Impact des acides gras sur le métabolisme osseux

Fabien Wauquier

UNIVERSITÉ BLAISE PASCAL **UNIVERSITÉ D'AUVERGNE**

Année 2011

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

N° d'ordre

THÈSE

Présentée à l'Université d'Auvergne
Pour l'obtention du grade de Docteur d'Université

Soutenue le 16 Décembre 2011

WAUQUIER Fabien

IMPACT DES ACIDES GRAS SUR LE METABOLISME OSSEUX

Rapporteurs: Pr Marie-Hélène Lafage-Proust (Université Jean Monnet, St-Etienne)
Pr Ambroise Martin (Université Claude Bernard, Lyon 1)

Membres examinateurs: Dr Jérôme Guicheux (Université de Nantes)
Dr Jean-François Landrier (Faculté de Médecine de la Timone, Marseille)
Dr Jean-Michel Chardigny (INRA Clermont-Ferrand / Theix)

Directeur de thèse: Dr Yohann Wittrant (INRA Clermont-Ferrand / Theix)

INRA, Unité de Nutrition Humaine (UNH) - Equipe ASM-UMR1019

UNIVERSITÉ BLAISE PASCAL **UNIVERSITÉ D'AUVERGNE**

Année 2011

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

N° d'ordre

THÈSE

Présentée à l'Université d'Auvergne
Pour l'obtention du grade de Docteur d'Université

Soutenue le 16 Décembre 2011

WAUQUIER Fabien

IMPACT DES ACIDES GRAS SUR LE METABOLISME OSSEUX

Rapporteurs: Pr Marie-Hélène Lafage-Proust (Université Jean Monnet, St-Etienne)
Pr Ambroise Martin (Université Claude Bernard, Lyon 1)

Membres examinateurs: Dr Jérôme Guicheux (Université de Nantes)
Dr Jean-François Landrier (Faculté de Médecine de la Timone, Marseille)
Dr Jean-Michel Chardigny (INRA Clermont-Ferrand / Theix)

Directeur de thèse: Dr Yohann Wittrant (INRA Clermont-Ferrand / Theix)

INRA, Unité de Nutrition Humaine (UNH) - Equipe ASM-UMR1019

« C'est en forgeant que
l'on devient forgeron…

… et c'est en sciant
que Léonard devint scie. »

Sommaire

Remerciements

Je tiens tout d'abord à adresser mes remerciements à **Véronique Coxam**, pour m'avoir accueilli au sein de son équipe, pour sa disponibilité, sa gentillesse et pour la confiance qu'elle m'a témoignée pendant ces trois années.

Je tiens à exprimer ma reconnaissance aux membres de mon jury de thèse. Je remercie **Marie-Hélène Lafage-Proust** ainsi qu'**Ambroise Martin** d'avoir acceptés d'être rapporteurs de ce travail. Merci également à **Jean-Michel Chardigny**, **Jérome Guicheux** et **Jean-François Landrier**, membres examinateurs. Merci beaucoup.

Merci également à **Jean-Marc Lobaccaro** pour avoir pensé à moi il y a trois ans et avoir proposé ma candidature pour la réalisation de ce travail de thèse.

Mes plus vifs remerciements sont bien sûr destinés à **Yohann Wittrant**, mon directeur de thèse, avec qui j'ai eu la chance de travailler en collaboration étroite pendant ces trois ans. Merci de m'avoir permis de participer à ce projet et de m'avoir fait confiance, d'avoir su me guider tout en tenant compte de mes opinions. Un très grand merci !

Un merci tout particulier à **Laurent Léotoing** pour sa gentillesse, pour ces précieux conseils qui m'ont beaucoup aidé ainsi que pour nos discussions sans fin sur la meilleure façon de procéder.

Un grand merci également à tous les autres membres de l'équipe ASM : **Marie-Jeanne**, **Patrice**, **Sylvie** et **Jean-Claude** pour votre important soutien aussi bien technique que moral et pour votre gentillesse au quotidien.

Je n'oublie pas mes collègues étudiants que j'ai croisés au sein de l'équipe pendant ces trois ans : **Anna**, **Véronique**, **Claire**, **Mélanie**, **Camille**, **Jérémy**, **Audrey**, **Fanny**. Merci à tous pour les bons moments partagés et bonne continuation au sein de l'équipe à **Claire, Mélanie, Camille et Audrey** (Ne riez pas ce sera bientôt votre tour…)

Globalement, un grand merci à l'équipe **ASM** dans son ensemble, pour sa convivialité et pour les soirées barbeuk, bowling, poker (merci de m'avoir laissé gagner à chaque fois !).

Merci aussi aux **footeux de l'INRA** : Gronaldo, Valentin, Nico, Sylvain, Julien, Chico, Souley, Audrey, Camille, Sghaier, Georges, Lahlou, Hamid, Souk, Jérémi, Titi, Vincent et les autres pour tous les bons moments partagés au cours de nos

joutes du jeudi soir, même si je pars avec le regret de ne pas avoir ramené avec vous cette fichue coupe….

Merci à tous les membres de l'**UNH** qui ont pu me fournir de l'aide à un moment ou à un autre dans mon travail ou avec qui j'ai simplement pu prendre plaisir à discuter.

Un merci tout particulier à mon **Aurélie** qui m'a soutenu au quotidien pendant ces trois années et qui part avec moi vers de lointaines contrées.

Merci enfin à toutes les personnes que je ne nommerai pas ici mais qui ont contribué, d'une manière ou d'une autre à ce travail.

Résumé

Rôle des acides gras dans le métabolisme osseux

Dans le contexte actuel d'allongement de l'espérance de vie, la prévalence des maladies liées à l'âge telles que l'ostéoporose est de plus en plus importante. Le coût de la prise en charge de ces pathologies constitue un problème de santé public majeur et la mise en place de stratégies de prévention nutritionnelle adaptées apparaît comme une excellente solution alternative aux traitements habituels. Pourtant, l'étude des activités biologiques des nutriments reste trop marginale pour certains tissus et certaines catégories de molécules, c'est notamment le cas du tissu osseux et des lipides, en particulier les acides gras. Ces derniers sont pourtant capables de moduler le devenir du tissu osseux que ce soit indirectement par des mécanismes systémiques ou directement au niveau de la cellule osseuse.

La perte osseuse liée au vieillissement est souvent associée à l'établissement progressif d'une inflammation chronique à bas bruit à l'échelle de l'organisme. Dans ce contexte, les niveaux de cytokines pro-inflammatoires telles que l'interleukine 6 ou le Tumor Necrosis Factor α sont augmentés et ces composés, qui sont connus pour induire la résorption osseuse, pourraient contribuer à la dégradation du squelette.

Notre hypothèse de travail était que certains Acides Gras Poly-Insaturés (AGPI) à propriétés anti-inflammatoires pouvaient éventuellement limiter l'inflammation chronique associée au vieillissement et ainsi contribuer à préserver le capital osseux. Dans cette optique, nous avons utilisé la souris SamP8 qui est un modèle de *progeria* présentant, à douze mois, un phénotype ostéoporotique. Dans ce modèle, l'administration d'un régime délétère à base d'huile de tournesol (ratio acide gras ω6 /ω3 très important) aggrave la perte osseuse en association avec une augmentation des marqueurs inflammatoires systémiques et osseux ainsi qu'une augmentation des marqueurs de la résorption osseuse. La supplémentation de ce régime tournesol avec de l'huile de bourrache ou de l'huile de poisson permet d'apporter des quantités importantes d'AGPI anti-inflammatoires (acide γ-linoléique pour l'huile de bourrache et ω3 pour l'huile de poisson). De manière intéressante, ces deux régimes supplémentés permettent, en plus de réduire les paramètres inflammatoires, de s'opposer à l'augmentation des marqueurs de résorption osseuse et de limiter la diminution de la densité minérale de l'os chez ces souris. Cette étude met donc en évidence le potentiel santé de certains AGPI au regard de la préservation du capital osseux et suggère un rôle déterminant de leurs propriétés anti-inflammatoires systémiques.

En parallèle, la description des effets directs des acides gras au niveau cellulaire par l'activation de récepteurs spécifiques occupent une place croissante dans la littérature. Récemment, le récepteur membranaire GPR40 (G Protein coupled Receptor 40) a été mis en évidence pour ses interactions avec les acides gras libres à longues chaînes et nous avons pu montrer son expression dans les précurseurs ostéoclastiques. Nous avons donc émis l'hypothèse que ce récepteur pourrait jouer un rôle dans la médiation des effets des acides gras sur les paramètres du remodelage osseux. Ce travail a permis de mettre en évidence l'existence d'un phénotype ostéoporotique chez la souris invalidée pour le GPR40. L'effet protecteur de ce récepteur semble lié principalement à un effet inhibiteur de son activation sur la différenciation des ostéoclastes. En effet, l'utilisation de l'agoniste spécifique du GPR40 empêche *in vitro* la différenciation ostéoclastique par RANKL de deux modèles cellulaires de manière GPR40-dépendante. De surcroit, cet agoniste est également capable *in vivo* de s'opposer à une perte osseuse induite chez la souris. Ces résultats révèlent pour la première fois l'implication du récepteur GPR40 dans la physiologie osseuse et apportent la connaissance d'une nouvelle possibilité de modulation directe des cellules osseuses par les acides gras.

Abstract

Role of fatty acids in bone metabolism

With increasing lifespan, prevalence of age-related complications has grown including skeletal defects such as osteoporosis. Socio-economic consequences of these disorders represent a major public health problem worldwide and in this context, nutritional prevention strategies may be considered as a good option to be associated with usual therapies. However, biological activities of some nutrients have been too poorly deciphered in regards to their bone health potential. This is notably true concerning fatty acids and both their direct and indirect relationships with the skeleton.

Age-related bone complications are often associated with a gradual establishment of a systemic low-grade inflammatory condition. This leads to high levels of pro-inflammatory cytokines such as interleukin 6 or tumor necrosis factor α which are known to favour osteoclast-induced bone resorption. Then, these high pro-inflammatory cytokines levels may contribute to age associated bone loss.

We hypothesize that in this context, poly-unsaturated fatty acids (PUFA) with known anti-inflammatory properties may counteract both the low-grade inflammation establishment and the associated bone loss. Thus, animals from the *progeria* mouse model SamP8 were used and were shown to exhibit osteoporosis at twelve months. Feeding these mice with a deletary (very high ω6/ω3 ratio) sunflower oil-based diet results in an exacerbated bone loss, associated with increased systemic and bone inflammation parameters as well as increased bone resorption markers. Either borage or fish oil supplementation of the sunflower oil-based diet result in high amounts of anti-inflammatory PUFAs in the diet (γ-linoleic acid with borage oil and ω3 with fish oil). Interestingly, mice fed with the two "supplemented" diets show reduced inflammatory parameters but also reduced levels of bone resorption markers and preserved bone mineral density. In this work, the bone health potential of some PUFAs was established and was linked to their systemic anti-inflammatory properties.

Besides, fatty acids are increasingly studied as signalling molecules, able to modulate cell signalling by their interactions with specific receptors. A few years ago, long chain fatty acids were shown to be ligands of the membrane bound fatty acid receptor GPR40 (G Protein coupled Receptor 40). As we showed GPR40 expression in osteoclast precursors, we hypothesized that it may be involved in fatty acid-induced bone remodelling regulation. In this study, an osteoporotic phenotype was found in GPR40-deficient mice. This GPR40-mediated bone protection seems to involve an anti-osteoclastogenic effect. As a matter of fact, a GPR40 specific agonist led to blunted RANKL-induced osteoclastic differentiation in a GPR40-dependant way. In addition, this agonist was also able *in vivo* to counteract ovariectomy-induced bone loss in mice. Thus, involvement of GPR40 in bone remodelling was established for the first time in this work, bringing to light a new mechanism in the fatty acid-induced modulation of bone metabolism.

Liste des publications

Articles Scientifiques :

* **Wauquier F,** Philippe C, Léotoing L, Poitout V, Mercier S, Davicco MJ, Lebecque P, Guicheux J, Pilet P, Poitout V, Coxam V, Wittrant Y. *The free fatty acid receptor GPR40 protects from bone loss through inhibition of osteoclast differentiation.* 2011 En Préparation ***(Article Numéro 3)***

* **Wauquier F,** Barquissau V, Léotoing L, Davicco MJ, Lebecque P, Mercier S, Philippe C, Miot-Noirault E, Chardigny JM, Morio B, Wittrant Y, Coxam V. *Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis.* Bone. 2011 Jun 6. ***(Article Numéro 2)***

***** Bourgine A, Beck L, Khoshniat S, **Wauquier F,** Oliver L, Hue E, Alliot-Licht B, Weiss P, Guicheux J, Wittrant Y. *Inorganic phosphate stimulates apoptosis in murine MO6-G3 odontoblast-like cells.* Arch Oral Biol. 2011 Mar 22. ***(Annexe 3)***

Revues de synthèse :

***** **Wauquier F,** Philippe C, Léotoing L, Spilmont M, Coxam V, Wittrant Y. *Fatty acids and bone relationships : "I love you… Neither do I"*. 2011 En préparation ***(Article Numéro 1)***

***** **Wauquier F,** Coxam V, Wittrant Y. *[Lipidic nutrition, inflammation and bone] Article in french.* Nutrition – Santé 2011

* **Wauquier F,** Leotoing L, Coxam V, Guicheux J, Wittrant Y. *Oxidative stress in bone remodelling and disease.* Trends Mol Med. 2009 Oct;15(10):468-77. ***(Annexe 1)***

* Coxam V, Davicco MJ, **Wauquier F,** Wittrant Y. *[Vitamin K and bone physiology] Article in french.* Cahiers de nutrition et de diététique (2009) 44, 163-172 ***(Annexe 2)***

Communications en congrès

09/2011 The role of the fatty acid receptor GPR40 in bone remodelling. *American Society for Bone and Mineral Research annual meeting,* San Diego, CA - **Poster**

09/2011 Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis. *American Society for Bone and Mineral Research annual meeting,* San Diego, CA - **Poster**

05/2011 Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis. *Société Française de Biologie des Tissus Minéralisés.* Paris, France - **Poster**

05/2011 The role of the fatty acid receptor GPR40 in bone remodelling. *Société Française de Biologie des Tissus Minéralisés,* Paris, France – **Communication orale**

04/2011 Implication of a membrane bound fatty acid receptor in bone remodelling. *Journées de l'école doctorale de Clermont Ferrand,* Clermont-Ferrand, France – **Communication orale**

10/2010 Implication of a membrane bound fatty acid receptor in bone remodelling. *Journée du Centre de Recherche en Nutrtion Humaine Auvergne,* Clermont-Ferrand, France – **Communication orale**

10/2010 Lipidic nutrition and age-related metabolic stress: Impact on locomotor system physiology. *Journée du Centre de Recherche en Nutrtion Humaine Auvergne,* Clermont-Ferrand, France – **Poster**

06/2010 Implication of a membrane bound fatty acid receptor in bone remodelling. *Société Française de Biologie des Tissus Minéralisés,* St Etienne, France - **Communication orale**

10/2009 Lipidic nutrition and bone lipotoxicity. *Journée du Centre de Recherche en Nutrtion Humaine Auvergne*. Clermont-Ferrand, France – **Poster**

03/2009 Implication of membrane-bound fatty acid receptors in bone remodelling: Bone lipotoxicity? *Société Française de Biologie des Tissus Minéralisés,* Nice, France - **Communication orale**

10/2008 Lipidic Nutrition and age-associated metabolic stress: Impact on bone physiology and adipose tissue interactions. *Journée du Centre de Recherche en Nutrtion Humaine Auvergne,* Clermont-Ferrand, France – **Communication orale**

Liste des abréviations

AGPI : Acide Gras Poly-Insaturé

AKT : Protéine Kinase B

AP-1 : Activator Protein 1

AR : Androgen Receptor (Récepteur des Androgènes)

ATP : Adénosine Triphosphate

BMU : Basic Multicellular Unit (Unité Multicellulaire de Base)

C/EBP : CCAAT/Enhancer Binding Protein

c-src : cellular-src

DEXA : Dual energy X-ray absorptiometry (absorptiométrie biphotonique à rayon X)

DHA : Docosahexaenoic Acid (Acide Docosahéxaénoique)

DLX : Distal-less homeobox

EPA : Eicosapentaenoic Acid (Acide Eicosapentaénoïque)

ERK : Extracellular signal-Regulated Kinase

FRAX : Fracture-Risk Assessment Tool

FSH : Follicle Stimulating Hormone

GPR : G Protein coupled Receptor

GPR40 : G Protein coupled Receptor 40

GPR120 : G Protein coupled Receptor 120

IGF-1 : Insulin-like Growth Factor 1

Iκ-B : Inhibitor of NF-κB

IKK : Iκ-B kinase

JNK : c-Jun N-terminal Kinase

JNKK : JNK-activating Kinase

LTCC : L-Type Calcium Channel (Canal Calcique de type L)

M-CSF : Macrophage-Colony Stimulating Factor

MAPK : Mitogen Activated Protein Kinase

MEF : Myeloid Elf-1-like Factor

MEK : MAPK/ERK kinase

MITF : Microphthalmia-associated Transcription Factor

MKK : Mitogen-Activated Protein Kinase Kinase

MRF : Muscle Regulatory Factor

MSX2 : Muscle Segment homeobox 2

NF-κB : Nuclear Factor Kappa B

NFATc1 : Nuclear Factor of Activated T cells, calcineurin-dependent 1

OMS : Organisation Mondiale de la Santé

OPG : Ostéoprotégérine

PDK1 : Pyruvate Dehydrogenase Kinase isozyme 1

PI3K : Phosphoinositide 3-kinase

PKB : Protéine Kinase B

PLC : Phospholipase C

PPAR : Proliferator-Activated Receptor

PTH : Parathormone

PUFA : Poly-unsaturated fatty acids

RANK : Receptor Activator of Nuclear Factor κ B

RANKL : Receptor Activator of Nuclear Factor κ B Ligand

Runx2 : Runt-domain transcription factor 2

SamP8 : Senescence Accelerated Mouse Prone 8

SERM : Selective estrogen receptor modulators

Sox : SRY-type HMG box

SP7 : = Osterix

TAB1 : TAK1 Binding Protein 1

TAK1 : TGF-β Activated Kinase 1

TGF-β : Transforming Growth Factor β

TLR : Toll-like Receptor

TNF-α : Tumor Necrosis Factor α

TRAF : TNF-Receptor Associated Factor

VDR : Vitamin D Receptor (Récepteur de la Vitamine D)

VLDL : Very Low Density Lipoprotein

Liste des illustrations

Première partie :

Analyse bibliographique

1- Le tissu osseux

1.1 Fonctions du tissu osseux

Que ce soit d'un point de vue biomécanique ou métabolique, le tissu osseux revêt une importance capitale pour l'organisme. C'est un tissu hautement spécialisé, caractérisé par sa dureté et une apparente rigidité mais qui n'est pas pour autant figé. A l'inverse, il s'agit d'une structure dynamique soumise à un remaniement perpétuel par le biais de cellules spécialisées : il est généré en continu par les ostéoblastes, contrôlé et modifié par l'action des ostéocytes et dégradé par les ostéoclastes. Ces propriétés lui permettent de s'autoréparer en cas de fractures, d'adapter sa masse, sa forme, et son architecture à différentes contraintes mécaniques et ainsi supporter une activité physique tout au long de la vie tout en gardant sa solidité. De fait, le tissu osseux est le support mécanique essentiel du squelette qui va permettre à cet organe d'assurer la locomotion, de transmettre les forces issues de la contraction musculaire pendant le mouvement et d'assurer la protection des organes internes (Pettifor J, Prentice A et al. 2003).

Par ailleurs, il joue également un rôle extrêmement important dans le maintien de l'homéostasie ionique de l'organisme. En effet, c'est un réservoir métabolique de calcium et de phosphore qui va pouvoir être sollicité en cas de besoin afin de réguler la composition du fluide extracellulaire.

Enfin, il a récemment été mis en évidence que ce tissu possédait également des activités endocrines lui conférant un rôle dans la régulation de l'excrétion rénale du phosphate et du métabolisme énergétique. (Fukumoto and Martin 2009)

1.2 Les différents types d'os du squelette humain

Sur la base de leur morphologie, on distingue principalement trois catégories d'os (Torotra and Grabowski 2002) **(Fig.1)** :

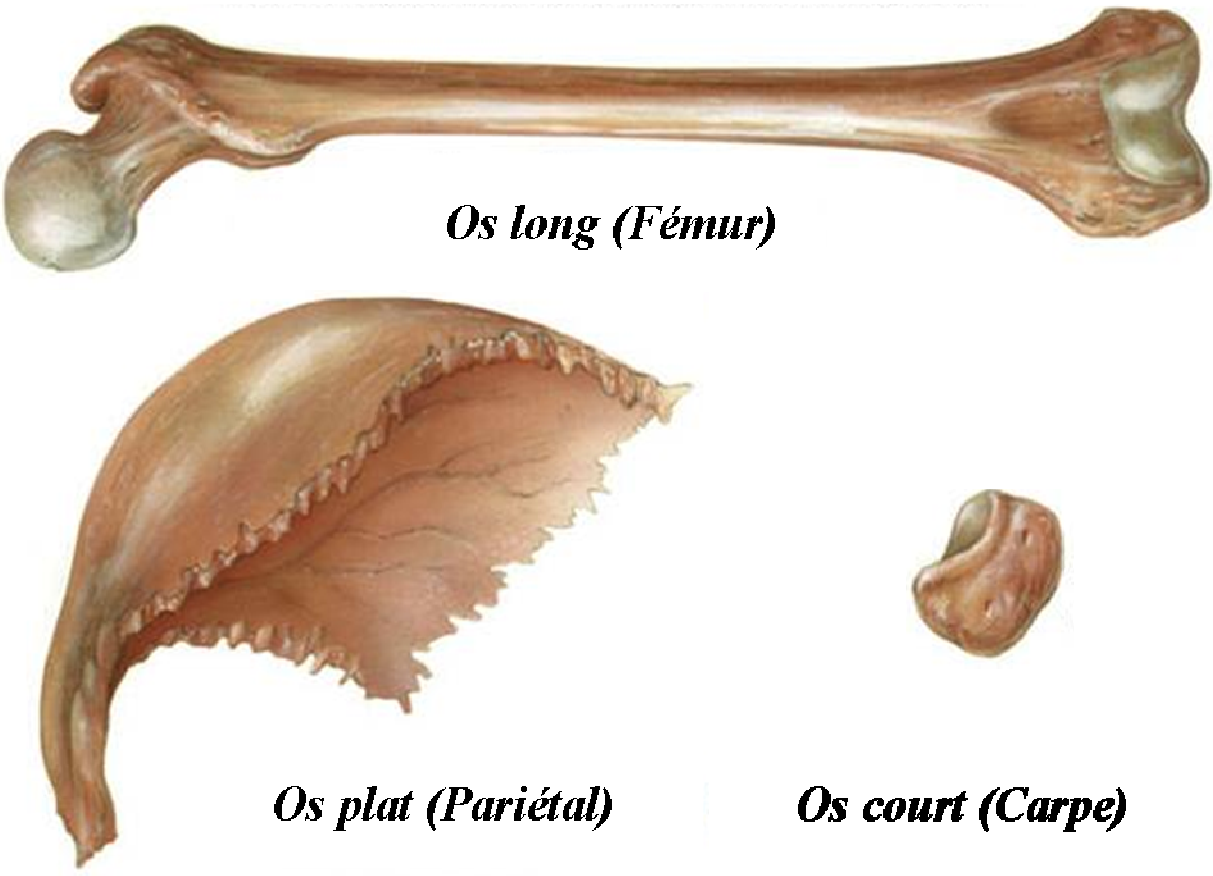


Figure 1 : Les différentes morphologies des os du squelette humain

- Les os courts qui ont leurs trois dimensions sensiblement égales.
- Les os plats qui ont une dimension nettement plus courte que les deux autres.
- Les os longs qui présentent une dimension bien plus importante que les deux autres. Ils sont constitués d'une partie centrale cylindrique appelée diaphyse, et de deux extrémités élargies et arrondies appelées épiphyses, couvertes de cartilage articulaire. Des régions coniques, appelées métaphyses, connectent la diaphyse à chaque épiphyse. Chez l'animal en croissance, l'épiphyse est séparée de la métaphyse par une couche intermédiaire de cartilage hyalin où les chondrocytes subissent d'intenses divisions. Il s'agit de la plaque de croissance

métaphysaire ou épiphysaire. Chez l'adulte, la plaque de croissance est remplacée par de l'os spongieux, ce qui provoque la fusion de l'épiphyse et de la métaphyse. La disparition du cartilage suite à la fusion de deux masses de tissu spongieux est appelée soudure (ou fermeture) des plaques de croissance **(Fig.2).**

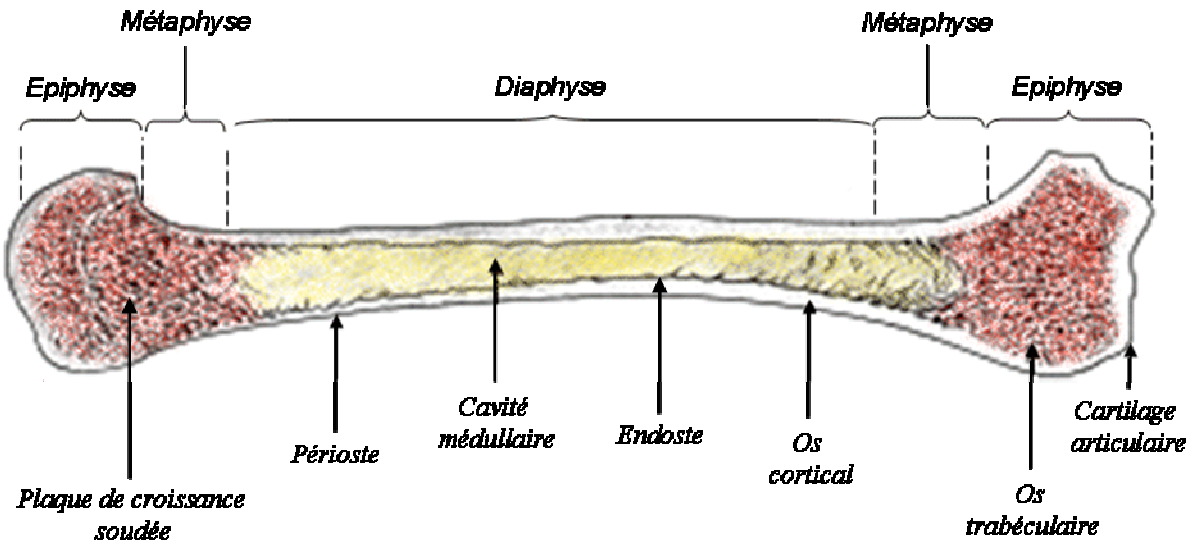


Figure 2 : Structure d'un os long

1.3 Structure macroscopique du tissu osseux

1.3.1 Structure interne

Au sein d'un même os, on peut distinguer deux types de structure osseuse sur la base de leur organisation et de leur porosité **(Fig.3)**.

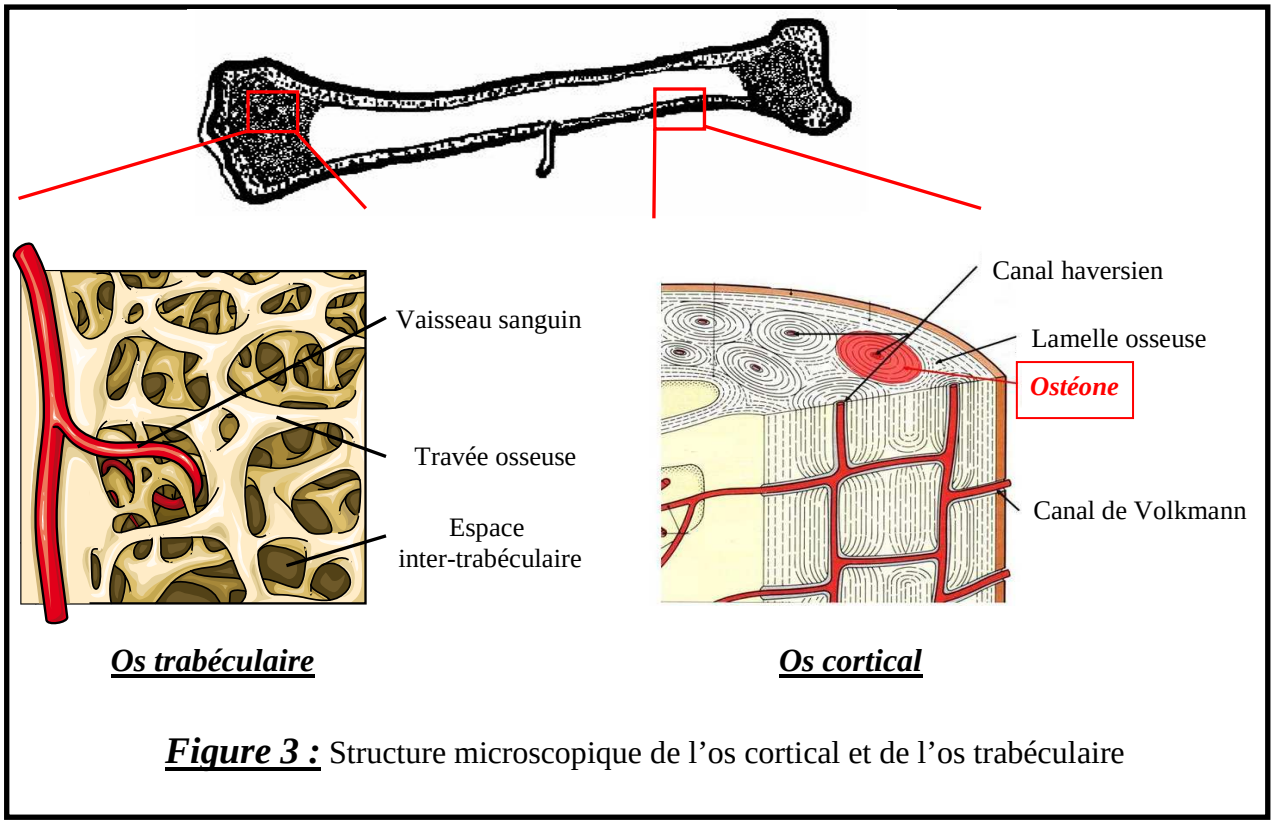


Figure 3 : Structure microscopique de l'os cortical et de l'os trabéculaire

L'os cortical est une structure compacte (porosité comprise entre 5 et 30 %) que l'on retrouve surtout au niveau de la « paroi » de la diaphyse des os longs et qui donne son aspect blanc, lisse et solide à l'os. La structure est constituée d'un réseau d'ostéones. Un ostéone est formé par des lames osseuses concentriques disposées autour d'un canal haversien par où passent des capillaires sanguins et des fibres nerveuses. Ces canaux haversiens sont reliés entre eux, avec la surface de l'os et avec la moelle osseuse par des canaux transversaux ou obliques (canaux de Volkmann). Chaque ostéone est aligné parallèlement à l'axe de la diaphyse avec un trajet légèrement hélicoïdal. Entre les ostéones, se trouvent des lamelles osseuses provenant d'ostéones plus anciens résorbés. L'os cortical représente 80 % de la masse d'un squelette adulte (Bringhurst, Demay et al. 2005).

