PTH.

7.1.3 Conjugated linoleic acid, PTH and bone mineral density in men

Objective 3: CLA status, as measured by red blood cell CLA content, in men will be positively related to body composition and bone mass.

Objective 4: To assess the effects of CLA on PTH in humans, specifically men.

CLA reduces (although not statistically significant) PTH in humans (8.0%, [195]) and in animals (40.0%, [63]), which could explain, in part, responses in bone and lean mass. Chronic high PTH concentrations are known to promote bone loss whereas intermittent or pulsatile PTH administration increases bone formation, BMD and reduce osteoporosis-related fractures [191]. Enhanced BMD and lean mass in human trials of calcium supplementation are ascribed to reduced PTH levels [241, 242, 321]. Therefore, in order to clarify the relationship between dietary CLA, BMD and lean mass it is essential to assess PTH response following CLA supplementation. A cross-sectional analysis investigating the relationship between erythrocyte c9, t11 CLA status and a dose response study to examine the effects of varying doses of c9, t11 CLA isomer supplementation on PTH in men was designed. Fifty-four community dwelling adult men from the greater Montreal area were studied for the cross-sectional analysis and screened for entrance criteria for the dose-

response study. Of the 54 men screened, 31 met the criteria and were included in the dose-response study then randomized to 1 of 3 groups: 1) 4.2 g olive oil, n=10), 2) 2.1 g olive oil and 2.1 g (1.5 g c9, t11 CLA, n=11) or 3) 4.2 g (3.0 g c9, t11 CLA, n=10). The results from the previous two manuscripts aided in ensuring an optimal design by supplementing the men with the preferential CLA in FFA form and by standardizing the timing of the blood sample collected (performed between 0800 -1000 h), fasting duration (12 h), and for thoroughness, PTH was assayed using both intact and bioactive PTH assays. Furthermore, calcium and alcohol intake was monitored and controlled for using a 24 h recall representative of the day prior to the blood sampling.

The cross-sectional analysis revealed that RBC c9, t11 CLA status, a reflection of long-term (~ 4 mo) dietary CLA intake, was positively related to BMD and WBL% and was negatively associated with BMI and WBF%. The hypothesis that CLA status would be associated with higher lean mass, and BMD as well as lower fat mass was accepted. However, based on the dose response branch of this study, c9, t11 CLA supplementation did not appear to affect PTH in healthy men, therefore, the hypothesis of 3 g/d of c9, t11 CLA supplementation for 16 wk would reduce PTH by 30% was rejected.

Despite showing an association between RBC CLA and body composition and bone-related outcomes, these changes appear to occur via a mechanism not directly involving PTH. Limitations of this study included the small number of participants and issues with outcome

variables (weight, BMI, intact and bioactive PTH, exercise) being significantly different among groups at baseline and across the study. However, this study demonstrated an adequate method for estimating dietary CLA intake based on its positive association with plasma CLA content, participant compliance was very good and the study only had one participant drop out. Based on other studies where PTH was reduced by CLA in humans (trend) [195] and rodents [63, 194], future studies should consider a larger scale study of the effects of CLA in older individuals with elevated PTH or in patients with secondary hyperparathyroidism or polycystic kidney disease.

7.1.4 Conjugated linoleic acid and the guinea pig aging model

> Objective 5: To determine if 16 wk of dietary c9, t11 CLA following orchidectomy-induced aging in the guinea pig model will:
> 1) prevent reductions in BMC and BMD of both trabecular and cortical in long bone and vertebrae; 2) elevate and preserve whole body lean mass despite lower testosterone; 3) prevent elevations in fat mass associated with withdrawal of testosterone; 4) reduce catabolism as indicated by change in lean mass and enhance fractional protein synthesis rate in gastrocnemius muscle; 5) reduce circulating concentrations of inflammatory cytokines (IL-6); 6) prevent losses in bone strength in long bones.