L'os spongieux ou trabéculaire est une structure plus lâche (porosité comprise entre 30 et 90 %) située au centre de la diaphyse, dans les régions épiphysaires et métaphysaires des os

longs ainsi qu'au sein des os courts et des os plats. La structure est formée par un réseau en trois dimensions de trabécules de tissu osseux, ramifiées et anastomosées. C'est dans ce réseau que prennent place la moelle osseuse et des vaisseaux. L'os trabéculaire ne représente donc que 20 % de la masse osseuse mais sa surface totale est environ dix fois supérieure à celle de l'os cortical (Bringhurst, Demay et al. 2005).

1.3.2 Enveloppes osseuses

Le périoste constitue une enveloppe externe de l'os qu'il recouvre entièrement à l'exception des zones correspondant aux points d'ancrages des ligaments et tendons au niveau des surfaces articulaires. C'est une structure très vascularisée, organisée en deux feuillets. Le feuillet externe correspond au périoste fibreux qui est formé par un réseau dense de fibres de collagène. Le feuillet interne quant à lui est appelé périoste cellulaire car il abrite des cellules souches mésenchymateuses, des ostéoblastes et des ostéoclastes. Ces populations cellulaires jouent un rôle important pendant le développement en permettant une croissance de l'os en épaisseur ainsi qu'au niveau de l'os « adulte » où elles assurent son renouvellement.

Au niveau de la face interne de l'os, on trouve une autre enveloppe qui est l'endoste. Il existe l'endoste cortical qui tapisse l'os compact au niveau de la cavité médullaire, l'endoste trabéculaire sur les travées osseuses bordant la moelle ainsi que l'endoste ostéonien qui tapisse les canaux haversiens. Dans tous les cas, on retrouve au niveau de cette enveloppe les mêmes populations cellulaires que celles observées au niveau du périoste cellulaire. Cette similitude vient de l'existence d'une continuité entre l'endoste et le périoste cellulaire via des canaux (canaux de Volkman) au niveau des canaux haversiens (Toppets, Pastoret et al. 2004).

1.4 Composition du tissu osseux

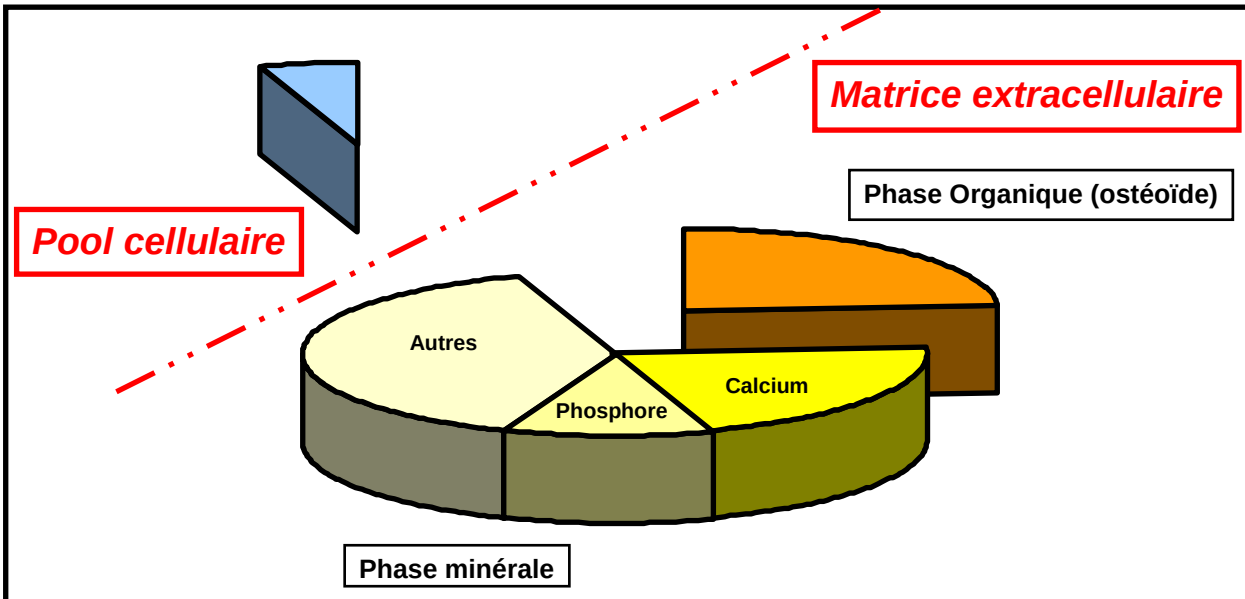


Figure 4 : Composition du tissu osseux

1.4.1 La matrice extracellulaire

Le tissu osseux est constitué, dans sa grande majorité, par de la matrice extracellulaire. Celle-ci occupe en effet entre 92 et 95 % du volume tissulaire. Elle est constituée d'une phase organique (25 %) et d'une phase inorganique (75 %) **(Fig.4).**

La phase organique de la matrice extracellulaire représente donc environ 20 % de la masse osseuse et forme l'ostéoïde qui est en fait la substance à partir de laquelle va pouvoir s'accomplir la minéralisation de l'os.

L'ostéoïde est constitué à 90 % de substance fibrillaire incluant des protéines structurales (collagène et élastine) ou adhérentes (fibronectine). Le collagène de type 1 est très largement majoritaire au sein de cette substance fibrillaire et se trouve donc être le constituant essentiel de l'ostéoïde. Il est formé de l'assemblage de trois chaînes alpha de polypeptides synthétisées par l'ostéoblaste et excrétées par celui-ci afin de former un réseau fibreux régulier. Ce réseau de collagène va servir de support à la minéralisation de l'os puisque c'est sur celui-ci que vont se fixer les cristaux d'hydroxyapatite. Par ailleurs l'orientation contrôlée des fibres de collagène va déterminer la résistance de l'os aux forces de tensions. La substance interfibrillaire correspond aux 10 % restants et englobe des glycosaminoglycanes, des protéoglycanes, quelques lipides et des petites protéines non collagéniques. Ces dernières,

comme l'ostéopontine, l'ostéonectine, l'ostéocalcine et les sialoprotéines ont un rôle clef dans la régulation de la formation de la matrice osseuse (Toppets, Pastoret et al. 2004).

Comme évoqué précédemment, une des grandes fonctions du tissu osseux est le stockage de sels minéraux. Cette réserve est en fait constituée par la partie inorganique de la matrice extracellulaire osseuse qui représente près de 70 % de la masse osseuse. Les sels minéraux qui y sont les plus abondants sont le calcium (27 %) et le phosphore (12 %) dans un ratio égal à 1,66 (Toppets, Pastoret et al. 2004). Cette phase inorganique est donc constituée par la minéralisation de l'ostéoïde formé au préalable. Le processus de minéralisation correspond à un dépôt de sels minéraux au sein de la matrice organique et majoritairement de calcium et de phosphate qui vont précipiter pour former les cristaux stables d'hydroxyapatite (Ca10 (PO4) 6(OH) 2). La formation de ces cristaux n'est possible qu'en présence de concentrations très élevées de Ca^{2+} et de PO_4^-.

Ces concentrations sont obtenues grâce à l'activité des cellules ostéoformatrices. Les ostéoblastes génèrent des vésicules qui vont bourgeonner au niveau de leur pôle apical, et se déposer dans la matrice **(Fig.5)**.

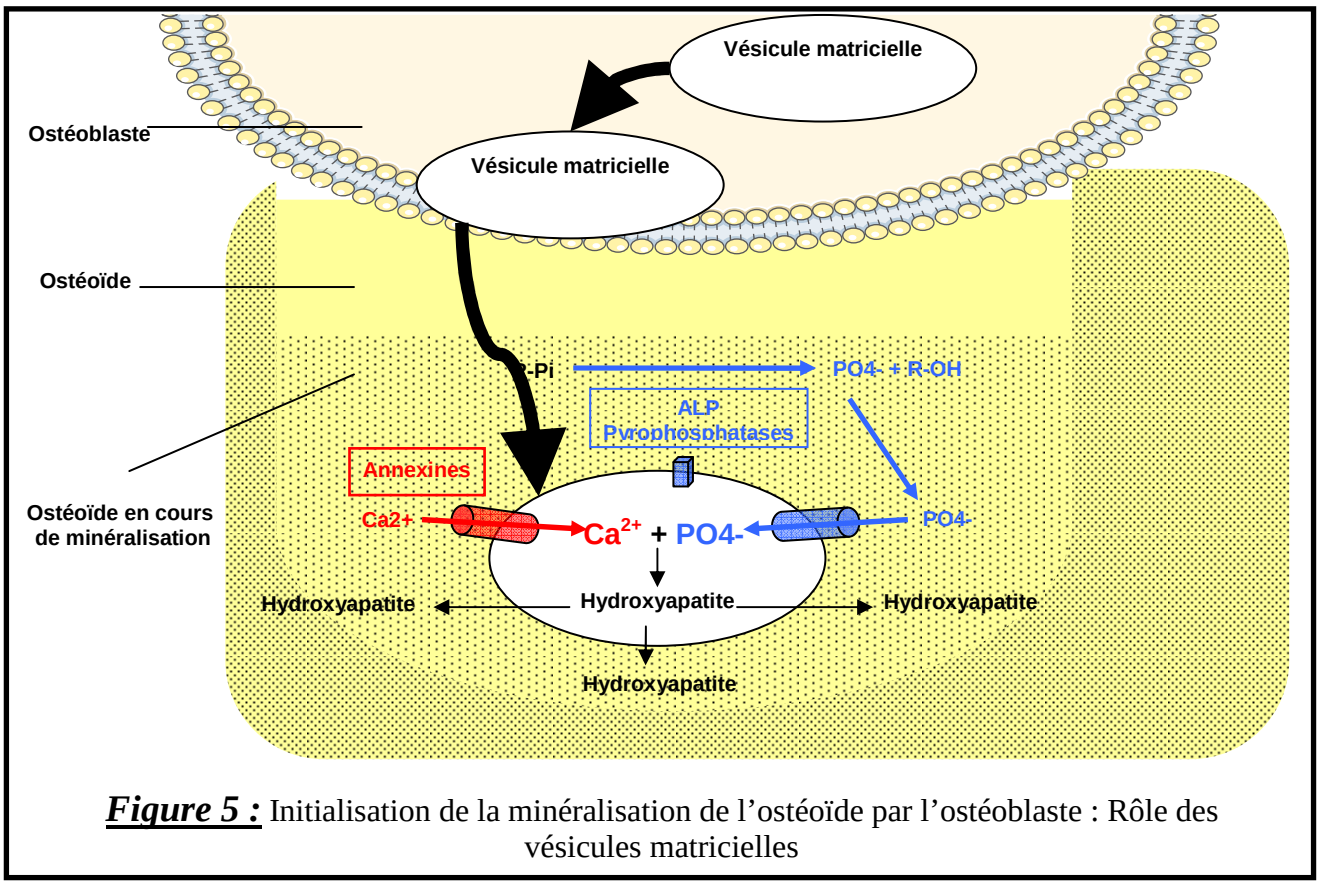


Figure 5 : Initialisation de la minéralisation de l'ostéoïde par l'ostéoblaste : Rôle des vésicules matricielles

Ces vésicules sont riches en phosphatases alcalines et en annexines ce qui va permettre l'accumulation de Ca^{2+} et de PO_4^-. La formation des cristaux commence au sein de la vésicule puis ceux-ci vont progressivement en sortir au cours de leur croissance et ainsi entrer en contact avec l'ostéoïde (Orimo 2010). Les vésicules contiennent également des pyrophosphatases qui vont « récupérer » les ions PO_4^- à partir de molécules plus grosses. Enfin, l'ostéocalcine ainsi que d'autres protéines non collagéniques, qui sont des composantes de l'ostéoïde sécrété préalablement, pourraient moduler le processus de minéralisation, notamment par la captation d'ions Ca^{2+} extracellulaires (Boskey 1998).

Ces différents mécanismes permettent donc la précipitation initiale des cristaux d'hydroxyapatite qui sera suivie par une augmentation de leur taille par un phénomène d'accrétion et par une confluence des différents foyers de cristaux.

Le calcium et le phosphate ne sont pas les seuls constituants de la matrice, on y trouve également du sodium, des carbonates du magnésium, du zinc ou du fluor (Toppets, Pastoret et al. 2004).

1.4.2 Les cellules osseuses

Il y a quatre principaux types cellulaires qui vont permettre la formation et l'entretien du tissu osseux. Il s'agit des ostéoblastes, des ostéocytes, des cellules bordantes et des ostéoclastes.

1.4.2.1 Les ostéoblastes

Les précurseurs des ostéoblastes sont les cellules souches mésenchymateuses du stroma médullaire. Ces dernières sont des cellules pluripotentes qui ont la capacité de se différencier en ostéoblastes, en chondrocytes, en adipocytes ou en myoblastes. C'est l'expression de facteurs de transcription spécifiques qui amène ces cellules à entrer dans la lignée ostéoblastique, devenant ainsi des cellules ostéoprogénitrices **(Fig.6)**. Celles-ci subissent une importante phase de différenciation au cours de laquelle elles expriment progressivement les gènes nécessaires à l'activité de la cellule mature. (Marie 2001) Au cours de cette maturation, ces précurseurs sont également amenés au contact de la matrice osseuse par migration.

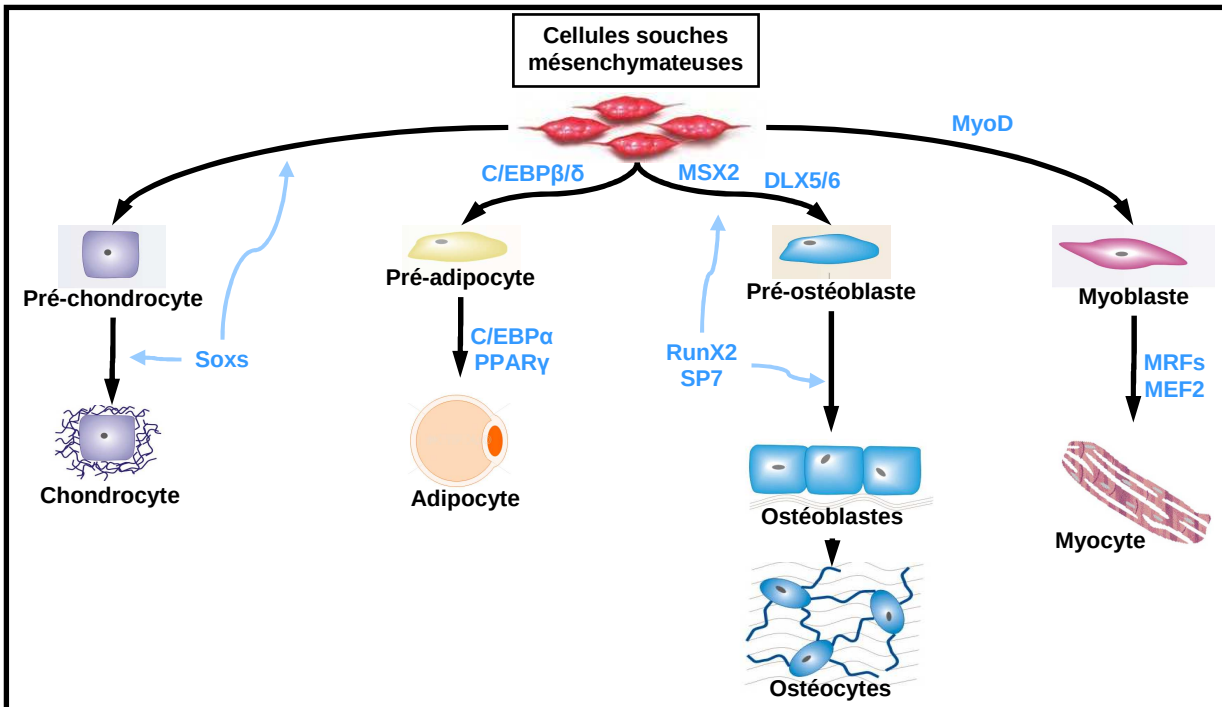


Figure 6 : Origine cellulaire des ostéoblastes, différenciation à partir des cellules souches mésenchymateuses

(C/EBP : CCAAT/Enhancer Binding Protein ; **PPAR :** Peroxysome Proliferator-Activated Receptor ; **Sox :** SRY-type HMG box ; **MSX2 :** Muscle Segment homeobox 2 ; **DLX :** Distal-less homeobox ; **RunX2 :** Runt-domain *transcription* factor 2 ; **SP7** = Osterix ; **MRF :** Muscle Regulatory Factor ; **MEF :** Myeloid Elf-1-like Factor **)**

Comme évoqué précédemment, l'ostéoblaste a pour fonction principale de synthétiser la matrice osseuse (production du collagène de type 1 et de protéines non collagéniques) et d'en contrôler la minéralisation (régulation des concentrations locales en Ca^{2+} et PO_4^- pour permettre la précipitation des cristaux d'hydroxyapatite) (Boskey 1998; Orimo 2010).

1.4.2.2 Les ostéocytes

Une fois leur tâche accomplie, une large majorité des ostéoblastes meurent par apoptose. Cependant, une partie d'entre eux se retrouvent emprisonnés dans la matrice qu'ils ont générée et se différencient alors en ostéocytes. Ces cellules sont reliées entre elles et avec les cellules de la surface osseuse par des extensions de la membrane cytoplasmique **(Fig.7)**.

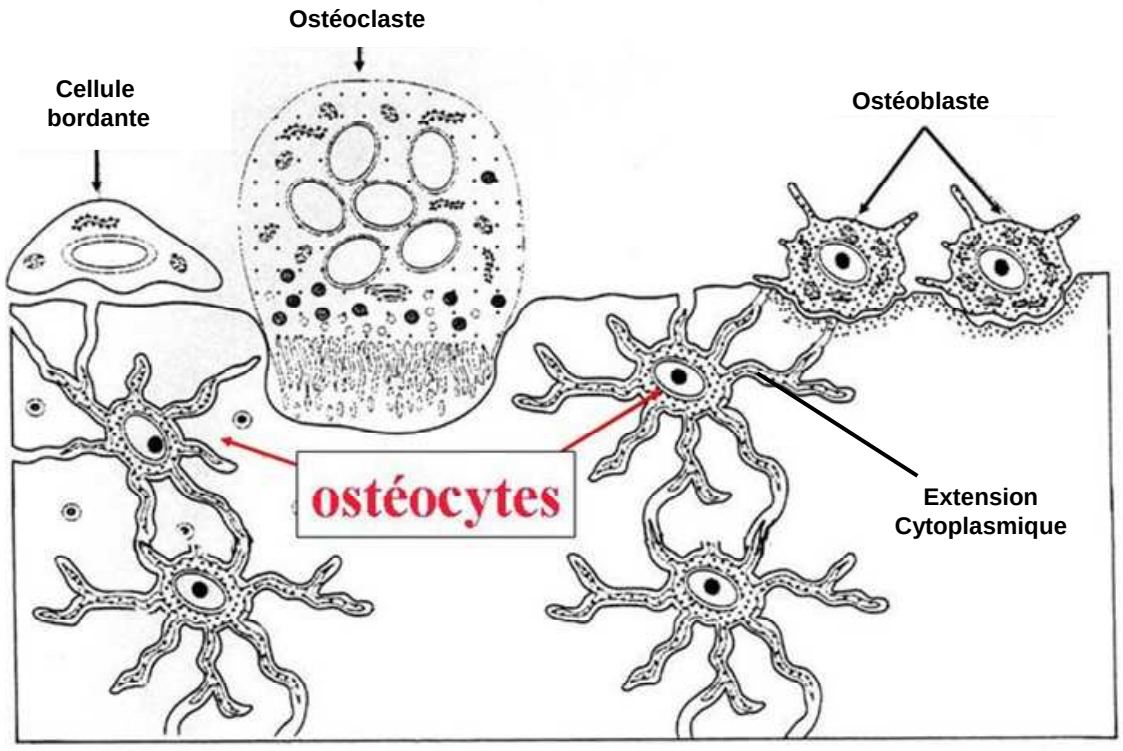


Figure 7 : Le réseau ostéocytaire (Nataf 2009)

Les ostéocytes sont les cellules les plus abondantes de l'os. Elles sont espacées régulièrement dans la matrice en formant un réseau de communication. Ce sont des cellules sensibles aux stimuli mécaniques qui détectent le besoin d'une augmentation ou d'une diminution de la formation osseuse dans le processus d'adaptation fonctionnelle ou en cas de micro fractures (Manolagas 2000).

1.4.2.3 Les cellules bordantes

Une autre partie des ostéoblastes qui ont accompli leur fonction d'ostéogénèse se transforme en cellules bordantes. Ce sont des cellules plates et allongées qui se trouvent à la surface de l'os à l'état quiescent. Ces cellules constituent une barrière entre l'os et la moelle osseuse. Elles sont en contact et peuvent communiquer avec les ostéocytes, les cellules stromales et les ostéoclastes. Elles constituent une réserve de cellules ostéoblastiques pouvant être mobilisées suite à l'action de facteurs locaux ou systémiques notamment au cours du remodelage et de la réparation osseuse (Parfitt, Mundy et al. 1996).

1.4.2.4 Les ostéoclastes

Les précurseurs des ostéoclastes sont des cellules souches hématopoïétiques de la lignée monocyte/macrophage. L'ostéoclaste mature est une cellule géante multinucléée, pouvant atteindre 100µM de diamètre qui est issue de la fusion de plusieurs préostéoclastes. L'ostéoclaste est amené sur son lieu d'action via la circulation sanguine. En son sein, on trouve des quantités importantes de phosphatases acides résistantes au tartrate contenues dans les lysosomes. La morphologie des ostéoclastes se caractérise par une bordure en brosse formée par des extensions "en doigts" de la membrane cytoplasmique.

La fonction de la bordure en brosse est la destruction de l'os. Autour d'elle se trouve une zone de la membrane plasmique appelée « zone de scellement » qui établit l'ancrage de la cellule à la matrice osseuse via des intégrines. Cette configuration crée un espace fermé qui va permettre l'établissement d'un micro-environnement nécessaire à la résorption osseuse.

La phase minérale de la matrice osseuse est dissoute par des ions H^+ sécrétés par des pompes H^+ - ATPase situées au niveau de la bordure en brosse alors que la phase organique de la matrice est dégradée par l'action des phosphatases acides, de métalloprotéinases et de cathepsines. La durée de vie des ostéoclastes est de 15 jours en moyenne, ils meurent ensuite par apoptose (Baron 2008) **(Fig.8).**

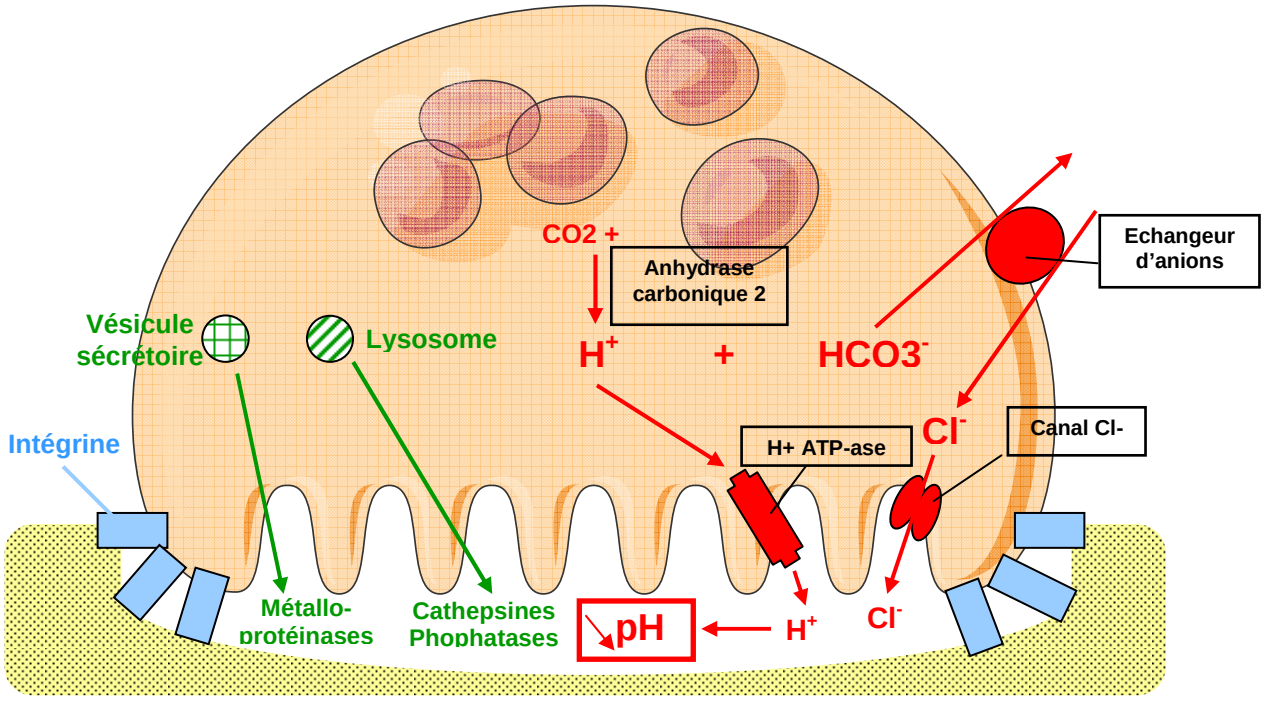


Figure 8 : Mécanismes de résorption de la matrice osseuse par l'ostéoclaste

(Les échangeurs d'anions pemrettent d'éviter l'alkalinisation du cytoplasme dû à l'expulsion des ions H+ par les H^+ ATPases. Les canaux Cl- de la bordure en brosse limitent le gradient électrochimique)

2- *Le remodelage osseux*

Le tissu osseux, outre la formation du squelette durant la croissance, doit assurer la conservation des propriétés mécaniques de celui-ci, ainsi que son adaptation aux contraintes mécaniques tout au long de la vie de l'organisme. Par ailleurs, il doit permettre la réparation d'éventuelles fractures et mettre à disposition les sels minéraux qu'il stocke. Ces différentes fonctions vitales nécessitent une grande plasticité qui est rendue possible par un perpétuel remaniement de l'os : le remodelage osseux.

Le remodelage osseux correspond à la destruction (ou résorption) par les ostéoclastes d'un tissu osseux ancien afin d'assurer son remplacement par de l'os nouveau généré par les ostéoblastes. Il se déroule au sein de structures bien définies qui sont les unités multicellulaires de base (BMU). On trouve environ 10^6 BMUs actives sur un squelette adulte en permanence. Le remodelage osseux conduit au renouvellement de 10 % du squelette chaque année.

2.1 Le cycle de remodelage au sein d'une BMU

La durée de vie d'une BMU est comprise entre 6 et 9 mois. Pendant cette période, les principaux acteurs cellulaires du remodelage, les ostéoblastes et les ostéoclastes, y sont recrutés de façon continue et vont agir d'une manière très contrôlée à la fois dans le temps et dans l'espace.