Finally, CLA has been shown to positively affect, lean mass [21], muscle mass [22], BMC, BMD [22], and bone microarchitecture in young male [23] and middle-aged female mice [22]. Furthermore, CLA decreased IL-6 by up to 20% in these same middle-aged females [22]. Despite this evidence showing the benefits of CLA and its isomers on muscle and bone, the studies used primarily female murine models. Since these animals are constantly growing, this may not reflect the hormonal changes seen with aging in the human. Also, whether CLA could prevent muscle and bone loss and during aging in males has not been thoroughly addressed. Hence, to account for these limitations, an animal model of accelerated aging was developed. Male, retired breeder guinea pigs (n=40; 70 – 72 wk; pigmented) were randomized to 4 groups: 1) SHAM+Control diet, 2) SHAM+CLA diet, 3) Orchidectomy (ORX)+Control diet, 4) ORX+CLA diet and were fed a control diet or the CLA diet where 1% of soybean oil was substituted with 1% CLA (4:1 c9, t11:t10, c12). The orchidectomy intervention resulted in the desired significant increase (640.3%) in proinflammatory cytokine (IL-6) and the significant reduction in total plasma testosterone (57.1%), body mass (4.0%), lean mass (4.2%), trabecular bone density (up to 37.7% in distal femur metaphysis), which are typical to aging in men. However, CLA had no effects on muscle protein fractional synthesis rates, sex hormones (total plasma testosterone and plasma estradiol), inflammatory cytokines (IL-6), body and bone composition, as well as bone microarchitecture of cortical bone, bone strength, and skeletal muscle protein fractional synthesis rate in an

accelerated aging model. Hence the hypothesis that CLA would prevent induced age-related losses in bone density, bone strength and muscle mass by reducing the effects of the withdrawal of anabolic stimuli (testosterone) and by reducing the catabolic effect of increased systemic inflammation (IL-6) was rejected. In the distal femur and proximal tibia metaphysis, vBMD was in the ORX CTRL compared to SHAM CTRL, however this loss in density was diminished in ORX CLA. Also BV/TV was lower in the ORX CTRL compared to SHAM CTRL whereas these losses were also reduced in ORX CLA. Similarly, Tb.N was lower in the ORX CTRL compared to SHAM CTRL, but losses were tempered in ORX CLA. Moreover, trabecular separation at the distal left femur metaphysis was increased significantly by in ORX CTRL, but did not reach significance in ORX CLA compared to the SHAM CTRL group. Additionally, cortical porosity was reduced by CLA at the mid-diaphysis. Together, these findings suggest that CLA has a modest effect in preventing bone loss observed with aging.

Limitations of this study included the limited availability of biochemical assays, with cross-reactivity to the guinea pig. Most assays attempted display little of no cross-reactivity with this species. Also, the guinea pigs in the CLA groups were receiving less omega-3 fatty acids and over time, the amount of ALA, EPA and DHA measured in RBC was reduced significantly suggesting a deficiency, which could have affected cytokine production and bone outcomes. However, the accelerated aging guinea pig model showed to be an appropriate human aging model, with

closer similarities than murine models in hormone, bone, and muscle outcomes. To clarify the significant bone and muscle differences observed in previous murine studies compared to the lack of changes observed with this guinea pig model of accelerated aging, future studies should be of longer duration and have a greater sample size. These studies would be further enhanced by a more mechanistic approach and focus on species-specific induction of PPAR responsive genes in bone and muscle following supplementation of various CLA isomer mixtures of varying dosages.

7.2 Conclusion

Human health and wellbeing is closely related to the foods consumed. These are an essential source of energy and can provide substrates needed for health benefits by altering pathophysiological processes and reducing the risk of chronic degenerative diseases such as osteoporosis and sarcopenia. CLA are found in functional foods and provide physiological benefits and may reduce the risk of certain chronic diseases beyond basic nutritional functions. CLA has recently gained popularity for its anticarcinogenic properties [33, 34] and its capacity to decrease fat mass as shown in several randomized placebo controlled clinical trials [35-38]. In addition, it was observed that CLA could benefit the musculoskeletal system by preventing bone and skeletal muscle loss incurred with aging [39-41].