Classiquement la séquence d'événement est décrite en quatre phases constituant un cycle de remodelage. On parle d'un cycle car le tissu osseux part d'un état quiescent pour y revenir une fois le remodelage effectué (Marie and Halbout 2008) **(Fig.9).**

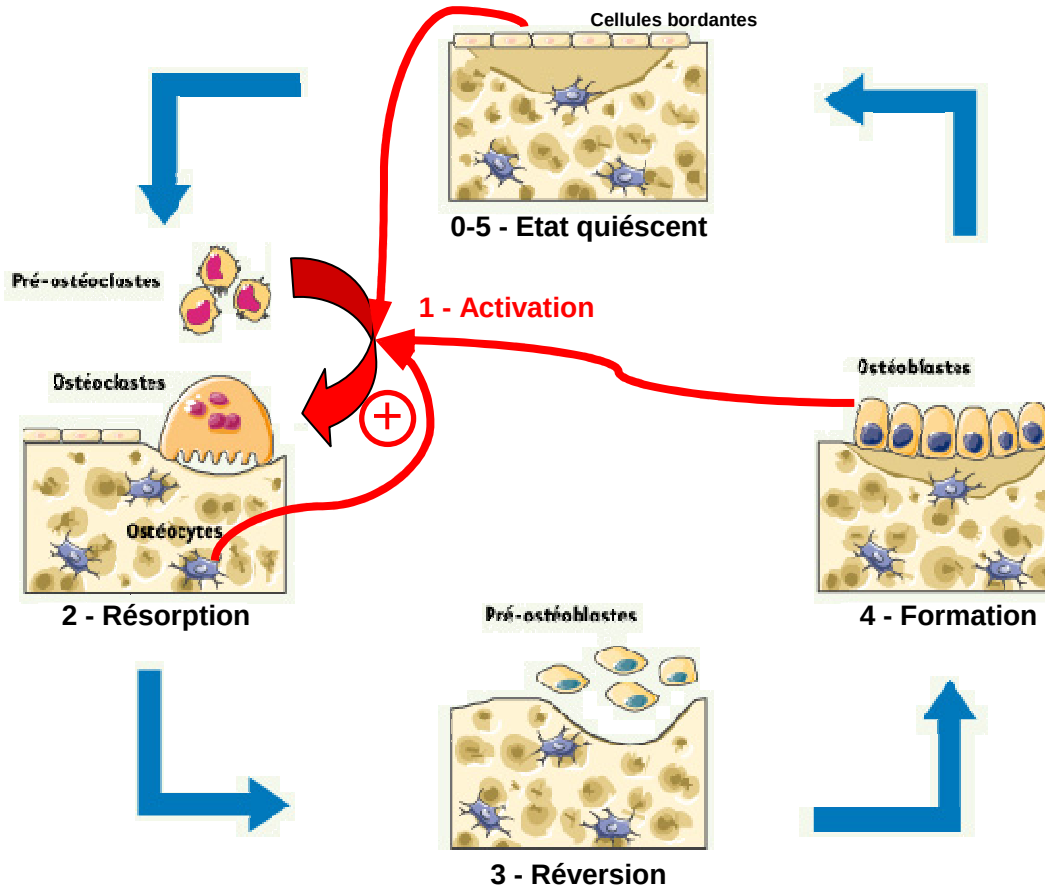


Figure 9 : Le cycle du remodelage osseux (d'après Marie et al, 2008) (Marie and Halbout)

- **Phase d'activation :** La séquence débute par l'activation, en un point donné du squelette, des cellules bordantes qui le recouvrent à l'état quiescent. Elles se rétractent alors et des précurseurs ostéoclastiques mononucléés sont recrutés au niveau de ce qui devient alors une BMU. Ces précurseurs vont alors fusionner pour former des ostéoclastes matures multinucléés. Ces derniers s'attachent alors à la matrice via les intégrines formant un anneau de scellement et créant un microenvironnement entre leur bordure en brosse et la matrice osseuse (Baron 2008).

- **Phase de résorption :** Elle correspond à l'activité ostéoclastique proprement dite avec la destruction des phases minérales et organiques de la matrice osseuse. Cette phase aboutit à la présence d'une cavité dans l'os appelée lacune de résorption ou lacune de Howship (Baron 2008).

- Phase de réversion : Cette phase correspond au détachement des ostéoclastes de la matrice osseuse et à leur remplacement, au niveau de la lacune, d'abord par des pré-ostéoblastes puis par des ostéoblastes actifs. La dégradation de la matrice osseuse entraîne la libération des éléments qui la constituent. Elle provoquerait notamment une augmentation des concentrations locales en calcium qui serait capable de perturber les phénomènes d'adhésion contribuant ainsi au détachement des ostéoclastes de la matrice (Lorget, Kamel et al. 2000). Par ailleurs, la phase organique de la matrice osseuse contiendrait également des facteurs favorisant la formation osseuse (voir *3 – La Régulation du remodelage osseux*) qui pourrait participer au recrutement et à l'activation des pré-ostéoblastes au niveau de la lacune de Howship (Devescovi, Leonardi et al. 2008).

- Phase de formation : Elle correspond à l'activité des ostéoblastes qui vont élaborer une nouvelle matrice osseuse pour combler la lacune. Une fois celle-ci synthétisée et minéralisée, une partie des ostéoblastes va mourir par apoptose, une autre, piégée au sein de la matrice va se différencier en ostéocytes alors qu'une dernière va venir constituer la couche de cellules bordantes qui recouvre l'os à l'état quiescent.

2.2 « Balance osseuse »

L'évolution de la structure osseuse va dépendre des niveaux d'activité respectifs des ostéoclastes et des ostéoblastes. La balance osseuse peut donc être considérée comme la résultante des passages consécutifs des ostéoblastes et des ostéoclastes au sein d'une BMU. Schématiquement, si l'activité des ostéoclastes surpasse celle des ostéoblastes, la lacune creusée n'est pas entièrement comblée et l'os perd en densité. A l'inverse, si l'activité ostéoblastique est dominante, le dépôt de matrice néoformée va au-delà du comblement de la lacune et l'os se densifie.

La balance osseuse varie au cours de la vie **(Fig.10)**. Au moment de la croissance, elle est naturellement fortement déséquilibrée en faveur de la formation osseuse ce qui conduit au gain de masse osseuse observé depuis la naissance jusqu'au début de l'âge adulte. Par la suite, les deux processus vont s'équilibrer conduisant à un maintien de la densité osseuse pendant plusieurs années. Durant cette phase, le remodelage est toujours actif permettant le maintien de l'architecture osseuse et l'homéostasie ionique mais se déroule sans perte ni gain de masse osseuse. Enfin, avec l'âge la balance va se déséquilibrer en faveur de la résorption en raison

de plusieurs facteurs (voir régulation du remodelage osseux) conduisant à une diminution progressive du capital osseux.

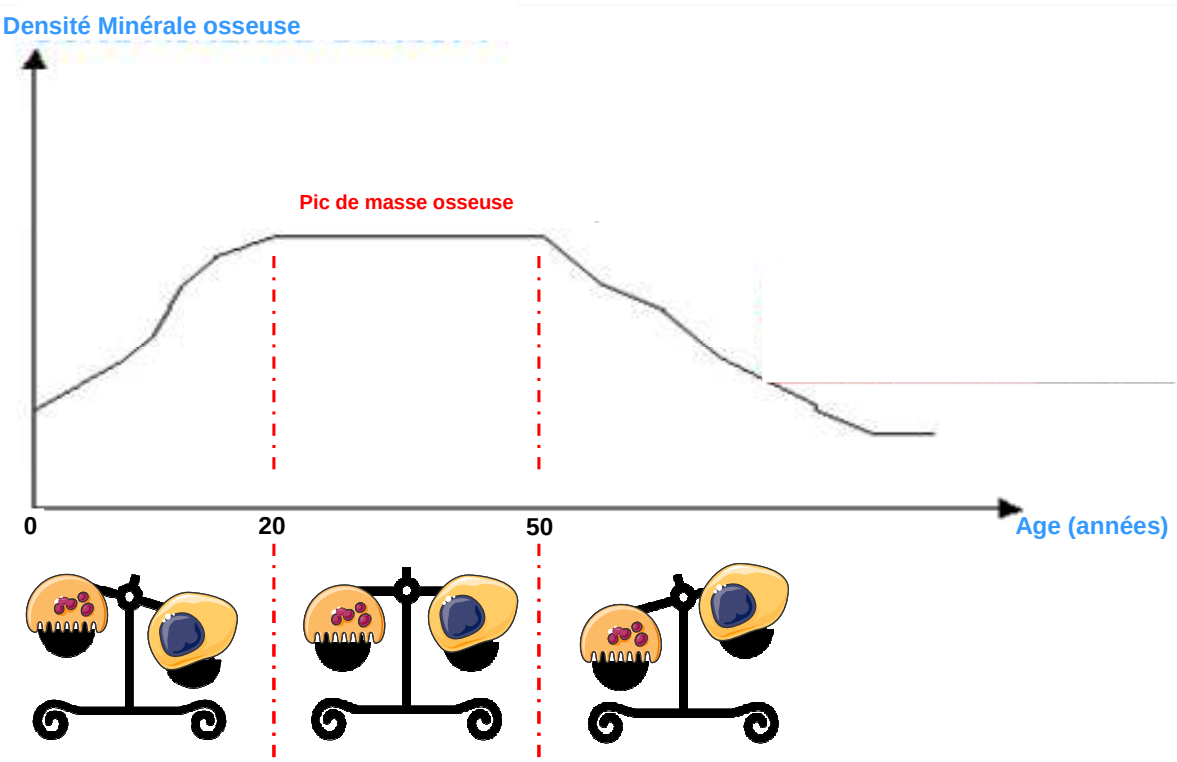


Figure 10 : Evolution de la densité minérale osseuse au cours de la vie

3- Régulation du remodelage osseux

La plasticité du squelette étant fondamentale pour lui permettre d'assurer ses fonctions, le remodelage osseux est un mécanisme très finement contrôlé via la modulation des activités des types cellulaires impliqués dans le processus. Cette régulation dépend aussi bien de facteurs locaux que de facteurs systémiques.

3.1 Facteurs locaux de régulation du remodelage osseux au sein d'une BMU

3.1.1 Initiation du cycle de remodelage / activation de la résorption ostéoclastique

Les phases d'activation et de résorption du remodelage reposent sur le recrutement, la différenciation et l'activation des pré-ostéoclastes afin d'induire la dégradation de la matrice osseuse en un point précis du squelette.

Les ostéocytes pourraient être les acteurs déterminants de ce « choix » de la zone de matrice osseuse à dégrader. Les ostéocytes sont capables de percevoir certains stress tels que les variations d'intensités de la stimulation mécanique exercée sur l'os ou la présence de micro-fractures. Ainsi, en réponse à ces dernières, ils entrent en apoptose (Verborgt, Gibson et al. 2000). La mort ou l'altération des ostéocytes étant capable d'induire la différenciation des ostéoclastes (Kurata, Heino et al. 2006) et la résorption osseuse (Noble, Peet et al. 2003) (Gu, Mulari et al. 2005) (Tatsumi, Ishii et al. 2007), ce mécanisme apparaît essentiel pour le recrutement des ostéoclastes au niveau de la matrice osseuse endommagée. De plus, il a été montré que les ostéoclastes résorbent préférentiellement de l'os « agé » (Henriksen, Leeming et al. 2007) qui est connu pour contenir moins d'ostéocytes viables (Noble, Stevens et al. 1997). D'un point de vue mécanistique, on pense que les ostéocytes envoient, via leurs prolongements cytoplasmiques, un ou plusieurs signaux aux cellules à la surface de l'os pour inhiber la différenciation ostéoclastique. Ainsi, leur apoptose induirait localement la perte de ce signal ce qui permettrait la différenciation des ostéoclastes. Le Transforming Growth Factor β (TGF-β) pourrait être un de ces signaux car cette cytokine est connue pour inhiber l'ostéoclastogénèse et son expression par les ostéocytes est diminuée lors de leur apoptose (Heino, Hentunen et al. 2002). Par ailleurs, l'induction de la résorption osseuse induite *in vivo*

par la mort programmée des ostéocytes chez la souris est associée à une augmentation de l'expression du Receptor Activator of Nuclear Factor κ B Ligand (RANKL) au niveau de leur moelle osseuse suggérant un contrôle indirect de l'ostéocyte sur l'ostéoclaste via la production de RANKL par les autres cellules de la lignée ostéoblastique (cellules bordantes et ostéoblastes)(Tatsumi, Ishii et al. 2007).

En effet, en dehors des ostéocytes, le contrôle de la différenciation des ostéoclastes est bien connu pour être largement sous le contrôle des autres cellules de la lignée ostéoblastique. Cela vient essentiellement de la capacité de ces cellules à produire des quantités importantes des facteurs RANKL et Macrophage-Colony Stimulating Factor (M-CSF), qui sont à la fois indispensables et suffisants pour induire l'ostéoclastogénèse (Wiktor-Jedrzejczak, Bartocci et al. 1990) (Lacey, Timms et al. 1998). M-CSF et RANKL travaillent de concert : M-CSF favorise la prolifération et la survie des précurseurs ostéoclastiques ainsi que l'organisation du cytosquelette des cellules matures (Insogna, Sahni et al. 1997). RANKL quant à lui coordonne la fusion des précurseurs aboutissant à la formation des ostéoclastes matures multinucléés tout en augmentant l'activité de résorption de ces cellules différenciées ainsi que leur durée de vie (Burgess, Qian et al. 1999). De façon intéressante, les souris déficientes pour le facteur de transcription Runt-domain transcription factor 2 (Runx2), qui sont totalement dépourvues d'ostéoblastes, présentent une ostéoclastogénèse très perturbée, mettant en exergue le rôle crucial des cellules de la lignée ostéoblastique dans le développement des ostéoclastes (Otto, Thornell et al. 1997; Hoshi, Komori et al. 1999). Par ailleurs, il a été montré que l'absence d'ostéoblastes matures avait peu d'effet sur le devenir des ostéoclastes, suggérant que les cellules supportant majoritairement l'ostéoclastogénèse sont les précurseurs de la lignée ostéoblastique correspondant aux cellules stromales ou aux cellules bordantes de l'os (Corral, Amling et al. 1998).

L'ostéoprotégérine (OPG) est également appelée « récepteur leurre ». En effet, elle est sécrétée sous la forme d'une protéine soluble qui va lier RANKL et ainsi empêcher son action. Ainsi, *in vivo*, les souris surexprimant l'OPG sont ostéopétrotiques alors que celles déficientes en OPG présentent une ostéoporose sévère associée à une activité plus importante des ostéoclastes. La production de l'OPG est également le fait des cellules de la lignée ostéoblastique mais à l'inverse de RANKL, son expression est d'autant plus forte que le degré de différenciation des cellules est important. **(Fig.11)** (Hofbauer, Khosla et al. 2000).

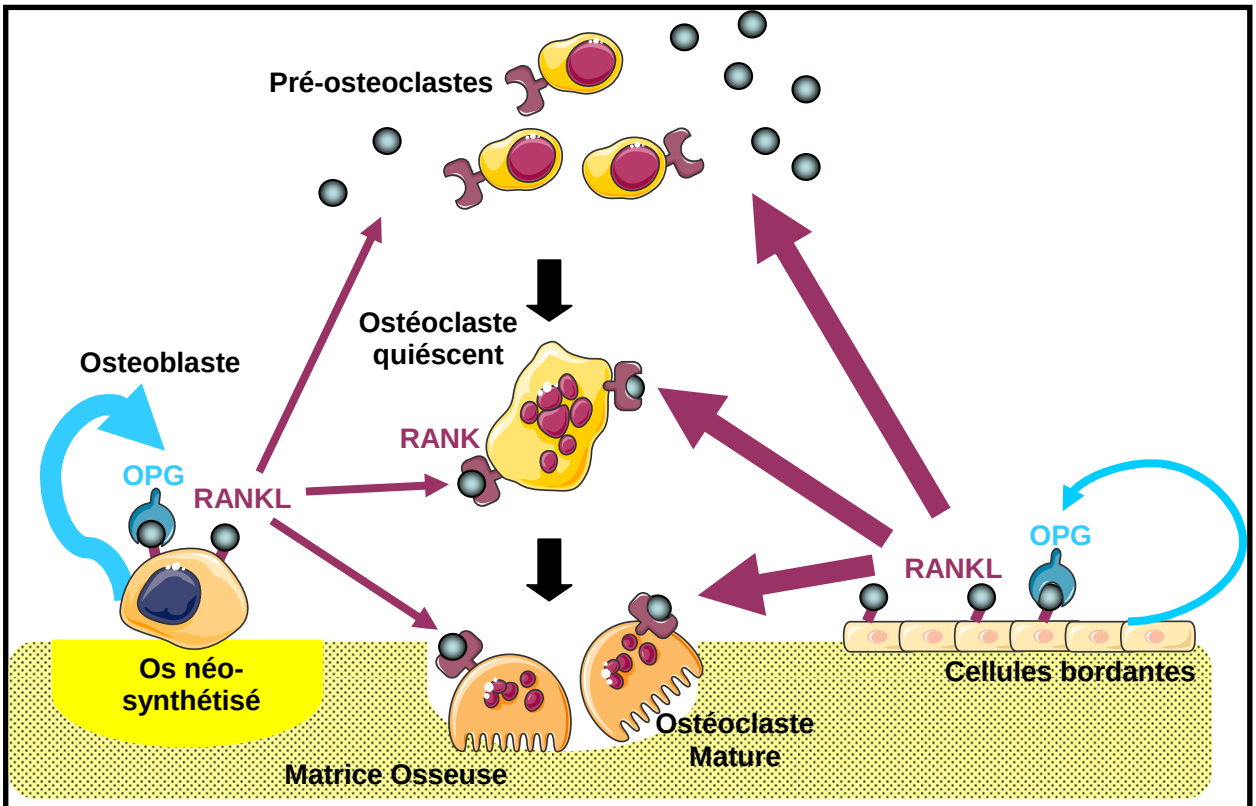


Figure 11 : Contrôle de l'ostéoclastogénèse par les cellules de la lignée ostéoblastique

Enfin, RANKL peut également être produit par les cellules immunitaires peuplant la moelle osseuse (Quinn and Saleh 2009) ainsi que directement par les ostéocytes (Zhao, Zhang et al. 2002) étendant ainsi la liste des acteurs cellulaires susceptibles de moduler directement la résorption osseuse.

3.1.2 Transduction du signal induit par RANKL

RANKL agit via la fixation sur le Receptor Activator of Nuclear Factor κ B (RANK) au niveau des précurseurs ostéoclastiques. Ce récepteur est logiquement également essentiel à la différenciation et la survie des ostéoclastes (Khosla 2001). La liaison de RANKL à RANK induit différentes voies de signalisation partiellement imbriquées qui vont conduire à l'activation de facteurs de transcription spécifiques permettant l'expression des gènes nécessaires à la fusion des précurseurs ainsi qu'à l'activité et à la survie de l'ostéoclaste mature **(Fig.12)**.

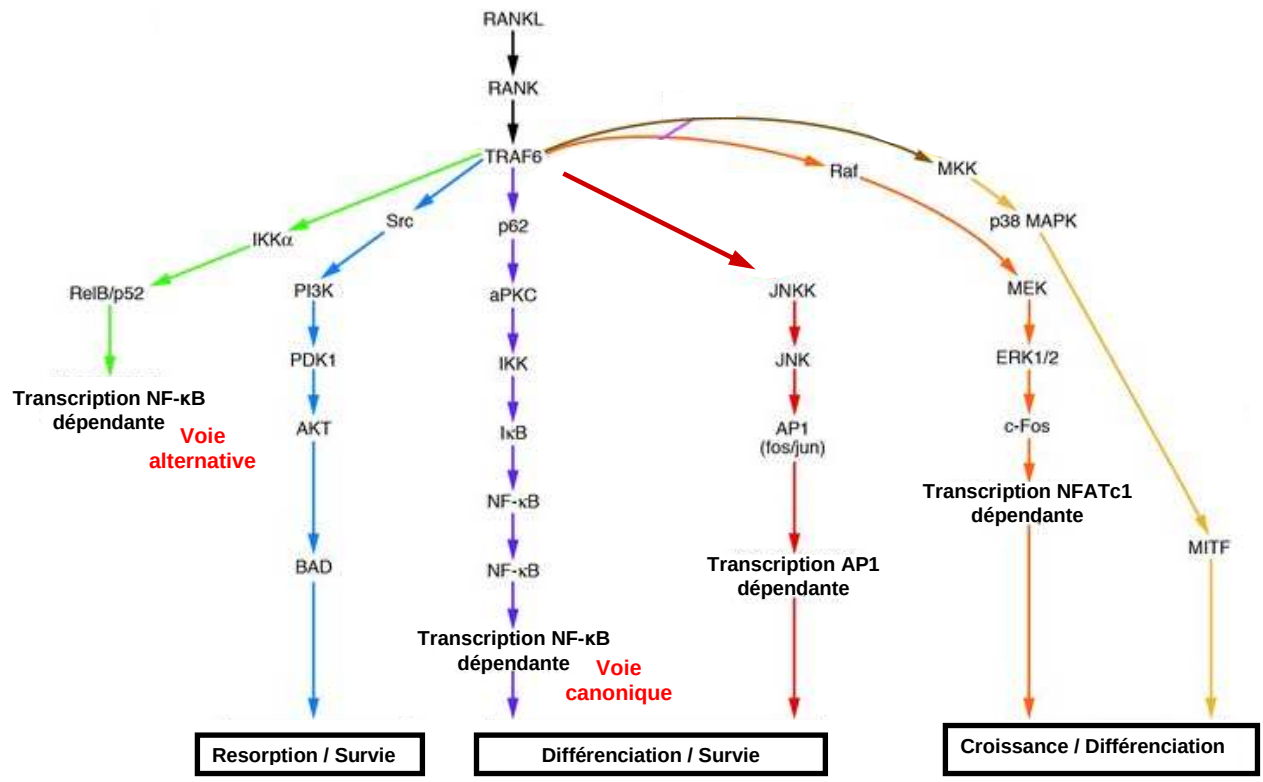


Figure 12 : Voies de signalisation induisant l'ostéoclastogénèse activées par la fixation de RANKL sur son récepteur RANK

(JNKK : JNK-activating Kinase ; **MEK :** MAPK/ERK kinase, **MKK :** Mitogen-Activated Protein Kinase Kinase ; **MITF :** Microphthalmia-associated Transcription Factor ; **PI3K :** Phosphoinositide 3-kinase ; **PDK1 :** Pyruvate Dehydrogenase Kinase isozyme 1 **)**

La voie du Nuclear Factor Kappa B (NF-κB) est très fortement induite par l'activation de RANK par RANKL. Le domaine cytoplasmique de RANK est associé à différents TNF-Receptor Associated Factors (TRAFs) au sein desquels TRAF6 est de loin le plus important au regard de la transmission du signal lié à la fixation de RANKL. En effet, TRAF6 est indispensable à l'activation de la voie NF-κB par RANK *in vitro* (Darnay, Haridas et al. 1998), et son absence se traduit par une ostéopétrose sévère chez la souris (Lomaga, Yeh et al. 1999). *In vivo*, l'absence des facteurs p50 et p52 conduisant à la double inactivation des voies NF-κB canonique et alternative induit une ostéopétrose sévère chez la souris (Iotsova, Caamano et al. 1997). L'analyse plus fine des acteurs en amont de ces voies a permis de mettre en évidence un rôle majeur de la voie canonique dans la différenciation ostéoclastique, celui de la voie alternative semblant plus marginal (Ruocco, Maeda et al. 2005) (Doffinger, Smahi et al. 2001).

En parallèle de la voie NF-κB, l'activation de RANK induit d'autres voies participant à la différenciation, à la fonction ou à la survie de l'ostéoclaste. Au sein de la famille des

Mitogen Activated Protein Kinases (MAPKs) RANKL entraîne l'activation des p38 MAPKs (Matsumoto, Sudo et al. 2000), des Extracellular signal-Regulated Kinases (ERK1/2) (Kim, Jin et al. 2009) et des c-Jun N-terminal Kinases (JNKs) (David, Sabapathy et al. 2002) (Yamamoto, Miyazaki et al. 2002) qui vont participer à la différenciation de l'ostéoclaste en activant des facteurs de transcription essentiels tels que le Nuclear Factor of Activated T cells, calcineurin-dependent 1 (NFATc1) (Takayanagi, Kim et al. 2002; Matsuo, Galson et al. 2004) ou les membres de la famille Activator Protein 1 (AP-1) tels que c-jun, c-fos, JunB ou Fra (Wagner 2002). L'activité de la kinase cellular-src (c-src) est également induite par RANKL permettant, entre autre, de favoriser la survie cellulaire via la phosphorylation de la protéine kinase B (PKB ou AKT) (Nakashima, Kobayashi et al. 2000).

3.1.3 Régulation des phases de réversion / formation

Afin de permettre au squelette de maintenir son intégrité, les lacunes de résorption laissées par les ostéoclastes doivent être comblées par l'activité ostéoblastique. Chez l'adulte en bonne santé, la formation osseuse se déroule 97 % du temps au sein d'une lacune de résorption (Takahashi, Epker et al. 1964). De même, de nombreuses études *in vitro* ont montré que les ostéoblastes forment de l'os préférentiellement dans les puits de résorption (Jones, Gray et al. 1994) (Mulari, Qu et al. 2004) (Schwartz, Lohmann et al. 2000).

Cette « préférence » des ostéoblastes pour les zones de matrice osseuse résorbées par les ostéoclastes correspond au phénomène de couplage du remodelage osseux. Il implique des acteurs de régulation locaux produits soit par les ostéoclastes, soit par la résorption osseuse qui conduisent au recrutement, à la différenciation et à l'activité des ostéoblastes.

La dégradation de la matrice osseuse par les ostéoclastes entraîne la libération des éléments qui la constituent. Des éléments se retrouvent donc libérés localement et parmi ceux-ci, on trouve l'Insulin-like Growth Factor 1 (IGF-1) et le TGF-β, deux cytokines qui pourraient favoriser à la fois le recrutement, la différenciation et l'activité des ostéoblastes (Mundy and Bonewald 1990) (Hayden, Mohan et al. 1995). Par ailleurs, en plus d'être relargués depuis la matrice osseuse, ces deux facteurs peuvent également être directement produits par les ostéoclastes (Lazowski, Fraher et al. 1994) (Robinson, Riggs et al. 1996).

En outre, une étude récente a permis de mettre en évidence que l'Ephrin B2, exprimée par les ostéoclastes, et l'Ephrin B4, exprimée par les ostéoblastes, étaient impliquées dans une communication réciproque en ces deux types cellulaires. L'expression de l'Ephrin B2 des ostéoclastes conduit à une induction de la formation osseuse via sa fixation sur l'Ephrin B4

des ostéoblastes. En parallèle l'Ephrin B2 est activée par l'Ephrin B4 conduisant à une inhibition de la différenciation ostéoclastique (Zhao, Irie et al. 2006). Etant donné que ce mécanisme implique un contact direct entre les deux types cellulaires, il a été suggéré qu'il serait impliqué dans la communication entre les cellules bordantes et les ostéoclastes, deux types cellulaires pouvant être au contact et connus pour se réguler réciproquement (Everts, Delaisse et al. 2002) (Karsdal, Fjording et al. 2001). L'expression de l'Ephrin B2 par les ostéoclastes contribuerait donc à la conversion des cellules bordantes en ostéoblastes actifs permettant l'initiation de la formation osseuse **(Fig.13)**.

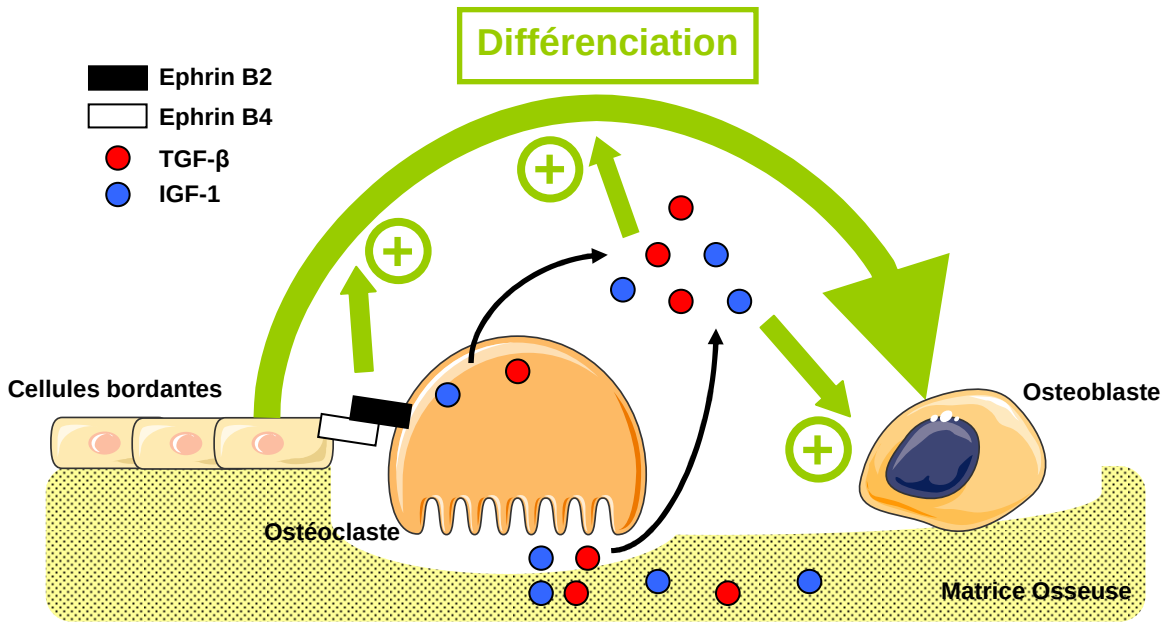


Figure 13 : Mécanismes impliqués dans le couplage entre résorption et formation osseuse

3.2 Régulation systémique du remodelage osseux

Les stress mécaniques détectés par les ostéocytes ne sont pas les seuls facteurs susceptibles de « déclencher » le processus de remodelage osseux. En effet, de part ces fonctions essentiels, le tissu osseux se trouve sous le contrôle d'un nombre important de facteurs hormonaux systémiques.

3.2.1 Régulation de l'homéostasie phospho-calcique

Les niveaux sanguins des deux composants minéraux majoritaires de l'os, le calcium et le phosphate, sont régulés au niveau de trois organes : l'intestin où ces ions sont absorbés, le rein où ils sont excrétés et l'os où ils sont stockés sous forme de réserve mobilisable par le processus de résorption osseuse par les ostéoclastes. Le maintien de cette homéostasie phospho-calcique est sous le contrôle de plusieurs hormones parmi lesquelles, la Parathormone (PTH), la 1a, 25-dihydroxy vitamine D (1,25(OH) $_2$-D, forme active de la vitamine D) et la calcitonine (Bikle 2008).

3.2.1.1 La PTH

La PTH est sécrétée par les parathyroïdes. Sa forme active est un peptide de 84 acides aminés et son rôle principal est de maintenir le niveau sérique de calcium en mobilisant les réserves osseuses, en augmentant son absorption intestinal et sa réabsorption rénale **(Fig.14)**. C'est la diminution des niveaux sanguins de calcium qui va déclencher la production de cette hormone via un récepteur transmembranaire « sensible au calcium » présent sur les cellules des parathyroïdes (Marx 2000). La PTH sécrétée est rapidement dégradée au niveau du foie avant d'être éliminée par les reins et certains fragments de la PTH peuvent s'accumuler en cas d'insuffisance rénale (John, Goodman et al. 1999). Au niveau du rein, l'hormone va permettre la réabsorption du calcium filtré et induire la production de la forme active de la vitamine D (Lambers, Bindels et al. 2006). En parallèle, elle va induire la résorption au niveau de l'os en augmentant la prolifération et la différenciation des ostéoclastes par le biais des cellules de la lignée ostéoblastique qui vont exprimer les facteurs de régulation locaux pro-ostéoclastiques tel que RANKL (Fu, Jilka et al. 2002). La PTH a également un effet stimulateur sur la formation osseuse en favorisant la conversion des cellules bordantes en ostéoblastes et en diminuant l'apoptose de ces derniers (Whitfield, Morley et al. 2002). En fait, l'effet osseux final de la PTH semble dépendre de son « rythme de production ». En effet, il a été montré, *in vitro* et *in vivo*, qu'une administration en continu de PTH est pro-résorptive alors qu'une administration intermittente s'avère anabolique pour l'os (Ishizuya, Yokose et al. 1997; Hodsman, Hanley et al. 2003).

3.2.1.2 La 1,25(OH) $_2$-D

Au niveau de l'organisme, la vitamine D peut provenir de deux sources : elle peut être formée par l'action des UVs sur le 7-dehydrocholesterol de la peau ou être apportée par l'alimentation. Ces deux modes de production conduisent à des précurseurs structurellement

légèrement différents (Vitamine D3 et Vitamine D2) mais aux devenirs biologiques identiques. Pour aboutir à la forme active, ces précurseurs subissent une première hydroxylation dans le foie pour aboutir à la 25(OH)-D puis une seconde dans le rein pour former le métabolite actif, la 1,25(OH) $_2$-D. C'est l'enzyme responsable de cette dernière transformation, la 1-α hydroxylase qui est activée par la PTH dans le rein pour augmenter la production de 1,25(OH) $_2$-D (Omdahl, Morris et al. 2002). Cette même enzyme est également inhibée par rétrocontrôle en cas d'augmentation des taux de 1,25(OH) $_2$-D (Omdahl, Morris et al. 2002). Le principal rôle de la 1,25(OH) $_2$-D est la stimulation de l'absorption intestinal de calcium **(Fig.14)** et de phosphate et elle joue également un rôle au niveau des parathyroïdes où elle limite la production de PTH (Perez, Picotto et al. 2008). Au niveau de l'os, la 1,25(OH) $_2$-D possède un effet anabolique par la stimulation de la différenciation des ostéoblastes et de leur minéralisation. Il faut noter que des doses très fortes conduisent à un effet inverse avec une augmentation de la production de RANKL par les ostéoblastes et donc à une augmentation de la résorption osseuse. De façon importante, des souris déficientes pour le récepteur de la vitamine D (VDR) mais supplémentées de manière adéquate en calcium et en phosphore ne présentent pas de phénotype osseux suggérant l'existence de mécanismes permettant de compenser l'effet anabolique de 1,25(OH) $_2$-D sur les ostéoblastes (Sutton and MacDonald 2003).

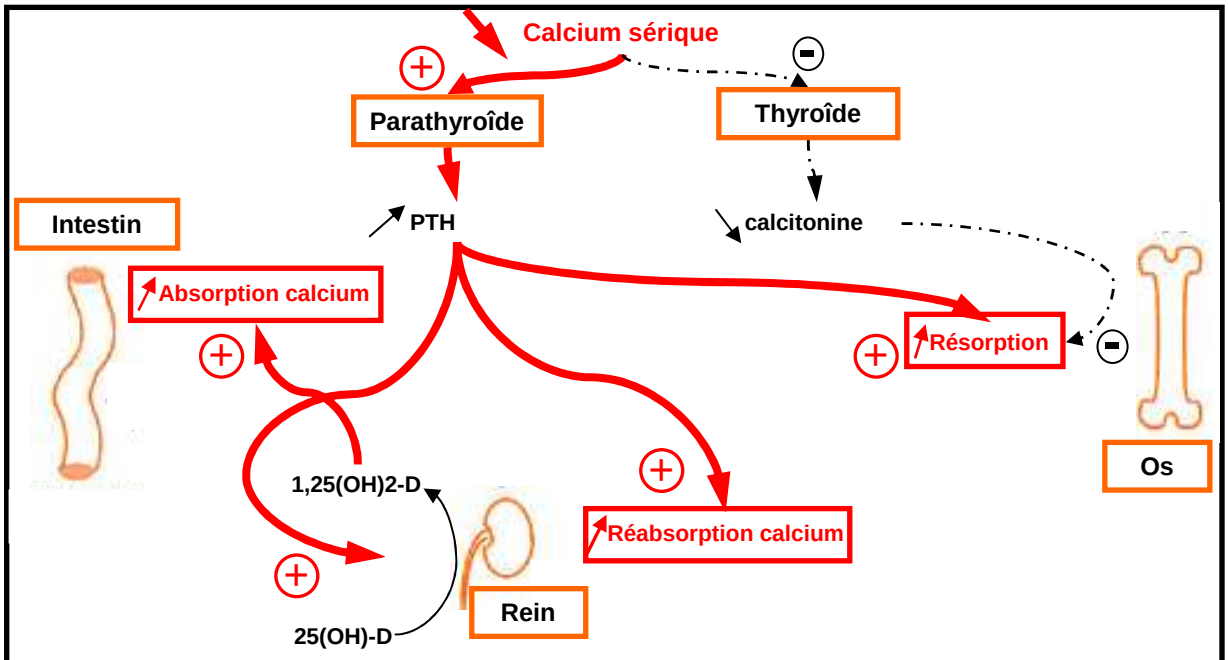


Figure 14 : Mécanismes de maintien de l'homéostasie calcique induits par la diminution du niveau sérique de calcium

3.2.1.3 La calcitonine

La calcitonine est un peptide de 32 acides aminés sécrété par les cellules C de la thyroïde. Cette hormone agit directement sur les ostéoclastes matures qui expriment son récepteur en diminuant leur activité de résorption et en augmentant leur apoptose (Turner, Tjahyono et al. 2010). A l'inverse de la PTH, sa production est déclenchée par une augmentation des taux sériques de calcium (Kantham, Quinn et al. 2009) **(Fig.14)**.

3.2.2 Régulations par les hormones sexuelles

3.2.2.1 Les œstrogènes

Les œstrogènes sont probablement les facteurs les plus importants pour le maintien d'un remodelage osseux adéquat. La déficience œstrogénique entraîne une augmentation de la résorption osseuse qui surpasse la formation induisant une perte de masse osseuse. Cela est observé chez la femme ménopausée, chez l'homme présentant une production œstrogénique déficiente (Jones, Boon et al. 2006) ainsi que dans les modèles d'animaux ovariectomisés (Nelson, Humphrey et al. 2002). Dans tous les cas, un traitement par les œstrogènes corrige cette fragilisation du squelette (Draper 2003) (Nelson, Humphrey et al. 2002).

Les mécanismes pouvant expliquer ce rôle ostéoprotecteur des œstrogènes sont nombreux **(Fig.15)**.

- Ils augmentent la production hépatique du facteur ostéogénique IGF-1 (Imai, Kondoh et al. 2010).

- Ils limitent la production de cytokines pro-inflammatoires pro-ostéoclastiques par certaines cellules immunitaires (Roggia, Gao et al. 2001) (Ryan, Shepherd et al. 2005).

- Le stress oxydant, connu pour favoriser l'ostéoclastogénèse, est également réduit par les œstrogènes (Wauquier, Leotoing et al. 2009).

- La Follicle Stimulating Hormone (FSH) qui est l'hormone pituitaire induisant la production d'œstrogènes par l'ovaire est un régulateur négatif du remodelage osseux. Or, les œstrogènes exercent un rétrocontrôle négatif sur la production de FSH dont les niveaux circulants se trouvent augmentés en cas de déficience œstrogénique (Sun, Peng et al. 2006).

- Ils agissent également directement au niveau des cellules osseuses où ils limitent l'activité et augmentent l'apoptose des ostéoclastes (Nakamura, Imai et al. 2007) alors qu'ils induisent au niveau de l'ostéoblaste, la production d'IGF-1 (Ernst, Heath et al. 1989), de TGF-β (Oursler, Cortese et al. 1991) et d'OPG (Hofbauer, Khosla et al. 1999).

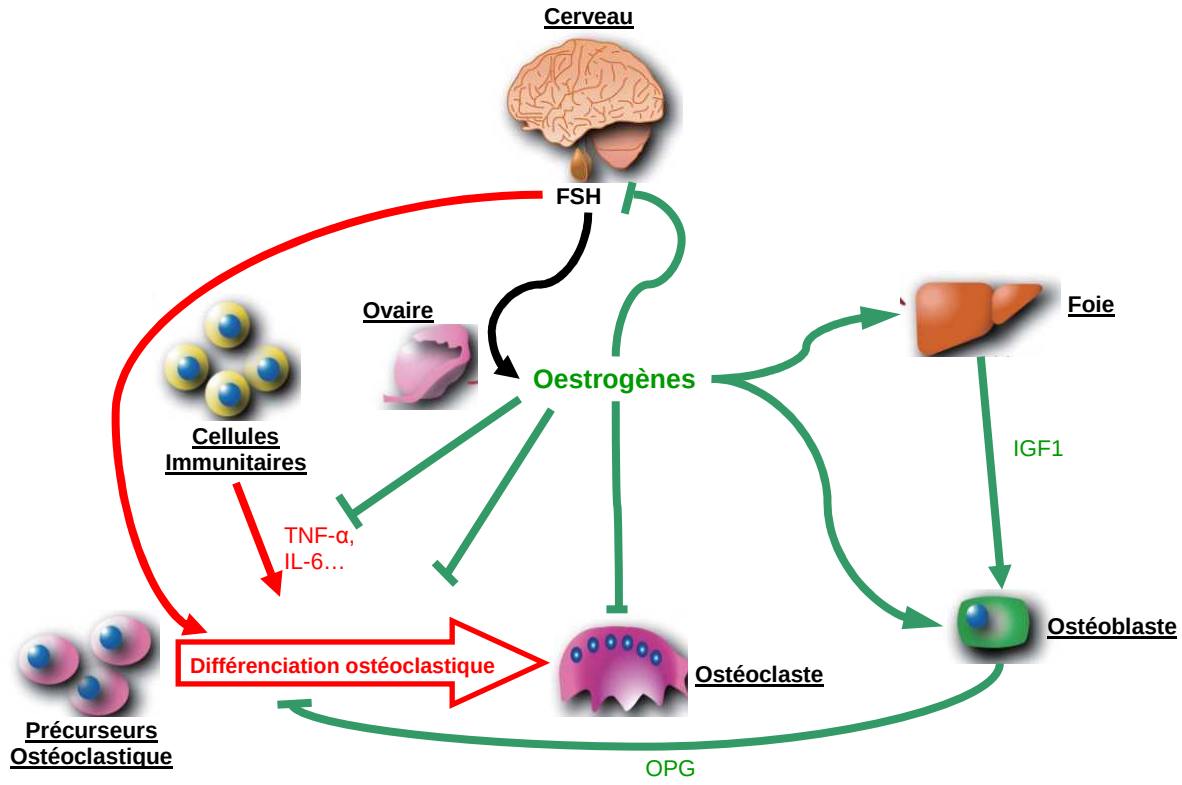


Figure 15 : Rôle ostéo-protecteur des œstrogènes

3.2.2.2 Les androgènes

Bien que les œstrogènes soient considérés comme les acteurs majeurs du remodelage de l'os, la testostérone aurait aussi un rôle important à jouer, chez l'homme, dans l'homéostasie osseuse. D'abord, l'hypogonadisme chez l'homme âgé est un facteur de risque connu de l'ostéoporose (Callewaert, Boonen et al. 2009). Par ailleurs, bien que les données de la littérature soient parfois conflictuelles (Snyder, Peachey et al. 1999), l'administration de testostérone chez l'homme hypogonadique, jeune ou âgé, a été montrée comme efficace, sous certaines conditions (Fink, Ewing et al. 2006), pour prévenir la perte osseuse (Behre, Kliesch et al. 1997) (Katznelson, Finkelstein et al. 1996).

Une partie de l'effet de la testostérone est probablement liée à sa capacité à être convertie en œstrogène par l'aromatase P 450 (Jones, Boon et al. 2006). Mais la génétique de la souris a permis de démontré que la testostérone avait un effet par elle-même notamment au niveau des ostéoblastes. Ainsi, les souris déficientes pour le récepteur aux androgènes (AR) spécifiquement au niveau des ostéoblastes présentent une diminution de leur masse osseuse

trabéculaire (Chiang, Chiu et al. 2009) alors que celles le surexprimant ont le phénotype trabéculaire inverse mais associé à une réduction de la masse osseuse corticale. (Wiren, Zhang et al. 2004) (Wiren, Semirale et al. 2008).

3.2.3 Le statut redox

Le statut redox de l'organisme est également un paramètre fondamental dans la détermination du statut osseux. En effet, une augmentation du stress oxydant se retrouve associée à de nombreuses situations de perte osseuse et l'augmentation de production d'espèces oxygénées réactives associée est connue à la fois pour activer la différenciation des ostéoclastes et inhiber la différenciation des ostéoblastes. (**Annexe 1 :** *« Oxydative stress in bone remodeling and diseases »* (Wauquier, Leotoing et al. 2009) **(page198)**)

3.2.4 Le statut inflammatoire

De la même manière, le processus inflammatoire génère de nombreuses molécules (Prostaglandines, Tumor Necrosis Factor α (TNF-α)…) susceptibles de déséquilibrer le remodelage osseux en faveur de la résorption et se retrouve donc également associé à des situations de perte osseuse. (**Article 1 :** *« Fatty acids and bone relationships : I love you… Neither do I »* D - Lipids, inflammation and bone **(page 97)**)

Ils existent de nombreux autres acteurs systémiques capables de moduler l'évolution de la masse osseuse. Ainsi, les glucocorticoïdes sont connus pour induire à long terme une diminution de la masse osseuse (Hong, Chen et al. 2008) alors que l'hormone de croissance entraîne une accélération des cycles de remodelage en favorisant à la fois la formation et la résorption osseuse, mais avec une résultante anabolique pour le squelette (Salles 2006). Le tissu osseux est également sous le contrôle des hormones du tube digestif, des hormones pancréatiques et d'adipokines. (**Article 1 :** *« Fatty acids and bone relationships : I love you… Neither do I »* C - Organs interactions **(page 81)**)

Le remodelage de la matrice osseuse est donc un processus très finement contrôlé, que ce soit au niveau local ou au niveau systémique. Une perturbation de ces mécanismes régulateurs conduit à une altération de la densité minérale osseuse et/ou une détérioration de l'architecture osseuse. Avec l'âge, le remodelage se déséquilibre en faveur de la résorption conduisant à une diminution progressive de la masse osseuse. En dessous d'un certain seuil, la fragilisation du squelette induit une augmentation importante du risque de fracture, on parle d'ostéoporose.

4- L'ostéoporose

4.1 Définition :

Selon la définition de l'Organisation Mondiale de la Santé (OMS), l'ostéoporose est « une maladie squelettique systémique, progressive, caractérisée par une faible masse osseuse et une détérioration de la micro-architecture osseuse avec pour conséquence une fragilité osseuse et un accroissement du risque fracturaire » (1991). Classiquement les fractures associées à l'ostéoporose sont celles des vertèbres, de l'avant bras et du col du fémur.

En se basant sur les études épidémiologiques démontrant une relation inverse entre le risque de fracture et la densité minérale de l'os évaluée par absorptiométrie biphotonique à rayon X (DEXA), l'OMS a établi deux seuils permettant de distinguer un contenu minéral osseux normal, une ostéopénie ou une ostéoporose. Ces seuils sont exprimés en T-score qui correspond à la déviation standard d'une valeur de densité minérale osseuse par rapport à la moyenne d'un groupe contrôle d'adultes jeunes du même sexe. Ainsi, un T-score > -1 correspond à un contenu minéral osseux normal, un T-score compris entre -1 et -2,5 correspond à une ostéopénie et un T-score < - 2,5 correspond à une situation d'ostéoporose **(Fig.16)**.

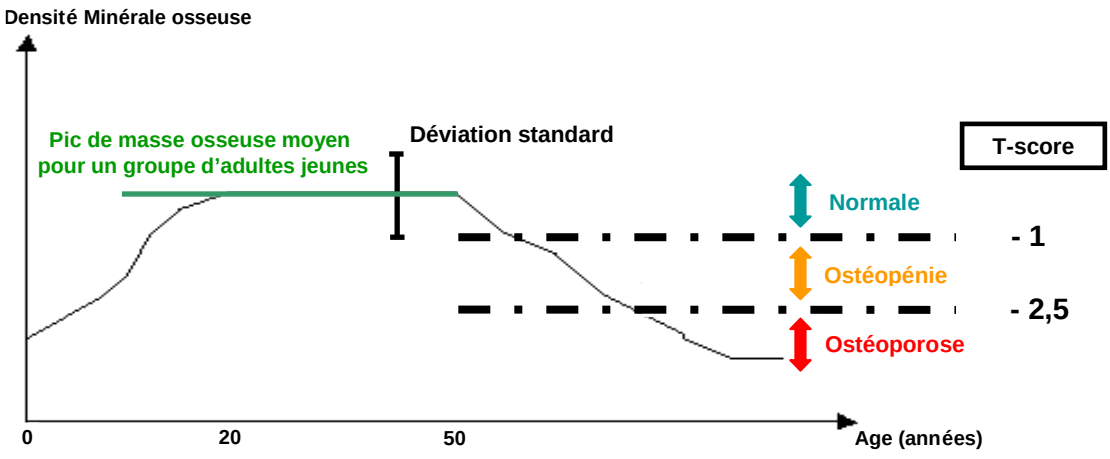


Figure 16 : Détermination du statut osseux en fonction de la densité minérale de l'os

Cette définition « densitométrique » de l'OMS établie en 1994, bien qu'imparfaite, reste encore bien acceptée et la DEXA reste la méthode de référence internationalement reconnue pour le diagnostic de l'ostéoporose (Bartl and Frisch 2009). La DEXA est dorénavant associée à la prise en compte d'autres facteurs de risque afin de déterminer le risque fracturaire d'un individu au sein du « Fracture-Risk Assessment Tool » (FRAX) (Rachner, Khosla et al. 2011)

4.2 Classification ?

En 1982, Riggs *et al* proposait de différencier deux types d'ostéoporose principalement sur la base de leur étiologie (Riggs, Wahner et al. 1982).

- L'ostéoporose de type 1, ou post-ménopausique qui serait principalement causée par la carence œstrogénique chez la femme suite à la ménopause et qui se caractériserait essentielement par une altération du tissu osseux trabéculaire.

- L'ostéoporose de type 2, ou sénile qui serait retrouvée plus particulièrement chez les sujets de plus de 70 ans, se caractériserait par une altération à la fois de l'os cortical et de l'os trabéculaire, et dont l'étiologie multifactorielle correspondrait aux mécanismes associés au vieillissement de l'organisme.

Il est très important de noter que ces deux types d'ostéoporose ne sont pas mutuellement exclusifs. En effet, ils cohabitent dans la plupart des cas et les mécanismes aboutissant à ces « deux ostéoporoses » sont, pour l'essentiel, communs. Ainsi, cette classification est de moins en moins utilisée et il est de plus en plus largement admis que l'ostéoporose de type 1 et de type 2 correspondent à des stades différents d'une même pathologie (Raisz 2005).

4.3 Facteurs de risques

En ce qui concerne l'ostéoporose, il faut distinguer les facteurs de risque de fragilité osseuse et les facteurs de risque de chute qui, le plus souvent, provoquent effectivement les fractures.

La fragilité osseuse caractéristique de l'ostéoporose est la conséquence d'un remodelage osseux qui se déséquilibre en faveur de la résorption osseuse au cours du vieillissement. De fait, l'âge est le premier facteur de risque de l'ostéoporose. Les antécédents de fractures constituent un autre paramètre essentiel qu'ils soient personnel ou familiaux.

Ainsi, le risque individuel d'une récidive après un premier tassement vertébral est multiplié par 8 et l'existence d'une fracture du col fémoral chez la mère double le risque d'avoir la même fracture chez la fille. Ce dernier point illustre l'importance souvent minorée de la composante génétique de l'ostéoporose. De part l'important rôle protecteur des œstrogènes, évoqué préalablement, la carence œstrogénique constitue une des causes principales de la fragilisation de l'os quelle que soit l'origine de cette carence (Ménopause, aménorrhée prolongée chez les sportives de haut niveau ou chez les anorexiques mentales). Une nutrition mal adaptée peut également participer à la fragilisation des os, notamment les carences en calcium et/ou phosphore (voir *5 – Nutrition et métabolisme osseux*). Il existe beaucoup d'autres facteurs de risque pouvant déséquilibrer le remodelage osseux en faveur de la résorption parmi lesquels le manque d'activité physique, le tabac, l'alcool, l'usage prolongé de glucocorticoïdes ainsi que certaines maladies chroniques de la thyroïde, du foie ou du rein.

Les facteurs de risque de chute sont également à prendre en compte et correspondent essentiellement à l'altération des fonctions musculaires et de la vue **(Fig.17)** (Meunier, Brantus et al. 2005).

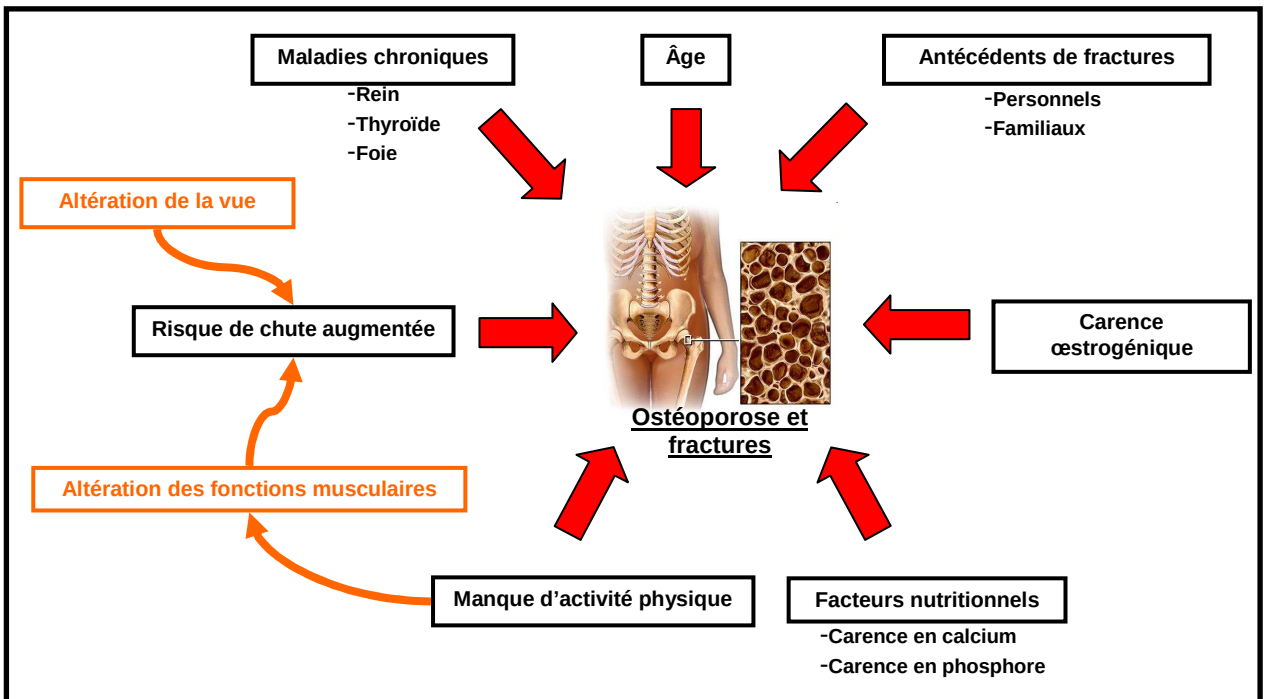


Figure 17 : Facteurs de risques de l'ostéoporose et des fractures ostéoporotiques

4.4 Un problème majeur de santé publique

A l'heure actuelle, on estime que sur 100 femmes, 40 présenteront, à un moment de leur vie, une ostéoporose. Par ailleurs, avec l'augmentation de l'espérance de vie, il est attendu que ce nombre ne cesse de croître dans un futur proche (Melton, Chrischilles et al. 1992) (Burge, Dawson-Hughes et al. 2007). Au sein des patients atteints d'ostéoporose, 40% auront une fracture ostéoporotique majeure (vertèbre, col du fémur ou avant-bras). Du point de vue du patient, ces fractures entraînent une perte de mobilité et d'autonomie affectant grandement leur qualité de vie. Plus grave encore, les fractures ostéoporotiques du col du fémur et les tassements vertébraux sont associés à une augmentation du taux de mortalité jusqu'à 20% en raison de l'hospitalisation requise qui génère une augmentation des risques pour d'autres complications (pneumonie ou pathologies thrombo-embolique causées par l'immobilisation) (Center, Nguyen et al. 1999).

D'un point de vue économique, les fractures ostéoporotiques génèrent de très importantes dépenses de santé publique. Celles-ci incluent les interventions médicamenteuses mais aussi les prises en charges hospitalières. Bien que difficiles à évaluer, les coûts engendrés ont été estimés à 10 milliards d'euros en Europe (Européenne 1999) et 17 milliards de dollars au Etats-Unis (Keen 2003).

4.5 Les traitements actuels

Les agents pharmacologiques actuellement disponibles dans le traitement de l'ostéoporose peuvent être classifiés en deux catégories : les anti-résorptifs qui agissent en inhibant la résorption osseuse par les ostéoclastes et les ostéo-anaboliques qui agissent en activant la formation osseuse par les ostéoblastes. Cependant, de par le couplage existant entre formation et résorption osseuse, le remodelage osseux est globalement freiné au cours d'un traitement par les anti-résorptifs et accéléré au cours d'un traitement avec les ostéo-anaboliques.

4.5.1 Les bisphosphonates

Les bisphosphonates sont des anti-résorptifs puissants qui possèdent une grande spécificité pour les cristaux d'hydroxyapatite constituant la phase minérale de la matrice osseuse. Il existe plusieurs formulations différentes avec des modes d'actions cellulaires et

moléculaires variables mais tous ces composés entraînent au final, une nette diminution du nombre d'ostéoclastes et de la résorption osseuse. L'efficacité de ces composés à limiter l'occurrence de certaines fractures ostéoporotiques a été démontrée (Black, Cummings et al. 1996) (Harris, Watts et al. 1999) (Chesnut, Skag et al. 2004) (Black, Delmas et al. 2007) (Lyles, Colon-Emeric et al. 2007)

Les bisphosphonates sont les médicaments les plus utilisés à l'heure actuelle dans le traitement de l'ostéoporose. Leur principal avantage est leur grande spécificité pour l'os qui évite les effets secondaires dans d'autres tissus. Cependant, il a été suggéré que les traitements à très long terme par les bisphosphonates pourraient induire une ostéonécrose de la mâchoire (Khosla, Burr et al. 2007) ainsi que des fractures du fémur (Lenart, Neviaser et al. 2009). Bien qu'il soit difficile à ce stade d'attribuer clairement ces complications aux bisphosphonates, des études complémentaires sont nécessaires pour clarifier la situation même si ces complications restent très rares.

4.5.2 Les œstrogènes

Les œstrogènes sont également classés dans la famille des anti-résorptifs. La carence œstrogénique étant un des principaux mécanismes impliqués dans l'établissement de l'ostéoporose, son utilisation thérapeutique pour la prévention de cette pathologie était naturelle. Cependant, il a été montré en 2003 que ces traitements de substitution hormonale étaient susceptibles d'augmenter les risques de cancer du sein et de maladies cardio-vasculaires (Cauley, Robbins et al. 2003). Depuis lors, leur utilisation a grandement diminué. Actuellement, on conseil leur utilisation à titre préventif pendant les 5 années suivant la ménopause avant de passer si besoin à un autre composé comme les bisphosphonates.