Most studies showing musculoskeletal benefits of CLA involved murine models, which most likely metabolize CLA differently than humans making it difficult to extrapolate these findings in humans. Moreover, the relative CLA intake per total fat intake across the present studies, which were typical intakes based on the literature, varied from 12.5% in the rats, to 5.6% in the guinea pigs, to 3.3% in the men. This further complicates the interpretation of the results in regards to significance for humans. However, the greater related intake of CLA in murine models could explain, in part, why these models usually yield greater responses.

The evidence provided in this dissertation, does not agree in that, despite several important findings regarding the effect (or lack thereof) of dietary supplementation of CLA and the assessment of outcomes associated with bone and muscle in men and selected animal models, small benefits of CLA on bone or muscle loss were observed. This is true for healthy young, middle-aged, and old animals and men where CLA supplementation did not lead to clinically relevant changes [323-324].

Future clinical studies investigating potential musculoskeletal benefits of CLA could focus on populations with muscle wasting and bone disease such as cancer cachexia, chronic obstructive pulmonary disease and osteoporosis. Also, possible synergistic effects of combining other nutrients or pharmacological agents with CLA to potentiate the desired effect could be explored. Other CLA isomers (notably t10, c12), combinations of different isomers or intake of CLA fortified foods (either artificially or by biomagnification) could also be tested.

Overall there continues to be insufficient evidence to support the use of CLA as an anabolic or anti-catabolic agent in muscle and bone across the lifespan.

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Appendix A – Bone microarchitecture definitions

Definition of outcomes for trabecular and cortical bone microarchitecture[1]

Abbreviation	Variable	Description	Standard unit
Trabecular measures			
BV/TV	Bone volume fraction	Ratio of the segmented bone volume of the total volume of the region of interest	mm^3
Conn.D	Connectivity density	A measure of the degree of connectivity of trabeculae normalized by TV	$1/mm^3$
SMI	Structure model index	An indicator of the structure of trabeculae; SMI will be 0 for parallel plates and 3 for cylindrical rods	N/A
Tb.N	Trabecular number	Measure of the average number of trabeculae per unit length	1/mm
Tb.Th	Trabecular thickness	Mean thickness of trabeculae, assessed using direct 3D methods	mm
Tb.Sp	Trabecular separation	Mean distance between trabeculae, assessed using direct 3D methods	mm
Cortical measures			
Ct.Ar/Tt.Ar	Cortical area fraction	The fraction of the area composed of cortical bone	%
Ct.Po	Cortical porosity	The volume of pores divided by the total volume of cortical bone	%

[1] From Bouxsein et al. [322].