4.5.3 Le raloxifène : un modulateur sélectif du récepteur des œstrogènes (SERM)

Le raloxifene est le SERM le plus utilisé dans le traitement de l'ostéoporose. Il s'avère efficace dans la prévention des fractures vertébrales, mais pas des autres (Ettinger, Black et al. 1999). Il est environ deux fois moins puissant que les œstrogènes ou les bisphosphonates pour augmenter la densité minérale de l'os (Johnell, Scheele et al. 2002). Tout comme les œstrogènes, la prise de raloxifène est associée à une augmentation des maladies thrombo-emboliques mais pas avec les pathologies coronaires cardiaques ni le cancer du sein (Barrett-Connor, Mosca et al. 2006).

4.5.4 La calcitonine

Les capacités antirésorptives du traitement à la calcitonine sont nettement moins importantes que celles des molécules décrites jusque là bien que certaines études montrent un effet bénéfique sur l'occurrence des fractures ostéoporotiques vertébrales (MacLean, Newberry et al. 2008). En clinique, son utilisation est de fait limitée aux patients ne pouvant pas utiliser les autres composés.

4.5.5 Teriparatide (Fragment 1-34 de la parathormone humaine)

Ce composé est un agent ostéo-anabolique qui stimule la formation osseuse au-delà des capacités de résorption des ostéoclastes, induisant un gain de masse osseuse. Il réduit à la fois l'occurrence des fractures vertébrales et des autres (Neer, Arnaud et al. 2001). De plus, il induit un gain de masse osseuse plus important que celui observé par un traitement avec des anti-résorptifs (Black, Greenspan et al. 2003).

Il a été montré chez le rat qu'un traitement très prolongé avec le teriparatide pouvait induire la formation d'ostéosarcomes (Vahle, Long et al. 2004). Bien que les doses utilisées dans cette étude correspondent à près de 40 fois celles utilisée chez l'homme, ces données ont conduits à la non-utilisation de ce composé chez les patients à risque pour les ostéosarcomes et à la réduction des durées de traitement. Un inconvénient de ce fragment de parathormone est que le gain de masse osseuse induit par le traitement peut être perdu en quelques mois une fois le traitement arrêté ce qui nécessite l'utilisation d'un anti-résorptif par la suite pour maintenir la densité minérale osseuse.

De part sont coût socio-économique déjà très lourd qui est en plus amené à augmenter dans les années à venir, l'ostéoporose représente un véritable défi de santé publique. Les traitements médicamenteux existent et ont démontré leur efficacité. Cependant, certains de ces agents pharmacologiques présentent des effets secondaires indésirables avérés ou qui restent à clarifier. Par ailleurs, en raison de la nature asymptomatique de la pathologie avant la première fracture, ils ne sont souvent utilisés qu'à la suite de celle-ci, pour prévenir les récidives. Ces différents points révèlent l'importance du développement de stratégies de prévention de cette maladie, au sein desquelles la nutrition trouve tout naturellement sa place.

5- *Nutrition et métabolisme osseux*

La première ligne de défense du tissu osseux par la nutrition repose sur une alimentation apportant, en quantités suffisantes, les principaux constituants des matrices organiques et minérales de l'os.

5.1 Le calcium

En tant que constituant majoritaire de la matrice osseuse, le calcium joue évidemment un rôle essentiel pour la santé osseuse (2006). Un apport suffisant de calcium alimentaire est fondamental à la fois pour le développement du squelette et l'atteinte d'un pic de masse osseuse optimal mais aussi pour limiter la perte osseuse liée à l'âge (Foundation 2008).

Des études épidémiologiques ont permis de mettre en évidence que des apports suffisants en calcium tout au long de la vie permettent de réduire les risques de fractures post-ménopause de 60% (Heaney 1992). Malheureusement, une proportion significative de la population des pays développés reste en dessous des apports nutritionnels conseillés (Cashman 2002). Il faut que les pertes normales de calcium soient compensées par des apports suffisants associés à une bonne absorption au niveau de l'intestin. Dans le cas contraire, l'os est utilisé comme source de calcium pour maintenir la concentration extracellulaire (Rizzoli 2008).

Dans une revue analysant les résultats de 52 études d'intervention consistant en une augmentation des apports nutritionnels calciques, il apparaît que 50 de ces études ont mis en évidence un effet positif de cette supplémentation associé à un remodelage osseux ralenti, une diminution de la perte osseuse liée à l'age et une diminution du risque de fracture (Heaney 2000).

Ces différents résultats placent le calcium comme un nutriment essentiel à la santé osseuse. Cependant, sa supplémentation peut aussi se trouver associé à des perturbations gastro-intestinales et interférer avec l'absorption d'autres constituants importants tels que le zinc et le fer (Rizzoli 2008). De plus, une étude de 2008 de supplémentation calcique sur des femmes ménopausées en bonne santé à révélé une association de cette supplémentation avec un risque augmenté d'accidents cardiovasculaire chez les femmes de plus de 80 ans ayant déjà connu ce type d'accidents (Bolland, Barber et al. 2008). Ces observations indiquent que

l'utilisation de la supplémentation calcique doit se limiter aux cas où les apports alimentaires sont en dessous des apports conseillés.

Au final le calcium est un nutriment essentiel pour l'acquisition et le maintien de la masse osseuse, il est d'ailleurs classiquement utilisé, en association avec la vitamine D, chez les patients présentant une densité minérale osseuse faible.

5.2 La vitamine D

La vitamine D est également très importante pour le devenir de la masse osseuse. Elle contribue à l'absorption du calcium au niveau intestinal et agit également directement sur le squelette pour favoriser sa constitution (Foundation 2006). La déficience en vitamine D est connue pour conduire à des défauts de minéralisation de l'os pouvant conduire à une ostéomalacie. Par ailleurs, une littérature très fournie permet de considérer la déficience en vitamine D comme un contributeur important de l'ostéoporose, notamment via une réduction de l'absorption du calcium, une perte osseuse accélérée, une altération musculaire et un risque augmenté d'hyperparathyroidisme (Foundation 2006).

Un méta-analyse du potentiel anti-fracturaire de la supplémentation en Vitamine D chez les personnes âgées a permis de montrer que des apports en vitamine D de l'ordre de 700- 800 IU/jour permettent de réduire le risque de fractures de la hanche de 26% (Bischoff-Ferrari, Willett et al. 2005).

Du point de vue de la densité minérale osseuse, on évalue entre 75 et 100 nmol/L la concentration sérique optimale en vitamine D (Bischoff-Ferrari, Giovannucci et al. 2006). Au niveau mondial, plus de 50 % de la population n'atteint pas ces niveaux circulants de vitamine D, et pourraient donc bénéficier d'une augmentation de leurs apports (Gaugris, Heaney et al. 2005). Pour ces personnes, corriger les niveaux sériques en vitamine D le plus vite possible est le but à atteindre. Il a été estimé que des apports journaliers entre 700 et 1000 IU sont nécessaires pour obtenir des concentrations comprises entre 75 et 100 nmol/L chez l'adulte (Mosekilde 2008).

La vitamine D est produite naturellement par l'organisme, par l'action des UVs provenant du rayonnement solaire sur la peau. Cependant, les études montrent que cette source de vitamine D n'est pas toujours suffisante, (Gaugris, Heaney et al. 2005) nécessitant un complément par l'alimentation. Les sources de vitamine D sont limitées dans notre alimentation. (Poissons gras, foie…) et certains pays ont opté pour l'autorisation de produits

enrichis en vitamine D (lait, margarine) qui constituent une option intéressante (Foundation 2006).

5.3 Les protéines

Les protéines sont d'autres constituants essentiels des tissus osseux que ce soit d'un point de vue structural ou fonctionnel. En ce sens, fort logiquement, l'importance d'apports protéiques alimentaires suffisants est cruciale dans l'établissement et le maintien du capital osseux (Promislow, Goodman-Gruen et al. 2002) (Kerstetter, Looker et al. 2000) (Hannan, Tucker et al. 2000).

L'augmentation de la quantité de protéines dans le régime induit une augmentation du facteur ostéo-anabolique IGF-1 ce qui pourrait constituer un mécanisme permettant d'expliquer l'effet bénéfiques des régimes hyper protéinés sur la masse osseuse (Bonjour, Schurch et al. 1997) (Dawson-Hughes, Harris et al. 2004).

A l'inverse, certaines études épidémiologiques suggèrent un effet plutôt délétère des régimes trop riches en protéines (Abelow, Holford et al. 1992). La consommation excessive de protéines pourrait conduire à une augmentation de la production d'acide dans l'organisme due au relarguage de protons intervenant au cours de l'oxydation des acides aminés soufrés (Rizzoli 2008). Cette acidose peut provoquer une activation de la résorption osseuse, les ostéoclastes étant activés de façon très efficace par les protons extracellulaires (Hoebertz, Arnett et al. 2003). De même un pH acide s'avère délétère pour l'activité ostéoblastique *in vitro* (Bushinsky 1995).

Ainsi, ce genre d'acidoses systémiques induit par une alimentation trop riche en protéines pourrait à long terme induire une hypercalciurie chronique associée à une déminéralisation du squelette. Ces régimes hyper protéinés, potentiellement acidogènes, sont bel et bien associés à une augmentation des taux de calcium urinaire, mais des études récentes tendent à montrer que ce calcium ne proviendrait pas de la dégradation du tissu osseux mais plutôt d'une absorption intestinale potentialisée (Kerstetter, O'Brien et al. 2005) (Hunt, Johnson et al. 2009).

Au final, la majorité des études à ce jour vont dans le sens d'un effet bénéfique de l'apport protéique sur la santé osseuse et mettent en lumière les risques associés à une nutrition protéique insuffisante.

5.4 Les nouvelles pistes de la prévention nutritionnelle de l'ostéoporose

Depuis maintenant plusieurs années, les stratégies de prévention nutritionnelle de la perte osseuse à l'origine de l'ostéoporose se sont bien développées. Ainsi, au-delà du calcium, de la vitamine D et des protéines, une littérature importante s'est développée autour de l'intérêt d'autres nutriments et micronutriments susceptibles de contribuer à l'acquisition et/ou au maintien du capital osseux.

Parmi les nutriments d'intérêt, la **vitamine K** occupe une place privilégiée. Les différents composés lipophiles regroupés sous l'appellation vitamine K présentent un effet bénéfique sur le squelette via une potentialisation du processus de minéralisation ostéocalcine-dépendant. Or la déficience vitaminique K, considéré comme un facteur de risque de l'ostéoporose, est observée de manière assez fréquente dans certaines tranches de la population suggérant qu'un renfort de la vigilance du suivi des recommandations nutritionnelles concernant cette vitamine pourrait s'avérer bénéfique. (**Annexe 2 :** *« Vitamine K et physiologie osseuse »* (Coxam, Davicco et al. 2009) **(page 209)**)

Le potentiel ostéoprotecteur de la **vitamine C** a également été bien démontré chez la femme ménopausée, notamment par le biais d'études épidémiologiques révélant une corrélation positive entre les apports nutritionnels en vitamine C et la densité minérale osseuse (Leveille, LaCroix et al. 1997) (Hall and Greendale 1998). Par ailleurs, une étude d'intervention a mis en évidence un effet potentialisateur de la prise de vitamine C sur un gain de masse osseuse induit par les œstrogènes et une supplémentation en calcium (Morton, Barrett-Connor et al. 2001). L'effet osseux de la vitamine C proviendrait essentiellement de ses vertus anti-oxydantes (Dennehy and Tsourounis 2010) et son action activatrice sur la synthèse de collagène.

Les **phyto-œstrogènes** sont des composés retrouvés dans le règne végétal qui possède, selon la molécule, des propriétés agonistes ou antagonistes des œstrogènes endogènes. Cela est dû à des analogies structurales de ces molécules avec l'œstradiol. Etant donnée l'importance de la carence œstrogénique dans l'établissement de l'ostéoporose, l'intérêt potentiel des phyto-œstrogènes dans la prévention de la déminéralisation du squelette est évidente. De plus, des études sur des modèles animaux d'ostéoporose ont permis de confirmer l'efficacité de certains de ces composés pour prévenir la perte osseuse (Branca 2003) (Setchell and Lydeking-Olsen 2003) et l'un d'entre eux, la génistéine, s'est avéré capable de d'améliorer la densité minérale osseuse chez la femme ménopausée (Morabito,

Crisafulli et al. 2002). Cependant, cet effet bénéfique sur la densité minérale osseuse est loin d'être retrouvé à chaque fois (Whelan, Jurgens et al. 2006) et l'impact de ces molécules sur le risque fracturaire est inconnu.

Les plantes contiennent encore d'autres micronutriments potentiellement protecteurs pour la santé osseuse. Les **polyphénols**, par exemple, constituent une grande famille de molécules (plusieurs milliers identifiées) qui possèdent un large spectre d'activités biologiques incluant notamment des vertus anti-oxydantes et anti-inflammatoires. L'impact de certains de ces composés sur la santé osseuse a été étudiée avec, dans certains cas, des résultats très encourageant. A titre d'exemple, la quercétine, un polyphénol de la classe des flavonols est capable de préserver les propriétés biomécaniques de fémurs de rates ovariectomisées (Horcajada-Molteni and Coxam 2001).

Le point communs de ces micronutriments est leur présence en quantité importante dans les plantes en général et dans les fruits et légumes en particulier. Leurs potentiels ostéoprotecteur est donc à corréler avec l'effet bénéfique de la consommation de produits d'origine végétale sur les paramètres osseux qui est très classiquement observé dans les études épidémiologiques (Tucker, Hannan et al. 1999) (New 2003). Ainsi, même si bien des choses restent à découvrir, la littérature s'est considérablement développée dans le domaine des interactions entre les produits d'origine végétale et le métabolisme osseux, appuyant l' importance fondamentale de cette classe d'aliments pour la santé de notre squelette et révélant certaines propriétés osseuses individuelles de leurs micro constituants.

Ainsi, l'évolution des concepts de prévention nutritionnelle de l'ostéoporose est en cours. Au-delà du calcium et de la vitamine D, de nombreuses autres nutriments et micronutriments sont à prendre en considération pour leurs capacités à influer sur le remodelage du squelette. Par ailleurs, pour les maladies évolutives liées à l'âge tel que l'ostéoporose, la pertinence du principe de nutrition préventive est bien reconnue. Cependant, l'étude des activités biologiques osseuses des nutriments reste trop marginale pour certaines catégories de molécules. C'est notamment le cas des lipides en général, et des acides gras en particulier. Actuellement, le nombre de personnes atteintes d'obésité est en nette augmentation et ceci est associé à une modulation importante au sein de la population, de la fraction lipidique de l'alimentation, en quantité et en qualité. Dans ce contexte, la littérature existante concernant les propriétés de ces nutriments sur le devenir du squelette, bien que de taille croissante, reste insuffisante.

6- *La nutrition lipidique*

Les lipides de l'alimentation humaine ou animale sont constitués de phospholipides, de stérols, et d'une très large majorité de triglycérides (90%). Ces derniers sont constitués d'une molécule de glycérol sur laquelle se trouvent fixés trois acides gras **(Fig.18)**. La spécificité des triglycérides alimentaires repose donc sur la nature des acides gras le constituant.

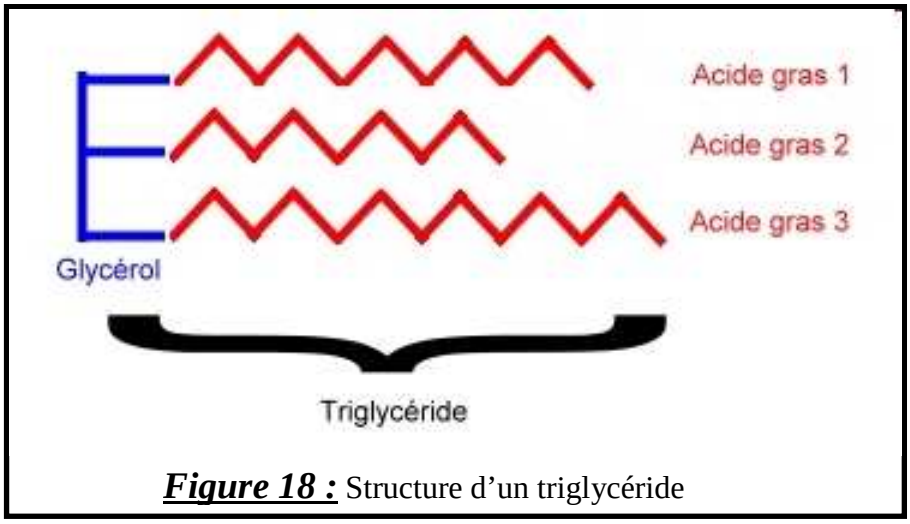


Figure 18 : Structure d'un triglycéride

6.1 Digestion et sources d'acides gras pour les tissus de l'organisme

Au niveau de l'intestin, l'action combinée des sels biliaires et de la lipase pancréatique aboutit à la formation de mycelles contenant des acides gras et des 2-mono-acylglycérols qui sont absorbables par les entérocytes. Les triglycérides seront reformés au sein de l'entérocyte et associés à des lipoprotéines pour constituer les chylomicrons qui vont assurer le transport de ces lipides alimentaires, via la circulation, vers le tissu adipeux pour les stocker et vers les tissus utilisateurs (Zinsou 2005).

Au niveau des tissus cibles, les triglycérides contenus dans les chylomicrons sont hydrolysés à nouveau par l'action d'une lipoprotéine lipase conduisant à la formation de deux acides gras et d'une molécule de 2-mono-acylglycérol. Ces composés vont pouvoir rentrer dans les cellules par diffusion en profitant du gradient de concentration existant entre les milieux intra et extra-cellulaires.

Les « restes » de chylomicrons sont captés par le foie. Au sein de cet organe, des acides gras peuvent être synthétisés à partir de glucose et d'acides aminés. Ces acides gras, sous forme de triglycérides, vont être assemblés avec ceux issues des « restes » de chylomicrons ainsi qu'avec des lipoprotéines pour constituer les Very Low Density Lipoproteins (VLDL) qui sont relarguées dans la circulation où elles peuvent, à l'image des chylomicrons, apporter des acides gras au tissu adipeux ou aux autres tissus utilisateurs.

Au niveau des adipocytes, les acides gras sont stockés sous forme de triglycérides constituants une réserve mobilisable. En cas de besoin, la lipase hormono-sensible est activée ce qui conduit à l'hydrolyse des triglycérides en acide gras libres qui vont être expulsés de l'adipocyte et rejoindre la circulation sanguine au sein de laquelle ils seront transportés vers les tissus cibles complexés à l'albumine **(Fig.19)**.

Au final les acides gras sont mis à la disposition des tissus sous trois formes majeures circulantes, les chylomicrons, les VLDLs et les acides gras libres complexés à l'albumine (Rennes 2009).

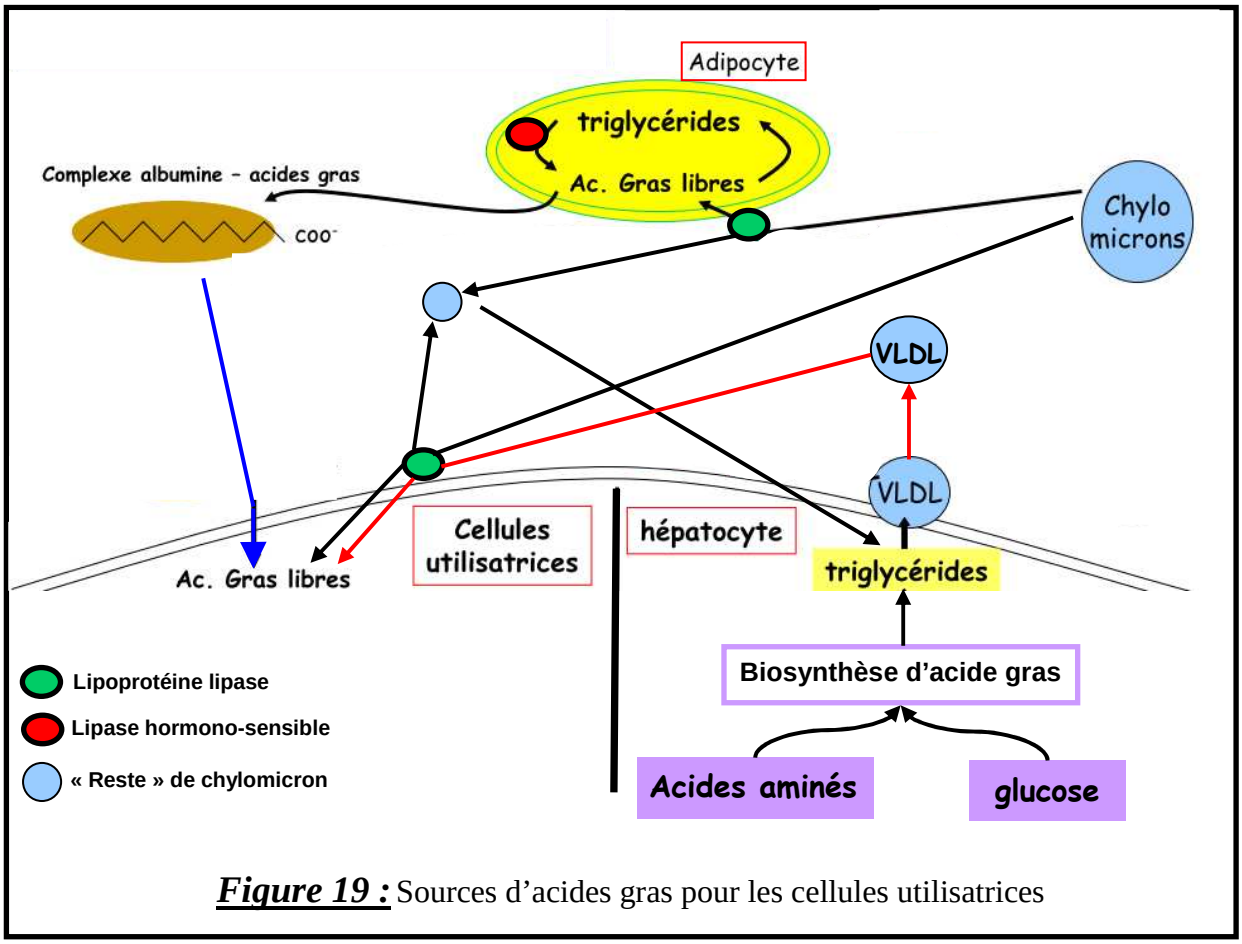


Figure 19 : Sources d'acides gras pour les cellules utilisatrices

6.2 Fonctions des acides gras

Les acides gras ont trois fonctions majeures à accomplir au sein de l'organisme :

- Rôle structural : les acides gras servent à la synthèse d'autres lipides, notamment les phospholipides qui forment les membranes autour des cellules et des organites.

- Rôle métabolique : les acides gras sont une source majeure d'énergie pour l'organisme. Leur dégradation par le processus de β-oxydation va permettre de générer des quantités importantes d'adénosine triphosphate (ATP)

- Rôle fonctionnel : les acides gras sont les précurseurs de plusieurs messagers intra- et extracellulaires. Par ailleurs, ils sont aussi par eux-mêmes des molécules de signalisation à part entière impliquées dans la régulation de processus biologiques fondamentaux.

De manière intéressante, ces différentes fonctions des acides gras vont dépendre à la fois de la quantité d'acides gras présents mais aussi de leur nature. Ainsi, une modulation des triglycérides alimentaire et donc des acides gras les constituants va avoir in fine de nombreuses conséquences pour la physiologie de l'organisme, y compris pour la physiologie osseuse.

7- ***Objectif du travail de thèse***

Le but de mes travaux a été de déterminer les bases des relations qui lient la nutrition lipidique et les processus de remodelage osseux afin d'en déduire les mécanismes fondamentaux nécessaires à l'élaboration de stratégies nutritionnelles rationnelles de prévention de l'ostéoporose.

Pour ce faire, une analyse bibliographique a été réalisée concernant les relations existantes entre les acides gras issus de l'alimentation et le métabolisme osseux. Le but était de définir un état de l'art et de caractériser, de la manière la plus exhaustive possible, les acides gras potentiellement intéressant pour la préservation du capital osseux ainsi que les mécanismes impliqués décrits jusqu'à présent. ***Publication numéro 1 : " Fatty acid and bone relationships : I love you… Neither do I »"***

Suite à cette revue de la littérature, une étude d'intervention préclinique a été entreprise visant à évaluer le potentiel protecteur de certains acides gras polyinsaturés (AGPI) à longue chaîne pour l'évolution de la santé osseuse avec l'âge. Les propriétés anti-inflammatoires, établies dans la littérature, de certains AGPI ont été testées pour leur capacité à tempérer une perte osseuse dans un modèle murin de *progeria*, où l'établissement d'un statut inflammatoire sub-chronique serait un des mécanismes à l'origine de la dégradation du tissu osseux. ***Publication numéro 2 :*** "***Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis"***

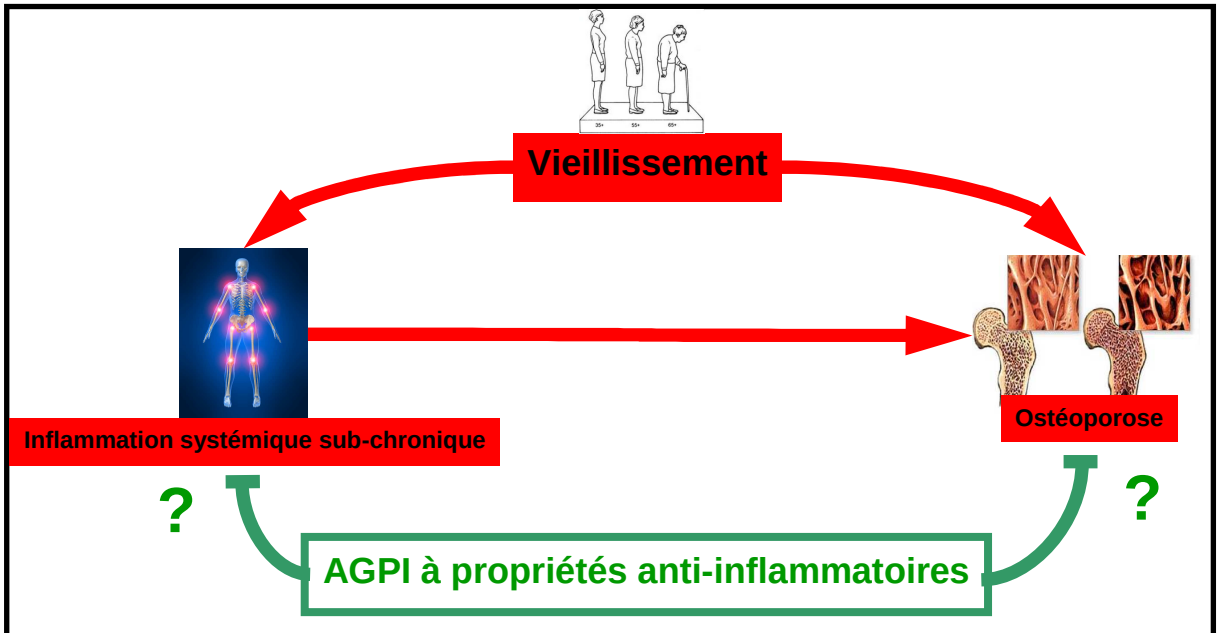


Figure 20 : Vieillissement, inflammation et perte osseuse : protection par les AGPIs ?

Par ailleurs, bien que la littérature se développe de manière très intéressante concernant l'impact des acides gras sur la santé osseuse, les données concernant les mécanismes par lesquels ces composés modifient le remodelage du squelette sont très parcellaires et ne permettent pas d'expliquer l'ensemble des phénomènes observés. Récemment, les acides gras à longues chaînes ont été décrits comme étant les ligands d'un récepteur couplé aux protéines G (GPR), le GPR40. Cette découverte confirme que le rôle des acides gras au sein de l'organisme va bien au-delà de leur rôle de substrat énergétique, ces molécules étant des molécules de signalisation à part entière, capables de moduler, de manière directe, la physiologie cellulaire. Ayant pu vérifier l'expression du GPR40 au niveau des cellules osseuses, nous avons cherché à déterminer si ce récepteur était, ou non, impliqué dans la régulation du remodelage du squelette. ***Publication numéro 3 :*** ***"The free fatty acid receptor GPR40 protects from bone loss through inhibition of osteoclast differentiation"***

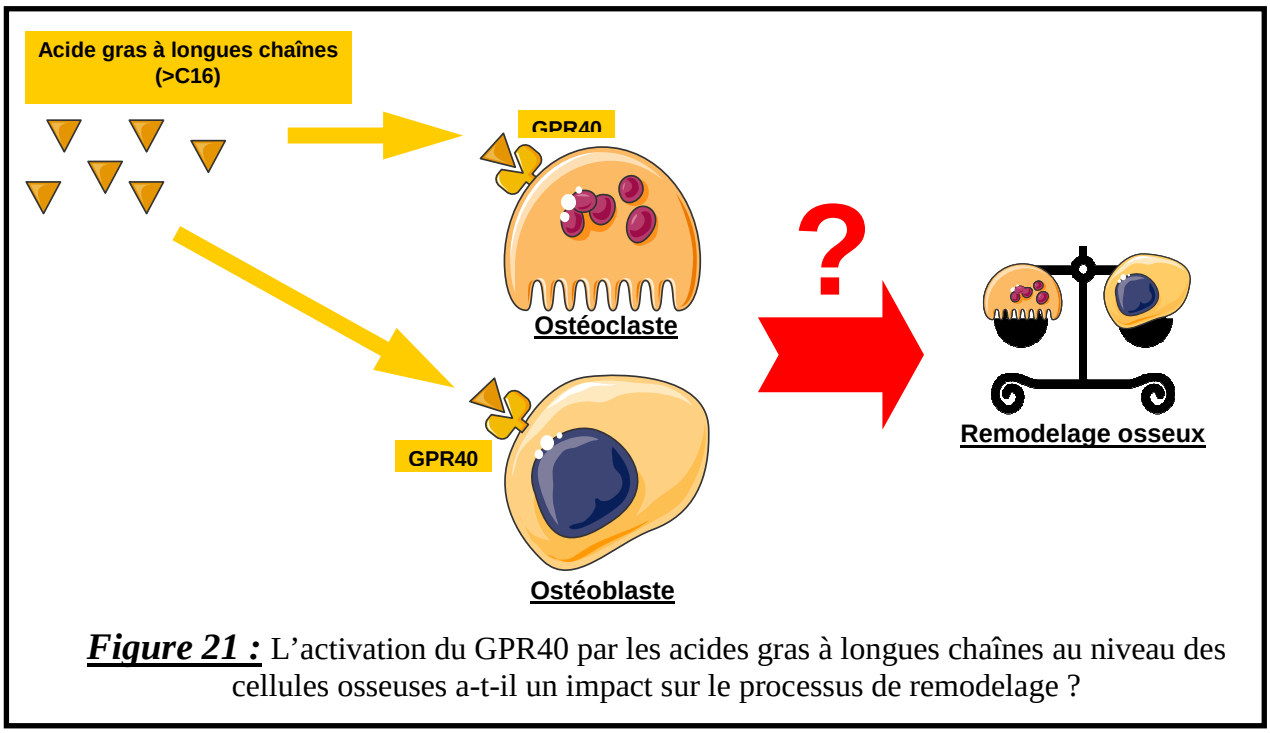


Figure 21 : L'activation du GPR40 par les acides gras à longues chaînes au niveau des cellules osseuses a-t-il un impact sur le processus de remodelage ?

Seconde partie :

Publications

Article numéro 1 :

Fatty acids and bone relationships: "I love you… Neither do I"

Fabien Wauquier, Claire Philippe, Laurent Léotoing, Mélanie Spilmont, Véronique Coxam, Yohann Wittrant

XXXX, 2011

Fatty acids and bone relationships: “I love you… Neither do I”

Fabien Wauquier[1,2,3], Claire Philippe[1,2,3], Laurent Léotoing[1,2,3], Mélanie Spilmont[1,2,3], Véronique Coxam[1,2,3], and Yohann Wittrant[1,2,3*]

1. INRA, UMR 1019, UNH, CRNH Auvergne, F-63009 CLERMONT-FERRAND, FRANCE
2. Clermont Université, Université d'Auvergne, Unité de Nutrition Humaine, BP 10448, F-63000 CLERMONT-FERRAND, FRANCE
3. Equipe Alimentation, Squelette et Métabolismes

* To whom correspondances should be addressed: e-mail: yohann.wittrant@clermont.inra.fr ; mail: UMR 1019 - Centre de Recherche INRA de Clermont-Ferrand/Theix; Phone: +33 (0)4 73 62 47 84; Fax: +33 (0)9 57 05 99 12

Keywords:

Fatty acids, bone, nutrition, osteoporosis

A - Introduction

Industrialized countries face an amplified prevalence of metabolic disorders as a consequence of increased amount of fat in daily food intake (James 2008). These disorders have reached record proportions during the last three decades and mostly include obesity and type 2 diabetes establishments. However, the main social and economic burdens of these pathologies lye in their morbitity. As a matter of fact, obesity and type 2 diabetes alter tissue functions and are associated with complications such as cardio-vascular diseases, cancers, sarcopenia and osteoporosis as well (Felson, Zhang et al. 1993).

Regarding locomotor dysfunctions, when associated with age-related bone loss, high fat diets have dramatical impact on osteoporosis establishment (Reid, Ames et al. 1992; Reid, Plank et al. 1992; Reid 2002), with an increase associated-mortality (Adachi, Adami et al. 2010). In fact, osteoporotic fractures can result in up to 20% excess mortality within one year. Additionally, up to 50% of patients will be disabled, half of them requiring long term nursing home care, while only one third fully recover from hip fracture. Current osteoporotic treatments are limited to drug-based strategies, however important side-effects have been reported, including a greater prevalence of breast cancer and in a lesser extend osteonecrosis of the jaw (Rizzoli, Reginster et al. 2011). Thus, these clinical observations strongly enlighten the need for alternative opportunities in the prevention of bone loss.

As a consequence, the epidemic of obesity has led to increased number of guidelines encouraging low fat and energy diets and the development of low fat products or fat substitutes as well, in order to reduce associated complications. However, from a “bone” point of view, mechanisms linking fat intake to bone alteration remain quite controversial.

Previous epidemiological studies have demonstrated a positive correlation between Body Mass Index (BMI) and Bone Mineral Density (BMD) (Reid, Ames et al. 1992; Felson, Zhang et al. 1993; Reid 2002). Nevertheless, this correlation may be attributed to mechanical stimulation of bone formation or obesity-related enhanced estrogen level (Gimble, Robinson et al. 1996; Reid 2002; Starcke and Vollmer 2006), independently of adiposity effect on bone. Indeed, when mechanical loading effect of total body weight was statistically removed, fat mass was negatively correlated with bone mass and positively associated with bone fractures, suggesting a detrimental effect of excess adiposity on bone (Blum, Harris et al. 2003; De Laet, Kanis et al. 2005; Hsu, Venners et al. 2006; Zhao, Liu et al. 2007).

In another hand, fat quality is also a crucial determinant for bone tissues behavior. Indeed, far from being only energy substrates, a growing body of evidence demonstrate that influence of fatty acids on bone metabolism widely relies on their origin, structure, relative concentration and metabolic context. As a matter of fact, depending on their family, fatty acids may either be beneficial or detrimental to bone (Raisz, Alander et al. 1989; Laneuville, Breuer et al. 1995). For instance, clinical observations, consistently with *in vivo* and *in vitro* data, rather support a bone loss induction by the ω-6 family, while ω-3 are believed to protect bone health (Corwin 2003; Ono, Kaneko et al. 2005; Calder 2006; Tsutsumi, Xie et al. 2009). Nevertheless, this debate is quite reductive regarding the wide range of fatty acid molecules present in human diet, and the mechanisms of action are poorly understood.

Then, according to the literature several downstream targets may be involved in mediating fatty acid impact on bone functions. Among them, inflammation status has been extensively investigated as a key parameter favoring bone degradation (De Benedetti, Rucci et al. 2006). Along with inflammation, oxidative stress plays a central role in bone cell behavior in the presence of high fatty acid concentration (Wauquier, Leotoing et al. 2009). In addition, in a context of integrated biology, several organs involved in fatty acids metabolism has been revealed as potent third protagonist in linking high fat diet to altered bone structure.

Finally, recent studies have shown that fatty acids may stand as relevant ligands for membrane-bound or nuclear receptors (Himes and Smith; Diascro, Vogel et al. 1998; Hwang 2001; Briscoe, Tadayyon et al. 2003; Brown, Goldsworthy et al. 2003; Maurin, Chavassieux et al. 2005), thus underlying the complexity of the system and the need for a systematic and timely review of the concepts linking fatty acids to bone health.

B - Lipids and bone: the clinical point of view

In addition to their role in energy production and storage, dietary fats influence physiological processes such as thermogenesis, insulin release and subsequent glucose homeostasis, global redox status and inflammatory parameters, thus impacting on organs and tissues behavior. As a matter of fact, fats, as fatty acids, are involved in the modulation of structural and functional properties at the cellular level. However, depending on the nature of the fatty acids (chain length, saturation, double bond tridimensional conformation…) they may either lead to beneficial or detrimental effects on bone tissues as revealed by a wide range of pre-clinical and clinical investigations.

1. Fatty acids terminology and biosynthesis

In tissues, fatty acids may either be found as triglycerides, phospholipids, chylomicrons, lipoprotein complexes or free. Free fatty acids, are carboxylic acids with an unbranched aliphatic tail that may differ in length (4 to 28 carbons) and unsaturation degree. Fatty acids are synthesized by plants, micro-organisms and animals as well, and origin from a *de novo* synthesis eventually followed by chain elongation and desaturation (Figure 1). The chain length and the degree of unsaturation give way to the specific properties of each fatty acid types (Legrand 2007).

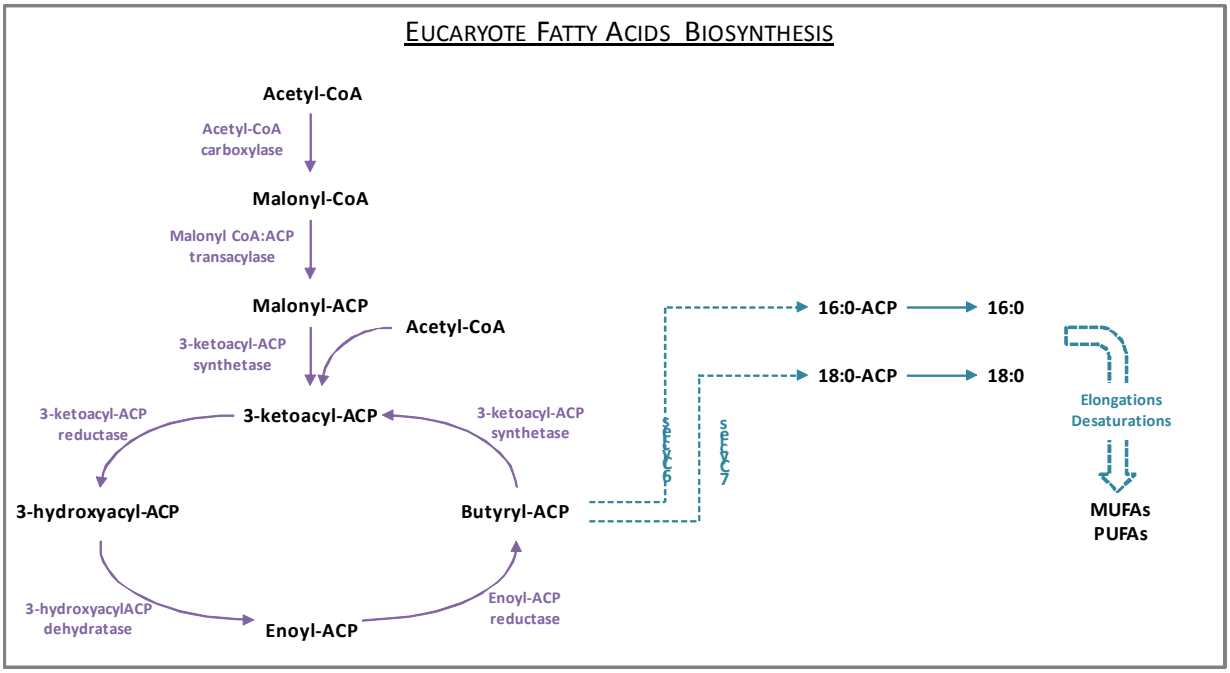


Figure 1 : Eucaryote fatty acids biosynthesis from Acetyl-CoA

Despite the wide range of fatty acids found in nature, only a few of them are currently associated with the human diet. Among those fatty acids, the linoleic acid and the α-linolenic acid are most notably required from the diet. Indeed, those two essential fatty acids are precursors of the ω-3 and ω-6 families respectively and, in contrast to others, they cannot be synthesized by humans from acetyl CoA due to the lack of dedicated enzymes (Figure 2) (Calder 2001; Nakamura, Cho et al. 2001; Guesnet, Alessandri et al. 2005; Legrand 2007).

2. Dietary sources

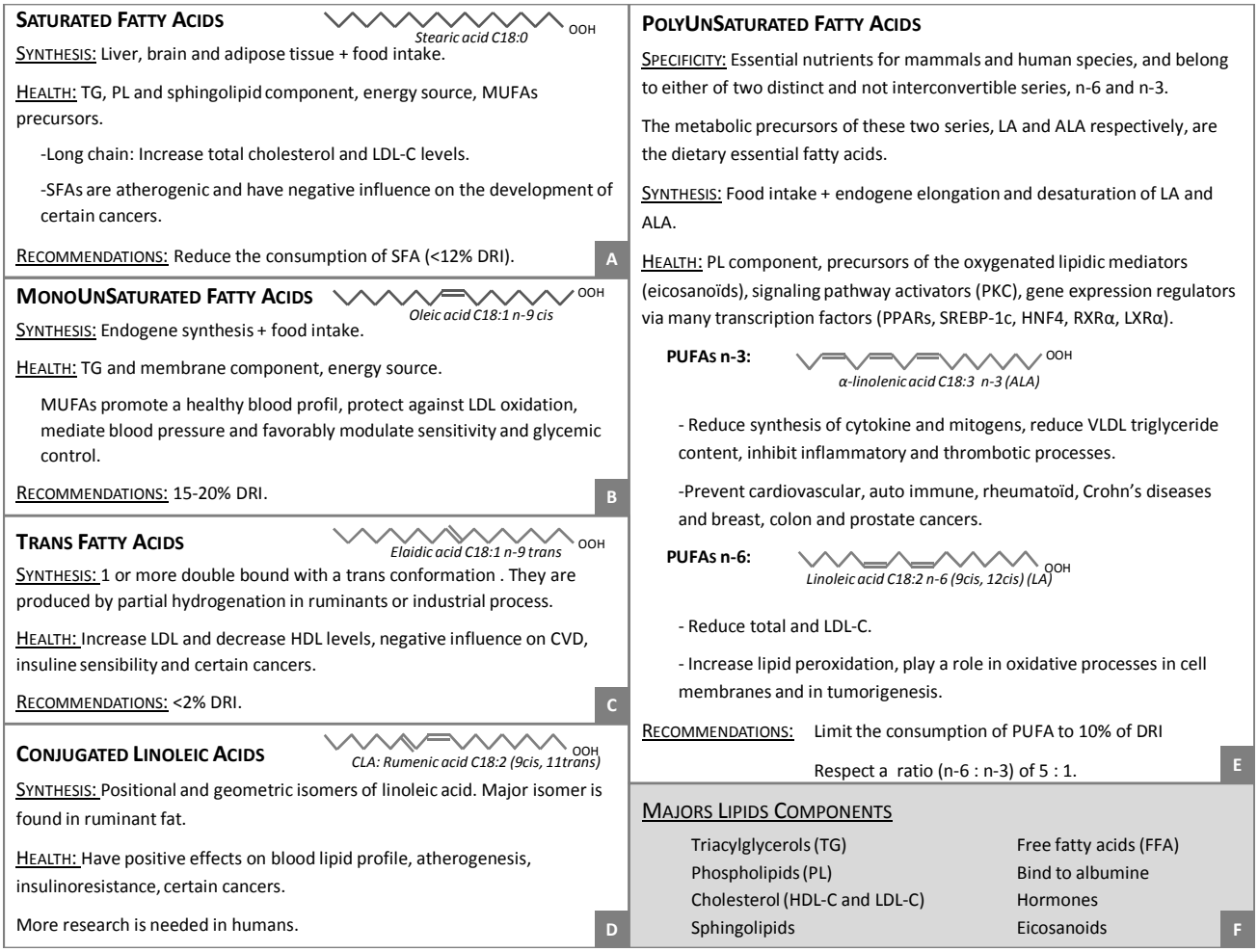
Table 1 describes the most common fatty acids found in daily food intake. They are grouped depending on their main dietary sources and the presence and number of double bound as saturated fatty acids (SFAs), mono unsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (Table 1).

SATURATED FATTY ACIDS (SFA)		
USUAL NAME	**FORMULA**	**SOURCES**
Butyric acid	C4 :0	Dietary fat
Caproic acid	C6 :0	Dietary fat
Caprylic acid	C8 :0	Dietary fat / coco oil / palm oil
Capric acid	C10 :0	Dietary fat / coco oil / palm oil
Lauric acid	C12 :0	Coco oil / palm oil
Myristic acid	C14 :0	Coco oil / palm oil / dietary fat
Palmitic acid	C16 :0	Most of oils and fats
Stearic acid	C18 :0	Most of oils and fats
Arachidic acid	C20 :0	Peanut oil
Beheric acid	C22 :0	Peanut oil
Lignoceric acid	C24 :0	Peanut oil
MONO UNSATURATED FATTY ACIDS (MUFA)		
USUAL NAME	**FORMULA**	**SOURCES**
Palmitoleic acid	C16 :1	Animal fats / vegetal oils / fish oils
Oleic acid	C18 :1	Fat especially olive oil and sunflower oil
Cis-vaccenic acid	C18 :1 cis	Most of vegetal oils
Gadoleic acid	C20 :1	Fish oil
Erucic acid	C22 :1	Mustard oil and rapeseed oil
Nervonic acid	C24 :1	Fish oil
POLY UNSATURATED FATTY ACIDS (PUFA)		
Ω-3		
USUAL NAME	**FORMULA**	**SOURCES**
α-linolenic acid	C18 :3 ω-3 (ALA)	Flax oil/ perilla oil / canola oil / soybean
Stearidonic acid	C18 :4 ω-3 (SA)	Fish oil / blackcurrant seed / soybean / hemp
Eicosapentaenoic acid	C20 :5 ω-3 (EPA)	Fish (salmon, herring, anchovy, smelt, mackerel)
Docosapentaenoic acid	C22 :5 ω-3 (DPA)	Fish (salmon, herring, anchovy, smelt, mackerel)
Docosahexaenoic acid	C22 :6 ω-3 (DHA)	Fish (salmon, herring, anchovy, smelt, mackerel)
Ω-6		
USUAL NAME	**FORMULA**	**SOURCES**
Linoleic acid	C18 :2 ω-6 (LA)	Most of vegetal oils
γ linoleic acid	C18 :3 ω-6 (GLA)	Primerose, borage and cassis oil
Arachidonic acid	C20 :4 ω-6 (AA)	Animal fat, liver, eggs, fish
CLA		
USUAL NAME	**FORMULA**	**SOURCES**
Rumenic acid	C18:2 9c, 11t	Dietary products
	C18:2 10t, 12c	Dietary products
TRANS FATTY ACIDS		
USUAL NAME	**FORMULA**	**SOURCES**
Elaidic acid	C18 :1 9t	Partially hydrogenated vegetable oils
Vaccenic acid	C18 :1 11t	Ruminant meat and milk
Linolelaidic	C18 : 2 ω-6 9t, 12t	Partially hydrogenated vegetable oils

Table1: Commons fatty acids: Classification, denomination and sources

3. General health impact

As mentioned above, beside their structural and energetic roles, fatty acids influence tissue behavior directly or indirectly through their derived metabolites. Their impact widely depends upon their structure (Box 1).



Box 1 : Fatty acids: Generalities, Biological Functions And Health Effects

Thus, according to the literature, **SFAs** are commonly described for their negative impact on health as they lead to higher risk of coronary heart disease. The correlation between SFAs consumption and cardiovascular disease establishment (CVD) emerged in the 1960s and 1970s. From this point, guidelines stressed out the importance to limit they proportion in daily food intake and to further investigate the impact of lipid quality and quantity on health (Wahrburg 2004; Woodside and Kromhout 2005; Micha 2010).

In contrast, **MUFAs** are considered to have a positive impact on health. The biological effects of MUFAs depend on whether the MUFAs are in the *cis* or *trans* configuration. The term MUFA reports to the *cis* geometry, their native form. MUFAs reduce the probability of metabolic syndrome and CVD development through a favorable modulation of blood lipid profile, blood pressure and insulin sensitivity, as well as a reduced inflammatory context (Gillingham, Harris-Janz et al. 2011).

Regarding **PUFAs**, their absorption leads to specific synthesis of long chain PUFAs (LCPUFAs) such as arachidonic acid for the ω-6 serie, eicosapentaenoic and docosahexaenoic acid for the ω-3 serie. Those LCPUFAs exhibit a crucial role in regulating cell membrane fluidity, eicosanoids production, inflammatory cytokine secretion and the expression of wide range of genes either involved in cell differentiation, metabolism and survival (Figure 2).

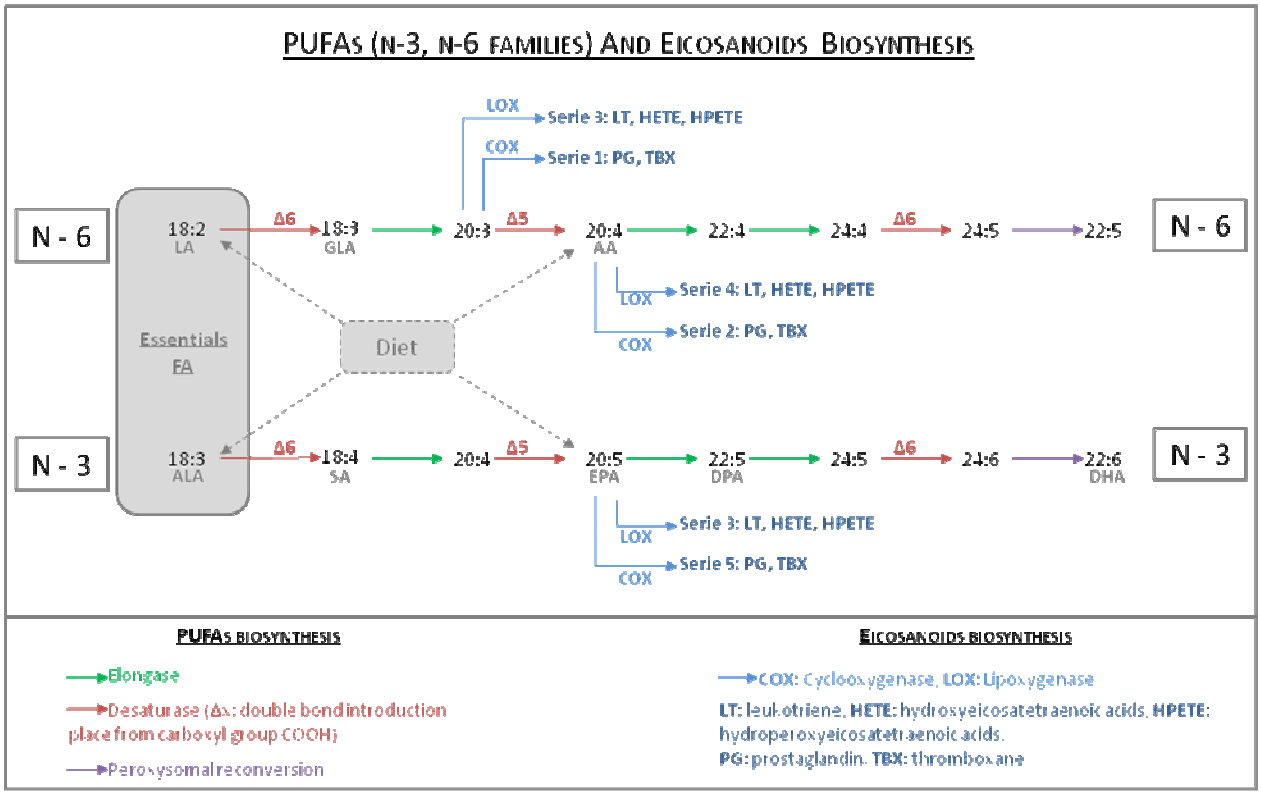


Figure 2 : Polyunsaturated fatty acids (ω-3 and ω-6 families) biosynthesis from linoleic and α-linolenic acids supplied by the diet and formation of eicosanoids from them.

To positively impact health, an optimal ratio of 5:1 to 3:1 (ω-6/ω-3) is now recommended. In these proportions, a benefic effect on pathologies such as CVD, autoimmune and inflammatory diseases, diabetes and obesity have been described. In contrast, when unbalanced, an excessive consumption of PUFAs ω-6 or a PUFAs ω-3 deficiency may promote inflammatory disorders establishment and related pathologies (Calder 2001; Nakamura, Cho et al. 2001; Guesnet, Alessandri et al. 2005).

Besides, although **trans FA** are commonly found in modest amount in meat and dairy products, their major consumption originate from industrial partially hydrogenated food. Since the 1990s trans fats have raised health concerns. Indeed, different studies have largely reported that their high levels in the diet lead to unfavorable effects on CVD, insulin sensitivity and cancers establishment. Then, most of health organizations now recommend reducing consumption of foods containing trans fatty acids (Hunter 2006; Gebauer, Psota et al. 2007; Reynolds and Roche 2010).

On the contrary, **CLA** isomers from milk and dairy products, have received considerable attention for their potent beneficial biological properties on health. To date, animal studies have revealed amelioration of blood lipid profile, reduction of atherosclerosis development and insulin-resistance. However, in humans, their interest is still debated and further investigations remain required (Banni, Petroni et al. 2004).

4. About lipids and bone health…

Literature has widely linked lipid intake and inflammation status, a key protagonist involved in bone resorption (Ono, Kaneko et al. 2005; Tsutsumi, Xie et al. 2009). As a matter of fact, inflammation favors bone degradation by stimulating osteoclast activity while inhibiting osteoblast bone formation leading to unbalanced bone remodeling and subsequent bone loss. Regarding inflammation, lipids exhibit a duality, with both pro- and anti-inflammatory effects depending on their structures and metabolism (Raisz, Alander et al. 1989; Laneuville, Breuer et al. 1995). In this light, a growing body of evidence has revealed that ω-6 increase bone loss while ω-3 are believed to protect bone health (Kruger and Horrobin 1997; Seifert, Bruce et al. 1997; Watkins, Li et al. 2001; Watkins, Li et al. 2001; Watkins, Lippman et al. 2001; Albertazzi and Coupland 2002; Corwin 2003; Fernandes, Lawrence et al. 2003; Watkins, Li et al. 2003; Ono, Kaneko et al. 2005; Calder 2006; Poulsen, Moughan et al. 2007; Salari, Rezaie et al. 2008; Tsutsumi, Xie et al. 2009; Kruger, Coetzee et al. 2010; Kim and Ilich 2011). Nevertheless, this debate remains controversial and rather reductive. Then, to make the way to an “as integrated and updated as possible” point of view

on the field, we grouped an “as exhaustive as possible” list of pre-clinical and clinical trials related to bone and lipids interactions.

To date, the **first evidence** that both quantity and quality of dietary lipids play an important role in bone health emerged in the early 1930’s with the Borland and Jackson’s study describing in rats, the establishment of a severe osteoporosis in a context of essential fatty acids deficiency (Borland and Jackson 1931). This finding was lately confirmed in the 1950’s with the demonstration that EFA deficiency was associated with a change in tissue lipid profile and histology as revealed by replacement of bone by adipose tissue and bone demineralization, respectively (Alfin Slater and Bernick 1958; Biran, Bartley et al. 1964). However, these studies were kept somehow anecdotic and the topic only became a field of research during the two last decades.

Indeed, the data from the literature have grown exponentially the last years, thus enlightening the growing importance of the field for health systems but underlying its complexity as well. The table 2 gathers the main clinical and *in vivo* studies linking fat diets to bone tissues behavior. Briefly, when taken together, data from the literature reveals that the studies were mainly focused on inflammation-induced bone loss, bone marrow adiposity and altered bone cells coupling.

In other words, when considering fatty acids and bone relationships type by type, most of the studies reveal that high-fat diets (HFD), most notably those rich in SFAs was negatively associated with BMD and positively with hip fracture risk (Corwin, Hartman et al. 2006; Jeong, Cho et al. 2010). In fact, the reduced bone density and mineral content is due to an increased bone adiposity that is correlated with a stimulated bone resorption and an impaired bone forming cell activity (Parhami, Tintut et al. 2001; Chen, Lazarenko et al. 2010; Xiao, Cui et al. 2010; Halade, El Jamali et al. 2011; Patsch, Kiefer et al. 2011).

In contrast, oleic acid, the most commonly used MUFAs, has been demonstrated as a relevant positive regulator of calcium and bone turnover markers levels in plasma thus counteracting osteoporosis features in rodents (Puel, Quintin et al. 2004; Rezq, Labib et al. 2010; Saleh and Saleh 2011). In humans, data parallel with animal studies in term of decreased hip fracture risk (Martinez-Ramirez, Palma et al. 2007; Orchard, Cauley et al. 2010), however controversies persist around this hypothesis (Macdonald, New et al. 2004).

With regard to PUFAs, their actions on bone remodeling are well documented either on clinical or animal studies. Three main hypotheses arising from the literature data have been particularly tested so far: (1) effects of high level of ω-3 PUFAs, (2) influence of the ω-6/ω-3 ratio modulation and (3) impact of different PUFAs combination within the diet.