Appendix B – Bone microarchitecture images

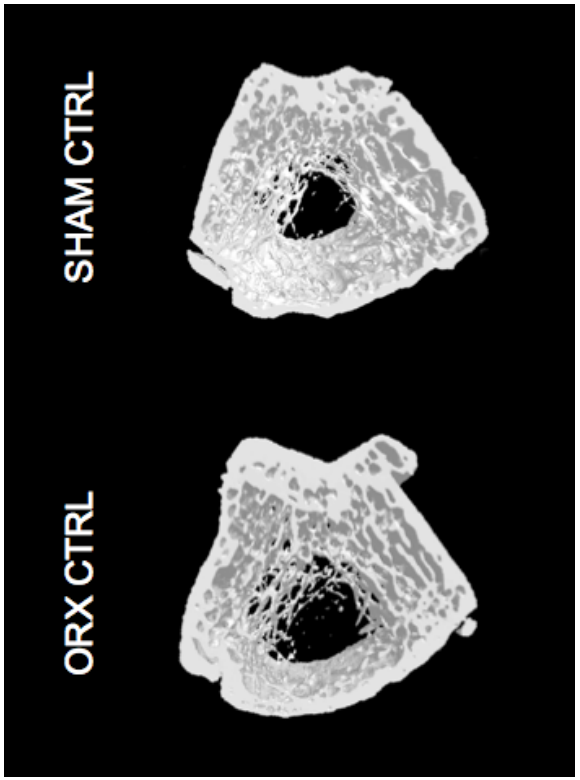

Figure B1. Femur metaphyseal region

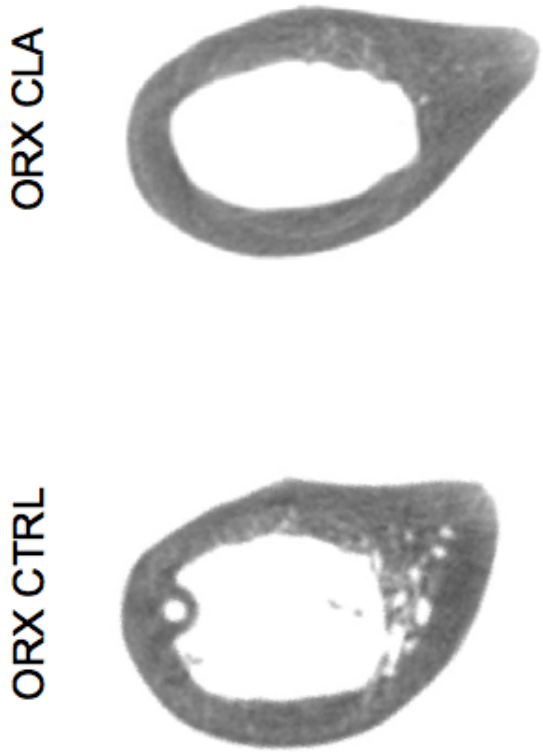

Figure B2. Femur mid-diaphyseal region

Appendix C – Chapter 6 supplemental tables

Table C.1. Red blood cell fatty acid profile at time 0, 2, 4, 8 and 16 of male guinea pigs