Experiments using fish oil enriched diet suggest a positive action of ω-3 fatty acids on bone health with increased bone mineral content, BMD, calcium balance and absorption, bone formation markers and decreased bone resorption's ones in mice (Sun, Krishnan et al. 2003; Zeitlin, Segev et al. 2003; Cohen and Ward 2005; Bhattacharya, Rahman et al. 2007), rats (Coetzer, Claassen et al. 1994; Sakaguchi, Morita et al. 1994; Kruger, Coetzer et al. 1995; Green, Wong et al. 2004; Kruger and Schollum 2005; Lukas, Gigliotti et al. 2011) and human (van Papendorp, Coetzer et al. 1995; Bassey, Littlewood et al. 2000; Zwart, Pierson et al. 2010; Farina, Kiel et al. 2011). Studies on the ω-3/ω-6 ratio modulation indicate that a high ratio protect bone health in all models (mice (Lau, Ward et al. 2009; Banu, Bhattacharya et al. 2010; Wauquier, Barquissau et al. 2011), rats (Claassen, Potgieter et al. 1995; Watkins, Li et al. 2006), piglets (Weiler and Fitzpatrick-Wong 2002; Mollard, Kovacs et al. 2005) and human (Hogstrom, Nordstrom et al. 2007)). Inversely, an unbalance ratio resulting from an overconsumption of ω-6 PUFAs involve detrimental effects on bone. Indeed the favorable effect of ω-3 PUFAs seem to rely in an increased rate of bone formation supported by both, a stimulatory effect on osteoblastic activity as demonstrated by plasmatic and transcriptomic markers analysis and an inhibitory effect on osteoclastic functions. Those effects are well documented *in vivo* on rodents (Sakaguchi, Morita et al. 1994; Yamada, Fushimi et al. 1995; Schlemmer, Coetzer et al. 1999; Poulsen and Kruger 2006; Shen, Yeh et al. 2006; Wauquier, Barquissau et al. 2011) and piglets (Watkins and Seifert 2000; Lucia, Fitzpatrick-Wong et al. 2003; Blanaru, Kohut et al. 2004). In human, most of prospective studies reveal a correlation between PUFAs consumption and a higher BMD as well as a decreased fracture risk (Griel, Kris-Etherton et al. 2007; Hogstrom, Nordstrom et al. 2007; Martinez-Ramirez, Palma et al. 2007; Farina, Kiel et al. 2011).

Regarding CLA and trans fats, their effects on bone health are poorly documented on humans. However, in rodents, CLA have a positive impact on bone formation, and BMD (Li, Seifert et al. 1999; Rahman, Bhattacharya et al. 2007; Halade, Rahman et al. 2011; Rahman, Halade et al. 2011) thus encouraging clinical trials.

According to these data, it becomes obvious that fat and bone are closely related however, mechanisms remain far from being fully elucidated and extended literature review have revealed additional tracks to further investigate the field (Gimble, Zvonic et al. 2006; Rosen and Bouxsein 2006; Duque 2008; Reid 2008; Rosen 2009).

BONE FORMATION	**BONE RESORPTION**
SERUM *Osteoblast enzyme* Total alkaline phosphatase (total ALP) Bone alkaline phosphatase *Matrix protein* Osteocalcin *Biomarkers of the synthesis of bone type I collagen* Procollagen type I C-terminal propeptide (PICP) Procollagen type I N-terminal propeptide (PINP)	**SERUM** *Osteoclast enzyme* Tartrate-resistant acid phosphatase (TRAP) Cathepsin K *Degradation products of type I collagen* C-terminal cross-link telopeptide generated by MMPs **SERUM / URINE** *Degradation products of type I collagen* C-terminal cross-link telopeptide (CTX) N-terminal cross-link telopeptide (NTX) *Markers of bone breakdown* Pyridinoline (PYD) Deoxypyridinoline (DPD) **URINE** *Collagen degradation products* Hydroxyproline

Box 2 : Bone turnover biomarkers

FAT IN THE DIET AND BONE: PRE-CLINICAL (RODENT AND PIGLETS) AND CLINICAL STUDIES (HUMANS)			
DATE	**AUTHORS**	**DESIGN**	**RESULTS**
MICE			
2001	(Parhami, Tintut et al. 2001)	Mice (4 weeks-old) for 17 weeks or for 30 weeks High-fat (HF) diet (including 1.25% cholesterol, 15.8% fat, and 0.5% cholate). Or a control diet (containing 6% fat)	HF diet after 7 months: ↘ femoral mineral (43%), ↘ mineral density (15%) and ↘ vertebral mineral content compared with mice on the control diet. Smaller deficits were observed after 4 months. Osteocalcin expression was reduced in the marrow of HF-fed mice.
2003	(Sun, Krishnan et al. 2003)	Mice (8 weeks-old) OVX or Sham operated Diet containing 5% corn oil (CO) or 5% fish oil (FO).	FO: ↗ plasma lipid C16:1 ω-6, C20:5 ω-3, and C22:6 ω-3 ↘ plasma lipid C20:4n6 and C18:2n6 Bone loss significantly higher ($p < 0.05$) in OVX mice fed with CO than FO fed mice
2003	(Zeitlin, Segev et al. 2003)	Mice (7 weeks-old) OVX or Sham operated for 13 weeks. Diet containing 7.55% of fat. ω-3 PUFA diet consisted in 0.55% of fish oil.	ω-3 diet: ↘ triglyceride levels in the serum ↘ IL-1 β levels ↗ secretion of IL-10, anti-inflammatory cytokine. Inhibit the increase in adipose tissue caused by OVX
2005	(Cohen and Ward 2005)	Mice postnatal 4 weeks to 13 weeks 10% fat-diet (Flaxseed oil FO or Corn oil CO)	FO diet ↗ EPA, DHA and ↘ LA and AA serum levels No significant change on IL-1β, IL-6 and TNF-α serum levels, on bone mass and strength in femur and lumbar vertebra.
2007	(Bhattacharya, Rahman et al. 2007)	Mice (52 weeks) for 26 weeks Diets containing 10% corn oil CO or 10% fish oil FO	FO: higher BMD in different bone regions ↗ bone formation markers ALP and osteocalcin in the serum
2007	. (Rahman, Bhattacharya et al. 2007)	Female mice (52 weeks-old) for 10 weeks Diet containing 10% CO or 9.5% CO+0.5% CLA	CLA: higher BMD in different bone regions ↘ proinflammatory cytokines (TNFα, IL-6, NF-κB) ↘ osteoclast (RANKL and TRAP). ↘ fat mass and ↗ muscle mass.
2009	(Lau, Ward et al. 2009)	Mouse *fat-1* (3 week-old), for 12 weeks Diet containing 10% safflower oil	*fat-1*: levels of ω-3 PUFA were higher in femurs vs control mice ω-6/ω-3 PUFA ratio in the femur was negatively correlated with BMC and peak load at femur midpoint and femur neck.

2010	(Banu, Bhattacharya et al. 2010)	Mouse *fat-1* or wild-type (8 week-old), OVX or Sham operated for 26 weeks Diet containing 10% safflower oil	Wild-type: OVX ↘ serum ALP and P1NP levels and loss of bone in the distal femur, femoral neck, proximal tibia, and fourth lumbar vertebra. *fat-1*: no bone lost after ovariectomy higher cancellous and cortical bone mass in the femur
2010	(Xiao, Cui et al. 2010)	Mice (3 week-old) for 12 weeks High fat diet HFD containing 21.2% fat (additional 17.5% lard and 0.5% cholesterol) or control diet containing 4.8% (w/w) fat.	HFD: ↘ BMD and maximum load of femur Down-regulation of most of the genes related to bone formation + up-regulation of those related to bone resorption with the changes in plasma bone metabolic biomarkers.
2011	(Halade, Rahman et al. 2011)	Mice (52 weeks-old) for 26 weeks Diet containing (10% corn oil) CO or (5% fish oil with 5% CO) FO or 9.5% CO + 0.5% CLA (50:50 of cis-9 trans-11 and trans-10 cis-12) CLA or combination of CLA and FO ad libitum	CLA: ↗ BMD FO: ↗ BMD CLA+FO: ↘ PPARγ and cathepsin K expression in bone marrow ↗ BMD ↘ bone marrow adiposity, inflammation, oxidative stress
2011	(Halade, El Jamali et al. 2011)	Mice (52 weeks-old) for 26 weeks Diet containing (10% corn oil) CO or standard	CO: ↗ number of osteoclasts per trabecular bone length ↘ trabecular bone volume in the distal femoral metaphysis Up-regulation of osteoclast-specific cathepsin k and RANKL expression and down-regulation of osteoblast-specific RUNX2 and OPG expression
2011	(Patsch, Kiefer et al. 2011)	Mice (7 week-old) for 24 weeks 3 different diets: low-fat diet (LF, 10% fat: control), or extended high-fat chow (eHF, 60% fat), or short-term high-fat chow (sHF: 21 weeks of LF and 3 weeks of eHF)	eHF ans sHF: ↗ CTX, body weight, insulin, and leptin and they show a negative correlation with bone density and bone volume. Bone loss and ↘ BMD vs control Changes in bone microarchitecture but trabecular and cortical thickness remained unaffected
2011	(Rahman, Halade et al. 2011)	Female mice (52 weeks-old) for 26 weeks Diet withCLA isomers: (9.5% CO + 0.5% c9t11 or + 0.5% t10c12 or + 0.25% c9t11 and 0.25% t10c12) or 10% corn oil (CO)	t10c12: higher BMD in femoral, tibial and lumbar regions than those fed CO or c9t11-CLA ↘ osteoclastogenic factors in serum (RANKL, TRAP, IL-6 and TNF-α)
2011	(Wauquier,	Female mice SAMP8 strain (8 weeks old) for 43	BO and FI prevent altered bone parameters of senile osteoporosis

	Barquissau et al. 2011)	weeks (SAMP8 mouse strain is a progeria model) Diet with different lipid sources: sunflower diet (high ω6/ω3 ratio: SU) or borage diet (high γ-linolenic acid BO) or fish diet (high in long chain ω3 FI)	Bone architecture (↗ BMD and bone volume) Inflammatory parameters (↘ CRP in plasma, ↘ expression of inflammation markers: IL-6, IL-6R, IFNgR1) Bone resorption (↘ CTX1 in plasma, ↘ expression of bone resorption markers: MMP2, MMP9, CTR, RANKL and ↗ OPG) ↗ osteoblasts markers (osterix, osteopontin)
RATS			
1931	(Borland and Jackson 1931)	Rats Fat-deficient diet (no fat in the diet)	Development of severe osteoporosis with an increase of renal and arterial calcification
1958	(Alfin Slater and Bernick 1958)	Rats Fat-deficient diet (no fat in the diet) for 12 weeks	↘ proliferation of cartilage cells at the tibialproximal head Development of an extensive osteoporosis
1994	(Coetzer, Claassen et al. 1994)	Rats (3 month-old) OVX 8% fat-diet for 6 weeks (fish or evening primrose oil)	Fish: ↗ EPA, DHA in red blood cell membrane ↗ femur calcium content ↘ urinary deoxypyridinoline excretion (bone resorption marker)
1994	(Sakaguchi, Morita et al. 1994)	Rats (17 weeks-old) OVX Diet ± EPA (11% of total FA) ± calcium for 35 days	EPA-enriched diet prevents the loss of bone weight and strength caused by oestrogen deficiency
1995	(Claassen, Potgieter et al. 1995)	Rats (5 weeks-old) (FA deficient diet) 8% fat-diet with different ratio of GLA :EPA for 84days	↘ urinary excretion of pyridinium correlated with hydroxyproline levels in the higher ratio GLA:EPA ↗ bone calcium content in the same group
1995	(Kruger, Coetzer et al. 1995)	Rats 8% fat-diet for 12 weeks (fish, evening primrose or sunflower oil)	Fish oil diet: ↘ faecal calcium excretion and ↗ calcium balance ↗ unsaturation of the intestinal membranes
1995	(Yamada, Fushimi et al. 1995)	Rats (6 weeks-old) treptozotocin-induced diabetic 6%fat-diet (soy bean oil ± EPA or DHA) for 7 weeks	EPA: ↗ bone strength and ↘ phosphate excretion and plasma inorganic phosphate concentration No significant change on plasma and urinary calcium

1999	(Schlemmer, Coetzer et al. 1999)	Rats (11 weeks-old) OVX Oestrogen implant at OVX for 2 groups and 1% LA as control or a diester with gamma-linolenic acid and eicosapentaenoic acid as part of a semi-synthetic diet (1g/kg) for 14 weeks	Oestrogen and diester alone ↗ calcium levels in femur in OVX animals In combinaison diester potentiated the effect of oestrogen on bone calcium Oestrogen alone or in cobinaison with diester ↘ urinary deoxypyridinoline and hydroxyproline excretion
1999	(Li, Seifert et al. 1999)	Rats Diet with 70g/kg of fat (soybean oil SBO or menhaden oil+safflower oil MSO ± 10g/kg CLA) for 42 days	CLA ↗ IGF binding protein levels in SBO group and ↘ in MSO group CLA ↘ tibial mineral apposition rate and bone formation rate No difference on serum osteocalcin levels
2002	(Sirois, Cheung et al. 2003)	Rats (21 days of age) for 5 weeks male or female Fish oil diet (60 g/kg menhaden oil plus 10 g/kg soybean oil) or control diet (70 g/kg soybean oil)	Fish oil had no effect on biomechanical strength properties of femurs and vertebrae in male. Females fed fish oil had reduced length growth and a lower vertebral peak load.
2004	(Green, Wong et al. 2004)	Rat (21 day-old) ± insulin deficiency for 35 days Diet containing soybean oil (70 g/kg diet) as control or menhaden oil (40 g/kg diet) + corn oil (30 g/kg diet) (CO+fish)	(CO+ fish): ↗ density of femur ↘ plasma osteocalcin and bone prostaglandin E2 Limit N-telopeptide, IGF-1, bone PGE2 and urinary Ca elevation and calcitriol diminution induced by the insulin deficiency
2005	(Kruger and Schollum 2005)	Rats (45 week-old) for 6 weeks Diet supplemented with oil: control: 5% corn oil and 3 test diets 5% evening primrose oil (PO), 4% fish oil plus 1% corn oil (FO), or 4% tuna oil plus 1% corn oil (TO)	TO: ↗ Ca absorption, BMD, and bone Ca content compared with control group Correlations between the DHA content of the erythrocyte membranes and bone density and bone calcium content.
2006	(Poulsen and Kruger 2006)	Rats (8 months-old) OVX 0.1 g (LOW) or 1.0 g (HIGH) of (EPA)/kg body weight for 9 weeks	↘ BMD in the HIGH group compared with OVX and SHAM controls No BMD differences between LOW and control OVX groups High-dose EPA supplementation exacerbated the effects of OVX on BMD
2006	(Shen, Yeh et	Rats (12 month-old) for 20 weeks	(ω-3): highest BMC and cortical + subcortical BMD

	al. 2006)	Diet with dietary lipid treatments (g/kg diet): - 167g safflower + 33g menhaden oils (ω-6 + ω-3), - 200g safflower oil (ω-6), - or 190g menhaden + 10g corn oisl (ω-3)	Higher sérum IGF-1, PTH, 1,25-(OH)2 vitamin D3 and ALP Lower bone NO production and urinary Ca, (ω-6): higher bone PGE2 production and serum Pyd
2006	(Watkins, Li et al. 2006)	Rat (3 months of age) OVX for 12 weeks Diet with a high-PUFA (HP) or a low-PUFA (LP) with a ratio of ω-6/ω-3 PUFAs of 5:1 (HP5 and LP5) or 10:1 (HP10 and LP10). Diets contain 110.4 g/kg of fat.	HP5 and LP5: lower bone loss in OVX rat (femur and tibia BMC and BMD). LP diets: lowest serum concentrations of the bone resorption biomarkers Pyd and DPD. HP groups: lowest bone formation marker osteocalcin. The 5:1 diets (HP5 and LP5) preserved rat femur BMC.
2010	(Chen, Lazarenko et al. 2010)	Rat (27 day-old) for 4 weeks Total enteral nutrition with a high fat diet: 45% corn oil (HFD) or a medium fat diet: 25% corn oil (MFD) Control: low fat diet LFD 14% fat *ad libitum*	HFD: ↘ bone mass compared to controls ↘ bone formation, ↗ bone resorption ↘ osteoblastogenic markers: osteocalcin and Runx2 ↗ bone marrow adiposity ↗ expression of adipogenic genes: PPARγ and aP2
2011	(Saleh and Saleh 2011)	Female rats (12-14 month-old) OVX or Sham Diet supplemented with extravirgin olive oil orally (1 ml/100 g Body Weight) for 12 weeks; 4 weeks before OVX and 8 weeks after.	OVX changes (↘ plasma calcium levels, ↗ plasma ALP, MDA, and nitrates levels, ↘ in the cortical bone thickness and the trabecular bone thickness of the tibia and ↗ osteoclast number) were attenuated by olive oil supplementation in the Olive-OVX rats.
2011	(Lukas, Gigliotti et al. 2011)	Female rat (28 day-old) for 8 weeks Different sources fat, in high fat diet (12%): corn oil (CO) or ω-3 PUFA rich, flaxseed (FO), krill (KO), menhaden (MO), salmon (SO) or tuna (TO)	TO: higher tibial BMD and BMC and lower lipid peroxidation compared to the CO-fed rats. FO or MO: improved bone microarchitecture compared to CO FO: higher serum osteocalcin compared to SO.
PIGLETS			
2000	(Watkins, Li et al. 2000)	Piglets (5 days of age) for 15 days Supplemented diet ± FA (0.8% of total FA as AA and 0.1% as DHA) ± PGE2 injections at 0.1mg/kg /day	PGE2: ↗ osteoblast activity (plasma osteocalcin and ↘ urinary calcium excretion) and ↗ femur mineral content FA: ↘ bone resorption (urinary NTX and ↘ bone PGE2) and ↗ femur mineral content

			PGE2 + FA: ↘ femur mineral content
2002	(Weiler and Fitzpatrick-Wong 2002)	Piglets (5 days of age) for 21 days 4 diets: - groups 1 and 2: 30 g fat/L + (ω-6):(ω-3) ratio (4.5:1) group 1 and (9:1) group 2 - groups 3 and 4: 60 g fat/L + (ω-6):(ω-3) ratio (4.5:1) group 3 and (9:1) group 4	Growth and bone mass did not differ among the four groups Diets modify levels of AA and DHA in plasma, brain, liver, and adipose tissue Higher plasma DHA in groups 3 and 4 was associated with less bone resorption.
2003	(Lucia, Fitzpatrick-Wong et al. 2003)	Piglets for 15 days Diet supplemented with fatty acid (0.8% of total fatty acids as AA and 0.1% of total fatty acids as DHA) ±PGE2 injections (0.1mg/kg/day)	PGE2: ↗ osteoblast activity => ↗ plasma osteocalcin and ↘ urinary calcium excretion Dietary FA: ↘ bone resorption => ↘ urinary N-telopeptide and ↘ bone PGE2
2004	(Blanaru, Kohut et al. 2004)	Piglets from days 5 to 20 of life Diet supplemented with AA (0.30%, 0.45%, 0.60%, or 0.75% of fat) plus DHA (0.1% of fat)	Diet modify levels of AA in plasma, liver, and adipose tissue No significant change on bone modeling ↗ wholebody bone mineral content with 0.60% and 0.75% AA
2005	(Mollard, Kovacs et al. 2005)	Piglets for 15 days Diets: control formula or the same formula with various levels of AA:DHA (0.5:0.1 g, 1.0:0.2 g or 2.0:0.4 g AA:DHA/100 g of fat)	Tissue AA and DHA increased in proportion to diet levels. Liver EPA correlated positively with whole body and femur BMC and BMD and lumbar spine BMC Low levels of AA:DHA (0.5:0.1) ↗ bone mass
HUMANS			
INTERVENTION STUDIES			
1995	(van Papendorp, Coetzer et al. 1995)	40 osteoporotic women (80 years ± 4 years) for 16 weeks Diet supplemented with 4g of oil: 4 groups Evening primrose oil (EPO: 73% LA + 10% GLA), Fish oil (FO: 16% EPA +11% DHA), Mixture EPO/FO (MIX: 60% LA +8% GLA + 4% EPA + 3% DHA) and Olive oil (OO: 10% palmitic acid + 78% oleic acid)	FO: ↗ serum calcium and urinary calcium clearance FO and MIX: ↗ osteocalcin and procollagen in serum from 1 to 16 weeks.
2000	(Bassey, Littlewood et	Randomized, double blind study. 43 premenopausal (25-40 years) and 42	<u>Pre-menopausal:</u> ↗ BMD in both groups (1%) ↘ serum markers of bone resorption (osteocalcin and ALP)

	al. 2000)	postmenopausal (50-65 years) women for 52 weeks Supplemented diet with 1,0g Ca ± 4g evening primrose oil and 440mg fish oil	Post-menopausal: ↘ BMD in both groups (1%) ↘ markers of bone turnover
2005	(Dodin, Lemay et al. 2005)	Randomized, double blind study. 198 healthy menopausal women (45-65 years) for 52 weeks Consumption of 40g flaxseed (n=101) or wheat germ placebo (n= 98) each day	Flaxseed ↘ serum total and HDL cholesterol concentrations No significant change in BMD between the two groups Both flaxseed and wheat germ ↘ the severity scores of menopausal symptoms (no significant changes)
2007	(Griel, Kris-Etherton et al. 2007)	Randomized, double-blind study with 3 crossover periods. 20 healthy male patients (48,6± 1,6 years) Consumption of 3 different diets: 1) Average American Diet (AAD: 34% total fat, 13% SFA, 13% MUFA, 9% PUFA), 2) Linoleic Acid Diet (LA: 37% total fat, 9% SFA, 12% MUFA, 16% PUFA), and 3) α-Linolenic Acid Diet (ALA: 38% total fat, 8% SFA, 12% MUFA, 17% PUFA)	NTx levels were significantly lower following the ALA diet and there was a similar trend for the LA diet compared to the AAD diet Levels of proinflammatory cytokines (IL-6, IL-1β and IL-4) did not change after consumption of the three test diets. Levels of serum TNF-α however were reduced following the ALA Diet, compared to the LA Diet and the AAD. The levels of NTx were positively correlated with the levels of TNF-α across all three diets. Levels of BSAP were unaffected by diet treatment
2011	(Appleton, Fraser et al. 2011)	Randomized controlled trial study 113 mild-moderately depressed individuals (26 males and 87 females, aged 18-67 years) Supplemented diet with 1,48 g EPA+DHA/day (n=53) or placebo (n=60) for 12 weeks	Change in β-CTX status (bone marker resorption) was not associated with change in ω-3 PUFA status
PROSPECTIVE STUDIES			
2004	(Macdonald, New et al. 2004)	***Aberdeen Prospective Osteoporosis Screening Study*** 891 healthy women aged 45-55 years at baseline and 50-59 years at follow-up 5 years later. Usual dietary intake was assessed by using food-	Total fat and SFA intakes did not appear to be associated with either BMD or BMD change. PUFAs and MUFAs were negatively correlated with FN BMD change, (significant after adjustment for confounders)

		frequency questionnaires BMD was measured by using DXA at the lumbar spine and femoral neck (FN)	
2005	(Weiss, Barrett-Connor et al. 2005)	***Rancho Bernardo Study*** 1532 men and womenaged 45–90 years : 643 men and 903 postmenopausal women (564 not using hormone therapy (HT), and 326 using HT) Dietary data were obtained through self-administered food-frequency questionnaires BMD was measured at the hip and spine with DEXA	Men had a significantly higher ratio of ω-6 to ω-3 fatty acids than did women. Negative and significant association between the ratio of ω-6 to ω-3 fatty acids with hip BMD in all groups and with lumbar spine BMD in women not using HT only
2006	(Corwin, Hartman et al. 2006)	***NHANES III Study*** 13 572 subjects 20-90 years old: 6547 men and 7025 women Dietary data were obtained from a single 24-h recall The recalls included all 7 d of the week BMD of the proximal femur (the hip region including the femoral neck, intertrochanter, trochanter, and total hip) was measured by DXA	No significant association between total fat intake and BMD at any of the sites analyzed. SFA intake was negatively associated with BMD in the femoral neck for all subjects. BMD decreased 2,3% from the lowest to the highest quintile of SFA intake The negative relation between saturated fats and BMD were more pronounced in men than in women. Across all men SFA intake was negatively associated with BMD. BMD decreased ~2-4% from Q1 to Q5 at these sites.
2007	(Hogstrom, Nordstrom et al. 2007)	***NO_2 Study*** 78 healthy young men (16,7 ±0,5 years) at baseline for 5 years and 11 months. BMD of total body, hip, and spine was measured by DXA at baseline and at the end of the study Fatty acid profile in serum phospholipids was analyzed (no dietary questionnaire)	Concentrations of ω-3 fatty acids were positively correlated with total and spine BMD at 22 years of age. And a positive correlation between ω-3 fatty acid concentrations and the changes in BMD at the spine was found between 16 and 22 years of age. Concentrations of DHA were positively associated with total and spine BMD at 22 y of age .And a positive correlation was found between DHA concentrations and the changes in BMD at the spine between 16 and 22 y of age.
2007	(Martinez-Ramirez,	***Hospital-based case–control study (Spain)*** Cases 167 patients (80% women) (<65 years) with	Participants in the two upper quartiles of PUFA intake showed a significant increased risk of fracture,

	Palma et al. 2007)	a low-energy fracture selected from the population Controls 167 patients without antecedents of any fracture matched to cases by sex and age Diet data were collected with semiquantitative food frequency questionnaire.	A higher ratio of MUFA to PUFA was associated with a reduced risk of fracture The intake of ω-6 fatty acids was associated with an elevated risk of fracture HDL-cholesterol levels were inversely associated with the risk of fracture
2010	(Genaro, Pereira et al. 2010)	***Cross-sectional study at São Paulo Hospital (Brazil)*** 70 osteoporotic postmenopausal women: mean age: 69,7 ± 6,4 years and weight: 56.3 ± 7.6 kg Body composition and BMD measurements were performed DXA The aim of the study is to evaluate the relationship of lean and fat mass to bone mass in osteoporotic postmenopausal women	Overweight women had significantly higher bone mass. Skeletal muscle index (SMI) showed a positive effect on BMD measurements and women with sarcopenia had significantly lower BMD measurements in total femur and femoral neck. In multiple regression analysis only lean mass and age, after adjustments to fat mass and BMI were able to predict total body BMC Lean mass adjusted to age and BMI were able to predict femoral neck BMD
2010	(Jeong, Cho et al. 2010)	***Retrospective analysis of data on women from Healthcare System Gangnam Center (Korea)*** The associations between lipid profiles (total cholesterol TC, LDL-C, HDL-C and triglyceride TG) and BMD at various skeletal sites (lumbar spine, proximal total hip, femoral neck, and trochanter) were explored Data on 4 613 premenopausal and 2 661 postmenopausal women (20–91 years)	No significant relationship between lipid profiles and BMD HDL-C was positively associated with BMD at only the lumbar spine in postmenopausal women TG were negatively associated with BMD at the total hip and trochanter in only premenopausal women.
2010	(Orchard, Cauley et al. 2010)	***Women's Health Initiative Study (WHI)*** 137 486 postmenopausal women (63 ± 7 years) between 1993 and 1998. Total fractures were identified by self-report; hip fractures were confirmed by medical record review.	Higher SFA consumption was associated with higher hip fracture risk Lower total fracture risk was associated with a higher MUFA and PUFA intake Higher consumption of marine ω-3 PUFA was associated with greater total fracture risk

		FA intake was estimated from food frequency questionnaires	Higher ω-6 PUFA intake was associated with a lower total fracture risk
2010	(Zwart, Pierson et al. 2010)	***Spaceflight: Short duration.*** 10 subjects (7 males, 3 females, 36-54 years) from four shuttle missions (12 to 16 days each) and 7 healthy control subjects (5 male and 2 female) ***Spaceflight: Long duration*** 24 subjects record the dietary intake once per week using a food frequency questionnaire ***Bed rest study*** 16 subjects Evaluation of various physiologic and nutritional changes during and after 60 or 90 days of 6-degree head-downtilt bed rest.	***Short-duration spaceflight*** ↗ NF-κB p65 expression (500%) after flight and it remained elevated for at least 14 days after flight. No significant difference the astronauts and the control group. ***Long-duration spaceflight*** A higher intake of fish was associated with a smaller decrease in whole-body BMD after flight. A higher intake of fish tended to decrease the change in wholebody bone mineral content ***Bed rest*** Intake of total ω-3 fatty acids during 60 days of bed rest was negatively correlated with the percent change in urinary NTX excretion from before bed rest to during bed rest
2011	(Arikan, Coskun et al. 2011)	***Study on Turkish women*** 107 postmenopausal women; 35 healthy (gp 1), 37 osteopenic (gp 2) and 35 osteoporotic (gp 3). BMI, BMD and serum lipid profile were measured	Positive correlation between BMI and lumbar vertebra, femur neck and femur total BMD Positive correlation between TG and femur neck and femur total BMD
2011	(Farina, Kiel et al. 2011)	***Framingham Osteoporosis Study*** 623 subjects between 1948 and 1988 (225 women and 397 men) mean age of 75 years BMD measurements were performed DXA Diet data were collected with food frequency questionnaire	High intake of fish were associated with maintenance of femoral neck BMD (FN-BMD) in men and in women ***In women:*** high intake of EPA+DHA with a high AA intakes were associated with higher FN- BMD LA intake tended to be associated with FN-BMD loss ***In men:*** low intake of EPA+DHA with a high intakes of AA were associated with a lower FN-BMD than did men with the lower intakes of AA

C - Organs interactions

It has been well acknowledged that bone is metabolically closely linked to other organs. Thus, fatty acids mediated bone modulation may be indirect through actions on bone affecting organs.