	Time (wk)	SHAM CTRL (n=10)	SHAM CLA (n=10)	ORX CTRL (n=10)	ORX CLA (n=10)
RBC (% total fatty acids)					
CLA (C18:2; c9, t11)	0	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	2	0.00 ± 0.00[a]	0.63 ± 0.07[b]	0.00 ± 0.00[a]	0.48 ± 0.07[a,b]
	4	0.00 ± 0.00[a]	0.79 ± 0.17[b]	0.00 ± 0.00[a]	1.04 ± 0.11[b,c]
	8	0.00 ± 0.00[a]	1.11 ± 0.21[b,c]	0.00 ± 0.00[a]	1.14 ± 0.21[c,d]
	16	0.00 ± 0.00[a]	1.40 ± 0.21[c]	0.00 ± 0.00[a]	1.67 ± 0.33[d]
LA (C18:2; n-6)	0	18.13 ± 0.78[a]	18.04 ± 0.55[a]	17.96 ± 0.74[a]	18.48 ± 0.77[a]
	2	16.71 ± 0.72[a]	18.80 ± 2.59[a]	18.16 ± 1.33[a]	16.21 ± 1.04[a]
	4	18.15 ± 1.12[a]	16.44 ± 0.74[a]	17.77 ± 0.57[a]	16.86 ± 1.05[a]
	8	18.27 ± 0.54[a]	17.44 ± 0.70[a]	18.35 ± 0.88[a]	17.38 ± 1.31[a]
	16	20.04 ± 1.23[a]	20.58 ± 0.86[a]	20.28 ± 1.29[a]	22.06 ± 2.26[a]
ALA (C18:3; n-3)	0	0.84 ± 0.10[a]	1.01 ± 0.11[a]	1.09 ± 0.09[a]	0.90 ± 0.10[a]
	2	0.79 ± 0.17[a]	0.74 ± 0.08[a,b]	0.94 ± 0.08[a]	0.56 ± 0.09[a]
	4	0.81 ± 0.08[a]	0.67 ± 0.13[a,b]	0.79 ± 0.10[a]	0.90 ± 0.11[a]
	8	0.51 ± 0.08[a]	0.41 ± 0.08[b]	0.64 ± 0.12[a]	0.64 ± 0.11[a]
	16	0.52 ± 0.08[a]	0.51 ± 0.09[a,b]	0.64 ± 0.13[a]	0.54 ± 0.15[a]
AA (C20:4; n-6)	0	15.55 ± 0.54[a]	14.73 ± 0.37[a]	14.98 ± 0.44[a]	14.87 ± 0.43[a]
	2	14.19 ± 0.29[a]	14.48 ± 0.43[a]	13.15 ± 0.49[a]	12.89 ± 1.09[a]
	4	14.02 ± 0.60[a]	14.22 ± 0.48[a]	14.29 ± 0.32[a]	13.97 ± 0.53[a]
	8	14.90 ± 0.28[a]	14.26 ± 0.58[a]	14.39 ± 0.53[a]	14.46 ± 0.85[a]
	16	15.33 ± 1.18[a]	12.68 ± 0.53[a]	13.26 ± 0.65[a]	12.21 ± 1.38[a]
EPA (C20:5; n-3)	0	1.64 ± 0.07[a]	1.41 ± 0.09[a]	1.60 ± 0.09[a]	1.42 ± 0.08[a]
	2	1.23 ± 0.18[a,b]	1.31 ± 0.15[a]	1.32 ± 0.08[a,b]	1.24 ± 0.16[a]
	4	1.20 ± 0.15[a,b]	1.15 ± 0.08[a,b]	1.07 ± 0.12[a,b]	1.09 ± 0.16[a]
	8	1.09 ± 0.14[a,b]	0.72 ± 0.06[b]	1.07 ± 0.10[a,b]	0.95 ± 0.09[a,b]
	16	0.92 ± 0.12[b]	0.66 ± 0.09[b]	0.75 ± 0.08[b]	0.48 ± 0.13[b]
DHA (C22:6; n-3)	0	0.57 ± 0.06[a]	0.48 ± 0.04[a]	0.45 ± 0.03[a]	0.47 ± 0.04[a]
	2	0.47 ± 0.05[a,b]	0.40 ± 0.06[a]	0.36 ± 0.04[a,b]	0.35 ± 0.06[a,b]
	4	0.48 ± 0.04[a,b]	0.38 ± 0.04[a,b]	0.37 ± 0.03[a,b]	0.47 ± 0.05[a]
	8	0.41 ± 0.02[a,b]	0.19 ± 0.04[b]	0.31 ± 0.04[a,b]	0.28 ± 0.04[a,b]
	16	0.34 ± 0.03[b]	0.19 ± 0.04[b]	0.17 ± 0.04[b]	0.19 ± 0.05[b]

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences (P<0.05).

Table C.2. Fatty acid profile of liver, quadriceps of male guinea pigs

	Time (wk)	SHAM CTRL (n=10)	SHAM CLA (n=10)	ORX CTRL (n=10)	ORX CLA (n=10)
Liver (% total fatty acids)					
CLA (C18:2; c9, t11)	16	0.00 ± 0.00[a]	2.69 ± 0.10[b]	0.00 ± 0.00[a]	2.61 ± 0.07[b]
CLA (C18:2; t10, c12)	16	0.00 ± 0.00[a]	0.38 ± 0.03[b]	0.00 ± 0.00[a]	0.35 ± 0.03[b]
LA (C18:2; n-6)	16	42.03 ± 0.43[a]	39.40 ± 0.52[b]	42.11 ± 0.49[a]	40.05 ± 0.64[a,b]
ALA (C18:3; n-3)	16	2.18 ± 0.26[a]	1.69 ± 0.15[a]	2.13 ± 0.14[a]	1.66 ± 0.15[a]
AA (C20:4; n-6)	16	7.53 ± 0.82[a]	7.84 ± 0.35[a]	7.53 ± 0.82[a]	7.87 ± 0.24[a]
EPA (C20:5; n-3)	16	0.013 ± 0.005[a]	0.014 ± 0.008[a]	0.006 ± 0.004[a]	0.008 ± 0.017[a]
DHA (C22:6; n-3)	16	0.54 ± 0.05[a]	0.52 ± 0.03[a]	0.61 ± 0.02[a]	0.55 ± 0.03[a]
Quadriceps (% total fatty acids)					
CLA (C18:2; c9, t11)	16	0.04 ± 0.02[a]	1.18 ± 0.19[b]	0.00 ± 0.00[a]	1.33 ± 0.10[b]
CLA (C18:2; t10, c12)	16	0.00 ± 0.00[a]	0.25 ± 0.03[b]	0.00 ± 0.00[a]	0.22 ± 0.02[b]
LA (C18:2; n-6)	16	24.88 ± 2.93[a]	25.02 ± 0.53[a]	26.57 ± 1.07[a]	25.64 ± 1.26[a]
ALA (C18:3; n-3)	16	1.56 ± 0.25[a]	1.18 ± 0.17[a]	0.98 ± 0.27[a]	1.44 ± 0.21[a]
AA (C20:4; n-6)	16	5.81 ± 0.66[a]	6.09 ± 0.57[a]	5.77 ± 0.67[a]	5.07 ± 0.44[a]
EPA (C20:5; n-3)	16	0.032 ± 0.002[b]	0.027 ± 0.003[b]	0.041 ± 0.002[a]	0.029 ± 0.001[b]
DHA (C22:6; n-3)	16	0.69 ± 0.09[a]	0.74 ± 0.09[a]	0.67 ± 0.08[a]	0.57 ± 0.07[a]

Data are mean±SEM. Significant differences determined using MIXED model with Tukey post hoc comparisons.
Different letters indicate significant differences ($P<0.05$).

Table C.3. Main effects of diet, surgery and time on whole body, appendicular and axial bone mineral content and density of male guinea pigs

	Diet		Surgery		Time (wk)				
	CTRL	CLA	SHAM	ORX	Baseline	2	4	8	16
Whole body									
WB BMC (g)	37.15±0.48	37.97±0.27	38.19±0.24[a]	36.93±0.49[b]	37.54±0.50	37.66±0.52	37.58±0.47	37.45±0.44	37.59±1.02
WB aBMD (g/cm^2)	0.296±0.014	0.286±0.002	0.284±0.001	0.297±0.014	0.280±0.003	0.321±0.003	0.285±0.003	0.284±0.002	0.283±0.036
Appendicular bone									
L Femur BMC (g)	1.11±0.01	1.12±0.01	1.14±0.01[a]	1.09±0.01[b]	1.12±0.01	1.12±0.02	1.10±0.01	1.12±0.01	1.10±0.02
L Femur aBMD (g/cm^2)	0.657±0.017	0.648±0.006	0.660±0.005	0.645±0.018	0.641±0.009	0.651±0.009	0.645±0.009	0.641±0.007	0.685±0.042
L Tibia BMC (g)	0.86±0.01	087±0.01	0.87±0.01	0.86±0.01	0.85±0.01[a]	0.86±0.01[a]	0.85±0.01[a]	0.86±0.01[a,b]	0.91±0.02[b]
L Tibia aBMD (g/cm^2)	0.409±0.020	0.390±0.002	0.390±0.002	0.410±0.020	0.389±0.004[a]	0.379±0.004[a]	0.383±0.003[a]	0.386±0.004[a]	0.463±0.049[b]
Axial bone									
LS 1-4 BMC (g)	0.78±0.01	0.78±0.01	0.79±0.01	0.77±0.01	0.78±0.01	0.78±0.01	0.78±0.01	0.77±0.01	0.78±0.02
LS 1-4 aBMD (g/cm^2)	0.350±0.002	0.345±0.002	0.351±0.002[a]	0.344±0.002[b]	0.341±0.004	0.349±0.009	0.353±0.009	0.345±0.007	0.351±0.004

Data are mean±SEM. Main effects determined using MIXED model with Tukey post hoc comparisons. Only main effects presented herein. Different letters indicate significant differences ($P<0.05$).