1. Adipose tissue

White adipose tissue (WAT) acts as an endocrine organ that secretes peptide molecules called adipokines. Among these hormones and cytokines, some have been shown to efficiently modulate bone metabolism. As the secretory function of WAT is widely known to be modulated by macronutrients in general and lipids in particular, this organ is likely to be a target of dietary fatty acid-induced bone modulation.

a. Leptin

Leptin effect on bone has been extensively studied since Ducy et al. (Ducy, Amling et al. 2000) showed that leptin-deficient and leptin receptor-deficient mice had an increased bone mass due to increased bone formation. Leptin mediated regulation of bone remodelling is complex; it may be either catabolic or anabolic depending of the presence or the absence of a central relay. Briefly, central leptin injection causes bone loss in leptin-deficient and wild type mice, (Ducy, Amling et al. 2000) via the activation of the sympathetic tone and subsequent osteoblast beta2-adrenergic receptors (Adrb2) mediated inhibition of bone formation (Takeda, Elefteriou et al. 2002). In addition, this sympathetic signalling leads to over expression of RANKL by osteoblast progenitors, therefore favouring bone resorption (Elefteriou, Ahn et al. 2005). In contrast, systemic leptin administration was shown to promote bone formation. Intra peritoneal injection of leptin reduces bone fragility in adult mice (Cornish, Callon et al. 2002) and rescues bone loss observed in tail-suspended rats by preventing both inhibition of bone formation and RANKL/OPG ratio increase (Martin, de Vittoris et al. 2005). This is thought to be due to direct activation of leptin receptor on osteoblasts, as *in vitro* studies have shown a leptin induced increase in osteoblast proliferation, differentiation and mineralisation (Cornish, Callon et al. 2002; Gordeladze, Drevon et al. 2002) as well as a decreased level of RANKL/OPG ratio. (Gordeladze, Drevon et al. 2002; Lamghari, Tavares et al. 2006). High fat diet induced obesity is always associated with an excessive WAT excretion of leptin both in humans and animals. However, the long

chain fatty acid (LCFA) composition of the diet has been shown to modulate the magnitude of this induction.

In human, changes in dietary intake of SFAs correlated positively to plasma leptin levels. (Reseland, Haugen et al. 2001). The opposite was observed for PUFAs intake. In parallel, wild-type rats fed a ω-3 PUFA-enriched diet (Reseland, Haugen et al. 2001) (Ukropec, Reseland et al. 2003) or fa/fa Zucker rats fed a dietary CLA supplemented diet (Noto, Zahradka et al. 2007; Gudbrandsen, Rodriguez et al. 2009), have lower plasma leptin concentration and reduced leptin mRNA expression in targeted adipose tissue as compared to high SFA containing diet. This inhibitory effect is likely to involve direct modulation of adipocytes by fatty acids as *in vitro* studies have shown that isolated rat adipocytes grown in presence of DHA, EPA or CLA have reduced levels of both basal and insulin-induced leptin secretion (Perez-Matute, Marti et al. 2007; Perez-Matute, Martinez et al. 2007). In addition, DHA and EPA were able to decrease human leptin promoter activity in a human trophoblast cell line (Reseland, Haugen et al. 2001). These results support a global down-regulation of circulating leptin levels produced by adipocytes in the presence of PUFAs, and contrariwise for SFAs. Given the impact of leptin on bone in absence of central influence, and the beneficial effect of ω-3 on isolated bone forming cells, these data support the complexity of the system. In fact, leptin induced regulation of bone mass is even more complex, with differential effects depending of the dose (Martin, David et al. 2007) and the skeletal site (Hamrick, Pennington et al. 2004; Guidobono, Pagani et al. 2006). Therefore, it remains difficult from a “leptin point of view” to further conclude on the relationship between high fat diets and bone altered metabolism.

b. Adiponectin

Interest in adiponectin involvement in bone homeostasis originates from the study of Berner et al., demonstrating that this hormone and its receptors AdipoR1 and AdipoR2 are expressed in bone-forming cells and that recombinant adiponectin enhances the proliferation of murine osteoblasts (Berner, Lyngstadaas et al. 2004). Thereafter, the positive role of adiponectin was confirmed both in human and murine osteoblasts where fixation of the hormone to its receptor AdipoR1 induces their proliferation via activation of JNK and their differentiation via p38 and AMP kinase respectively (Luo, Guo et al. 2005) (Kanazawa, Yamaguchi et al. 2007). Moreover, adiponectin counteracts osteoclastogenesis by inhibiting TNF-α/RANKL-stimulated NFATc1 expression (Yamaguchi, Kukita et al. 2008). However, in spite of these promising *in vitro* results, adiponectin-deficient (Ad-/-) mice do not exhibit

abnormality in bone mass or turnover (Shinoda, Yamaguchi et al. 2006). In addition, adiponectin has been widely negatively associated with BMD in obese humans (Lenchik, Register et al. 2003; Peng, Xie et al. 2008; Napoli, Pedone et al. 2010). In growing adiponectin transgenic mice, elevated circulating adiponectin led to lower femur BMC at 8 and 16 weeks of age (Ealey, Kaludjerovic et al. 2008). In addition, in a human co-culture system consisting of PBMCs, adiponectin stimulated osteoblast-induced osteoclast differentiation by promoting the expression of the pro-resorbing cytokine RANKL while inhibiting OPG expression via the AdipoR1/p38 pathway (Luo, Guo et al. 2006).

Unlike leptin and most other adipokines, adiponectin levels are decreased in obesity. In rats, SFAs and trans fatty acids WAT content were negatively correlated with both adiponectin plasma concentration and WAT RNA expression while MUFAs and PUFAs content showed the opposite (Perez de Heredia, Sanchez et al. 2009) (Saravanan, Haseeb et al. 2005). Interestingly, the decreased expression of adiponectin in WAT of mice fed a high fat (mostly SFAs)/high sucrose diet was counteracted by the incorporation of ω-3 PUFAs in the diet but not by the incorporation of ω-6 PUFAs (Todoric, Loffler et al. 2006). In addition, EPA alone was able to abolish the high fat diet-induced reduction in adiponectin plasma concentration when added to the diet in C57/BL6j mice. From a mechanistic point of view, both EPA and DHA were shown to induce adiponectin secretion by adipocytes, in a PPARγ dependant and a PPARγ independent fashion for DHA and EPA respectively (Oster, Tishinsky et al. 2010; Tishinsky, Ma et al. 2010).

Concerning CLA, t10,c12 isomer treatment results in a reduced adiponectin expression in WAT both in daily gavaged mice (Poirier, Shapiro et al. 2006) and in fa/fa rats fed a t10,c12-CLA supplemented diet (Gudbrandsen, Rodriguez et al. 2009). *In vitro* however, t10,c12-CLA was unable to modulate the secretion of adiponectin in 3T3L1 adypocytes whereas the c9,t11 isomer induced a significant increase (Ahn, Choi et al. 2006).

Taken together, the global impact of adiponectin on bone remains difficult to determine and may depend on the model and context (inflammatory, oxidative…). Thus, understanding the bone consequences of adiponectin regulation by fatty acids (upregulation by ω3 PUFAs and MUFAs and downregulation by SFAs and trans FAs) requires further integrated investigations.

c. Inflammatory cytokines

An important aspect of WAT/bone interactions comes from the capacity of WAT to secrete a wide range of inflammation-modulatory cytokines. Among them, classical pro-

inflammatory factors such as TNF-α, IL-6 or IL-1 are found and are known to be key mediators in the process of osteoclast differentiation and bone resorption both *in vitro* and *in vivo* (Khosla 2001; Mundy 2007). In addition, the accelerated bone loss at menopause is thought to be linked to the increased production of these cytokines as mice lacking IL-1β and TNF genes (Vargas, Naprta et al. 1996), over-expressing soluble TNF-α decoy receptor (Ammann, Rizzoli et al. 1997) or treated with an IL-1 receptor antagonist(Kimble, Matayoshi et al. 1995) are resistant to ovariectomy-induced bone loss.

During obesity, as WAT expands, adipocytes increase in both size and number, leading to adipocyte dysfunction (Guilherme, Virbasius et al. 2008). Secretion of pro-inflammatory factors by hypertrophied adipocytes is greatly increased, and furthermore, lipid-engorged adipocytes die and release their contents, which recruit macrophages (Weisberg, McCann et al. 2003) that further increase pro-inflammatory cytokines production. Overconsumption of fatty acids contributes to weight gain and inflammation (Lundman, Boquist et al. 2007), but SFAs and Trans fatty acids including t10,c12-CLA have a particularly strong effect on the inflammatory capacity of white adipose tissue (WAT). This has been demonstrated both in humans (Riserus 2008) and animals (Poirier, Shapiro et al. 2006; Kalupahana, Claycombe et al. 2010). This capacity of SFAs and t10,c12-CLA to promote WAT inflammation involves : 1) Increased pro-inflammatory cytokines production by adipocytes via NF-κB dependant mechanism (Poirier, Shapiro et al. 2006; Schaeffler, Gross et al. 2009). 2) Promotion of macrophage recruitment to WAT via JNK and NF-κB induced overproduction of monocyte chemoattractant protein-1 (MCP-1) (Takahashi, Yamaguchi et al. 2008) (Weisberg, Hunter et al. 2006). 3) Increased pro-inflammatory cytokines production by resident macrophages (Suganami, Tanimoto-Koyama et al. 2007).

Interestingly, some classes of fatty acids have in contrast shown protective properties in regard to WAT inflammation. For instance, in mice, ω-3 PUFAs supplementation in a high fat diet rich in SFAs were able to prevent both the increase in WAT macrophage infiltration and in inflammatory factors gene expression (Todoric, Loffler et al. 2006; Kalupahana, Claycombe et al. 2010). These anti-inflammatory properties on WAT activities were also driven by MUFAs (Kennedy, Martinez et al. 2009; van Dijk, Feskens et al. 2009).

Besides, it is worth noting that leptin and adiponectin may also be involved in this modulation of WAT inflammation as leptin has been found to stimulate inflammatory responses (van Dielen, van't Veer et al. 2001) while adiponectin acts as an anti-inflammatory cytokine (Ouchi, Kihara et al. 2000).

Taken together, these wide literature data enlighten complex relationships between fatty acids, adipose tissue metabolism and bone remodelling that cannot by itself fully explain the global effects of fatty acids intake on bone and thus require a further integrated point of view.

2. Digestive tract

Gut is an endocrine organ and secretes a variety of hormones involved in many physiological processes, including bone remodelling. With regard to the close relationships between digestive tissues and food intake, it is no surprise that stomac, gut and colon hormones are regulated by nutrients. This paragraph will focus on hormones known for their bone regulating properties whose production or action may be modulated by fatty acids.

a. Ghrelin

Ghrelin is a growth hormone secretagogue produced by the gastric fundus in response to fasting which synthesis is inhibited by food intake. This orexigenic peptide acts via a hypothalamus relay. Interestingly, ghrelin receptor is expressed by osteoblast and its stimulation leads to increased differentiation and proliferation of these bone forming cells, thus suggesting an anabolic role of ghrelin in bone remodelling (Maccarinelli, Sibilia et al. 2005; Delhanty, van der Eerden et al. 2006). In animal models, ghrelin administration in rats increases BMD independently of food intake or weight gain (Fukushima, Hanada et al. 2005). In addition, ghrelin was effective in promoting the intramembranous bone repair of a rat calvaria defect in association with an increase of the RNA expression of ALP, Oc and Col1a (Deng, Ling et al. 2008). In contrast to leptin, central infusion of ghrelin results in a decrease in sympathetic tone which may contribute to the anabolic effect of ghrelin (Yasuda, Masaki et al. 2003). However, ghrelin-null knockout mice exhibit normal bone mass (Sun, Ahmed et al. 2003). In humans, fasting serum ghrelin was negatively correlated with serum CTX, a marker of bone degradation, thus supporting its bone anabolic potential (Huda, Durham et al. 2007). These data correlate with a slight, but significant, increase in femoral neck BMD after treatment with an oral ghrelin mimetic (Nass, Pezzoli et al. 2008).

Lower fasting ghrelin level have been associated with high percentage of body fat (Tschop, Weyer et al. 2001), as well as overfeeding and high-fat dietary supplements (Robertson, Henderson et al. 2004), thus rising the possibility of a deletary impact of high fat diet on ghrelin production. This hypothesize was confirmed in rodent studies demonstrating that high fat diets induce hypoghrelinemia in both rats (Ebal, Cavalie et al. 2007; Handjieva-

Darlenska and Boyadjieva 2009) and mice (Moesgaard, Ahren et al. 2004). Alternatively, the class of the fatty acids in the diet seems another important factor. A recent work from Delhanty et al. suggests that unsaturated fatty acids may modulate ghrelin signal without affecting its synthesis. In fact, OA and LA were able to cause a significant increase in the sensitivity of the GHSR and to suppress the inhibitory effect of a ghrelin pretreatment on subsequent ghrelin responsiveness, probably through an increase of cellular membrane fluidity (Delhanty, van Kerkwijk et al. 2010). In addition, mice fed with a low n6/n3 ratio show better fasting ghrelin serum concentration compared to mice fed high n6/n3 ratio (Burghardt, Kemmerer et al. 2010). In this context, ghrelin appear to be a potent candidate for mediating the various effects of fatty acids on bone.

b. Incretins

GIP (glucose-dependent insulinotropic peptide) and GLP-1 (Glucagon-like Peptide-1) are both secreted by the intestine (small and large) in response to feeding. They are incretins that increase the insulin production in the pancreas. The GIP receptor is expressed by both osteoblasts (Bollag, Zhong et al. 2000) and osteoclasts (Zhong, Itokawa et al. 2007) and its activation leads to an increased osteoblast number and activity and prevents PTH-induced osteoclast activation *in vitro* (Zhong, Itokawa et al. 2007), suggesting a protective effect of GIP regarding bone degradation. As a matter of fact, intermittent administration of GIP to ovariectomised rats attenuates bone loss (Bollag, Zhong et al. 2001). However, in osteoblasts, continued GIP exposure leads to a decreased expression of the GIP receptor, underlining the importance of a pulsatile delivery in order to achieve anabolic effects. Consistently, transgenic mice over-expressing GIP have increased bone mass due to increased bone formation and decreased bone resorption as well as reduced bone loss with aging (Xie, Zhong et al. 2007; Ding, Shi et al. 2008). In the same way, GIP receptor knockout mice reveals, along with the expected altered insulin response to food intake (Miyawaki, Yamada et al. 1999), a low bone mass phenotype due to reduced bone formation and increased osteoclast number and activity (Tsukiyama, Yamada et al. 2006).

The GLP-1 receptor knockout mice display somehow a similar phenotype (Yamada, Yamada et al. 2008). However GLP-1 has no direct effect on bone cells. Indeed, although GLP-1 receptor has been reported to stimulate proliferation of mesenchymal stem cells and inhibits differentiation to adipocytes (Sanz, Vazquez et al. 2010), bone defect is rather thought to be caused by reduced calcitonin levels, as GLP-1 receptor is expressed on thyroid C cells and as its stimulation by GLP-1 increases secretion of calcitonin (Yamada, Yamada et al.

2008). In addition, administration of GLP-1 improves trabecular bone mass and microarchitecture in rats with streptozotocin-induced diabetes suggesting an insulin-independent action (Nuche-Berenguer, Moreno et al. 2009).

GIP and GLP-1 impact on bone may be regulated by fat ingestion. In fact, triglycerides triggers a greater and longer lasting GIP-response compared to carbohydrate (Krarup, Holst et al. 1985). More precisely, they have to be transformed to long-chain free fatty acids to influence GIP- or GLP-1 secretion (Ellrichmann, Kapelle et al. 2008) (Enc, Ones et al. 2009). In addition, a more potent effect of MUFAs as compared to SFAs has been observed both *in vitro* (Rocca, LaGreca et al. 2001) and *in vivo* (Thomsen, Rasmussen et al. 1999; Rocca, LaGreca et al. 2001). In fact, in rats, a prolonged high saturated-fat diet results in an impaired GIP secretion associated with an increase apoptosis of duodenal enterocytes, suggesting that a lipoapoptotic phenomenon may explain the less potent effect of SFAs on incretin production (Gniuli, Dalla Libera et al. 2008). From a mechanistic point of view, release of incretins by free fatty acids may involve G-protein coupled receptors. Both GPR40 and GPR120, two receptors for LCFAs (Briscoe, Tadayyon et al. 2003; Hirasawa, Tsumaya et al. 2005), are expressed by incretin secreting cells (Parker, Habib et al. 2009). However, GPR120 seems more important in regard to the secretion of incretins in response to free fatty acids (Hirasawa, Tsumaya et al. 2005; Hara, Hirasawa et al. 2009). In another hand, GLP-2 has been extensively described for its effects on bone, however, although it is produce by the same cells as GLP-1, the literature does not depict any influence of its expression by free fatty acids. Thus, as for ghrelin, GIP and GLP-1 could contribute to mediate the different effects of fatty acids on bone.

c. Calcium absorption

Impaired calcium absorption is a frequent feature of aged-related or estrogen deficiency-associated bone loss (Wright, Proctor et al. 2008). In human, high saturated fat diets have been reported to interfere with intestinal calcium absorption. As a matter of fact, free fatty acids may form unabsorbable insoluble calcium soaps and therefore contribute to low calcium absorption (Carnielli, Luijendijk et al. 1996; Koo, Hammami et al. 2003). However, this reaction may depend on fatty acid class and suffers a few discrepancies. Indeed, Merrill et al. showed that treatment of rabbit isolated brush border membrane from rabbit small intestine with unsaturated fatty acids results in a stimulation of Ca^{2+} uptake whereas treatment with SFAs has no effect (Merrill, Proulx et al. 1986). In the same way, small intestine from rats fed either high PUFA or high MUFAs diets show increased ion

transport in Ussing chamber whereas high SFA diet reduces it (Cartwright-Shamoon, Dodge et al. 1995). At a molecular level, n3 PUFAs such as DHA and EPA increase total transepithelial Ca transport involving the regulation of membrane ATPases (Haag, Magada et al. 2003) and enhancement of transcellular Ca transport (Gilman and Cashman 2007). Consequently, the modulation of calcium absorption by fatty acids intake may partially explain the impact of fat diets of bone mineral density and content.

3. Pancreas

Pancreas impacts bone health by secreting peptides with anabolic properties through modulation of bone cells activities. Indeed, insulin receptors are expressed on both osteoblasts and osteoclasts, allowing insulin to increase bone formation and decrease bone resorption both *in vitro* and *in vivo* (Pun, Lau et al. 1989; Cornish, Callon et al. 1996; Thomas, Udagawa et al. 1998). In addition to its classical receptor, insulin also binds and activates the IGF-1 receptor expressed by osteoblasts to exert anabolic actions. However, this latter signalling pathway is likely to be of lesser importance as chemical or genetic inhibition of IGF-1 receptor signalling does not affect osteoblast sensitivity to insulin (Fulzele, DiGirolamo et al. 2007).

a. Insulin deficiency and hyperglycemia effects on bone

In type 1 diabetes, insulin deficiency is associated with reduced bone mass, suggesting that insulin plays an important role on the regulation of bone remodelling (Bouillon, Bex et al. 1995; Munoz-Torres, Jodar et al. 1996; Thrailkill, Liu et al. 2005). Accordingly, studies on insulin receptor substrates (IRS-1 and IRS-2) deficient mice have revealed osteoporotic phenotypes in both cases with reduced insulin or IGF-1 induced osteoblastic proliferation and differentiation (Ogata, Chikazu et al. 2000; Akune, Ogata et al. 2002). IRS-2 deficient osteoblasts also exhibit an increase in RANKL production leading to increase osteoclastogenesis in co-culture with bone marrow cells (Akune, Ogata et al. 2002). However, in 2006 Irwin et al. established the absence of bone phenotype in mice with a specific bone deletion of the insulin receptor, strongly suggesting a rather systemic cause to explain the bone defect associated with insulin dependant diabetes mellitus (Irwin, Lin et al. 2006).

Indeed, another obvious possible contributor to this diabetic bone loss is hyperglycaemia itself (Brownlee, Vlassara et al. 1984) as high glucose levels have been shown to induce a couple of bone-deletorious mechanisms. Briefly, hyperglycaemia has been linked to increased oxidative stress, increased bone marrow adiposity and renal defects with hypercalciuria

(Giaccari, Sorice et al. 2009). In parallel, it leads to high osteoclast activity (Williams, Blair et al. 1997) and reduced osteoblast differentiation (McCabe 2007).

In summary, insulin appears as a key regulator of bone metabolism both directly and/or via its hypoglycaemic properties. In this regard, fatty acids are very important factors for bone, as they modulate both the pancreatic insulin production (Prentki, Joly et al. 2002) (Nolan, Madiraju et al. 2006) and the insulin transduction signal in the targeted cells (Samuel, Petersen et al. 2010). Besides, FFAs may have different effects on bone through alteration of pancreatic function depending on whether islets exposure is acute or chronic. The latter leads to either reduced insulin biosynthesis (Poitout, Hagman et al. 2006), secretion (Elks 1993; Zhou and Grill 1994) and induces β-cell apoptosis (Kawai, Hirose et al. 2001; Maedler, Spinas et al. 2001). Importantly, the chornic effects of FFA on insulin secretion, insulin biosynthesis and B-cell viability are thought to be mediated by, at least partially, distinct pathways (Poitout and Robertson 2008).

b. Fatty acids and insulin expression and release

Fatty acid deprivation of islet tissue causes loss of glucose-stimulated insulin secretion (GSIS), a process rapidly reversible by replacement with exogenous FFAs (Stein, Esser et al. 1996). In the same way, acutely elevated FFA supply augments GSIS both *in vivo* and *in vitro* (Stein, Esser et al. 1996; Roduit, Nolan et al. 2004). It has been shown on perfused rat pancreas that the magnitude of the FFA-induced increase in insulin release depends on their length and degree of saturation. Briefly, short chain FFAs are less potent than long chain FFAs; SFAs are more potent than MUFAs or PUFAs (Stein, Stevenson et al. 1997), and among PUFAs, AA have been found more active than OA, LA, LnA, EPA and DHA in inducing insulin release from isolated rat islets (Metz 1988). According to available data, enhancement of GSIS by fatty acids involves both direct activation of the membrane bound fatty acid recptor GPR40 (Briscoe, Tadayyon et al. 2003; Itoh, Kawamata et al. 2003; Fujiwara, Maekawa et al. 2005; Nagasumi, Esaki et al. 2009) and modulation of intracellular metabolism of FFA leading to accumulation of lipid signalling molecules such as long-chain acyl-CoA and diacylglycerol known to favour exocytosis (Nolan, Madiraju et al. 2006).

However, in contrast to acute effects, chronic exposure to high fatty acids concentration lead to altered pancreatic activity and decrease insulin release. As suggested by the observed insensitiveness of islets from GPR40 KO mice to chronical fatty acids exposure, GPR40 was first thought to be a stepstone of the process (Steneberg, Rubins et al. 2005). However, as following studies were unable to reproduce these findings (Latour, Alquier et al.

2007), it is likely that the role of GPR40 in the pancreas may be limited to the acute effects of FA. Other mechanisms have been suggested including a FFA-mediated upregulation of Uncoupling protein-2 (Lameloise, Muzzin et al. 2001; Briaud, Kelpe et al. 2002). This mitochondrial carrier was demonstrated to impair insulin secretion (Chan, De Leo et al. 2001; Joseph, Koshkin et al. 2002) and enhance FFA-mediated perturbation of the late stage of insulin exocytosis, thus preventing the hormone to be released from the fusion pore (Olofsson, Collins et al. 2007). This inhibition of insulin secretion is driven to the same extent by oleate or palmitate suggesting that only the amount of fatty acid may account for it to occur (Moore, Ugas et al. 2004).

Unlike the hormone secretion, the inhibition of insulin gene expression by fatty acid seems limited to palmitate (Moore, Ugas et al. 2004). Thus, it was hypothesized to involve *de novo* synthesis of ceramide, a process using palmitate as an exclusive substrate. Indeed, isolated islets show elevated level of ceramide in the presence of palmitate and when the *de novo* cermaide synthesis is inhibited, the decrease in insulin gene expression is prevented (Kelpe, Moore et al. 2003). This ceramide-induced inhibition of insulin gene expression may involve an activation of the JNK signalling pathway (Mathias, Pena et al. 1998; Solinas, Naugler et al. 2006).

c. Lipids and β-cells apoptosis

The last consequence of chronic fatty acid islet exposure is the induction of β-cell apoptosis. Many mechanisms have been proposed to contribute to this FFA induced β-cell death including modulation of intracellular lipid metabolism (El-Assaad, Buteau et al. 2003; Ruderman, Cacicedo et al. 2003), ceramide formation (Shimabukuro, Higa et al. 1998; Lupi, Dotta et al. 2002), oxidative stress (Piro, Anello et al. 2002; Maestre, Jordan et al. 2003), inflammation (Busch, Cordery et al. 2002) and endoplasmic reticulum stress (Laybutt, Preston et al. 2007).

The ability of FFA to induce β-cell apoptosis varies greatly depending of their structure. Even if chronic exposure of β-cell to excessively high levels of any fatty acid leads to decreased viability *in vitro* (Azevedo-Martins, Monteiro et al. 2006) irrespective of the fatty acid structural features, very important differences exist between FFA species. On the one hand, long chain SFAs have been shown to exert a stronger toxicity than shorter chain SFAs (Eitel, Staiger et al. 2002). On the other hand, the differences are even more pronounced between saturated and unsaturated FA, the latter even exerting protective properties under

appropriate conditions (Maedler, Oberholzer et al. 2003; Welters, Tadayyon et al. 2004; Furstova, Kopska et al. 2008; Wei, Li et al. 2010).

For instance, it has been shown that the MUFA oleate is much less efficient that palmitate in inducing β-cell apoptosis, needing very prolonged exposure to reach a mild loss of viability (Cnop 2008). In addition, the combination of the two FFAs leads to reduce apoptosis as compared to palmitate alone (Dhayal, Welters et al. 2008). This protective effect of oleate (C18) can be reproduced by palmitoleate (C16) but at a lesser extent by myristoleate (C14) and is further reduced with shorter molecules suggesting an importance of the chain length in the cytoprotective efficacy (Dhayal, Welters et al. 2008). It is worth noting that the trans counterparts of the naturally occurring cis MUFAs are less effective in preventing the palmitate-induced β-cell apoptosis (Dhayal, Welters et al. 2008).

β-cell anti-apoptotic properties have also been found concerning PUFAs including LA, LnA, AA or EPA (Papadimitriou, King et al. 2007; Wei, Li et al. 2010) (Beeharry, Lowe et al. 2003). Finally, islets from the transgenic mouse model fat-1, endogenously producing ω-3 PUFAs, have been shown to be protected against pro-inflammatory cytokine-induced (Wei, Li et al. 2010) and STZ-induced (Bellenger, Bellenger et al. 2011) β-cell death in association with ω-3 dependant production of anti-inflammatory eicosanoids.

d. Amylin

Along with insulin, the pancreas secretes a wide range of potentially bone-modulating compounds. Among them, amylin is a peptide hormone related to calcitonin that is secreted by β-cells in response to feeding. *In vitro*, it has been shown to inhibit osteoclast differentiation and motility (Alam, Moonga et al. 1993) by binding the amylin receptor which is formed by the calcitonin receptor dimerized with receptor activity modifying proteins (RAMPS)(Naot and Cornish 2008). In addition, a positive role of amylin in inducing osteoblast differentiation was also found (Cornish, Callon et al. 1998; Cornish, Callon et al. 1999).

Analyse of amylin knockout mice reveals a low bone mass phenotype mainly associated with increased osteoclast number and activity with no change in osteoblast parameters (Dacquin, Davey et al. 2004). Furthermore, systemic amylin administration results in bone anabolism in “normal” mice (Cornish, Callon et al. 1995; Cornish, Callon et al. 1998) and counteracts type 1 diabetes (Horcajada-Molteni, Chanteranne et al. 2001) or ovariectomy-induced (Horcajada-Molteni, Davicco et al. 2000) bone loss by inducing bone formation and reducing bone resorption in mice and rats respectively. Finally reduced amylin levels were

found in patients with osteoporosis as compared to healthy controls (Bronsky and Prusa 2004).

Amylin blood basal concentration is higher in obese than in lean human subjects (Reda, Geliebter et al. 2002) as well as in rodents fed a high fat diet (Pieber, Roitelman et al. 1994; Westermark, Leckstrom et al. 1998). In addition, fatty acids have been shown to induce both amylin secretion (co secreted with insulin) and its expression at a transcriptional level in murine β-cells (Qi, Cai et al. 2010). Considering the bone anabolic properties of amylin, its upregulation might represent a mechanism explaining the high bone mass sometimes associated with obesity. However, in human, amylin is able to polymerize to form cytotoxic amyloid fibrils in the islets. These cytotoxic fibrils are thought to contribute to the loss of β-cell mass observed in type 2 diabete (Guardado-Mendoza, Davalli et al. 2009) and their formation requires both high amylin levels and high fat feeding (Westermark, Arora et al. 1995). In this context, it is worth noting that, saturated fatty acids are more potent than MUFAs and PUFAs in inducing amyloid formation both *in vivo* and *in vitro* (Ma and Westermark 2002).

In addition to insulin and amylin, β-cells also produce another peptide hormone: the preptin. This compounds increases osteoblast proliferation and survival *in vitro* and was found to be bone anabolic when administered to normal mice (Cornish, Callon et al. 2007). However, the association between preptin, fatty acids and bone health remains to be investigated.

Finally, given the role of bone regulating factors released by pancreatic cells, modulation of β-cell functions by fatty acids has to be taken into account when considering bone and lipids relationships.

4. Other tissues

A growing body of evidences suggests that besides adipose and digestive tissues, bone metabolism may be altered in case of several organs dysregulations including liver, kidney or skeletal muscles. The link between these three organs and fatty acids may involve a cluster of life-shortening obesity-related commorbidities named metabolic syndrome. In most of patients, this metabolic status results from a lifestyle-induced caloric mismatch due to both an insufficient physical activity and an excessive energy intake. The caloric excess leads to an overwhelming of WAT lipid storage capacities and subsequent ectopic accumulation of these lipids (steatosis) that damage nonadipose organs including skeletal muscle, liver and kidney

and which, in return, may impact bone health (Unger and Orci 2001; McGavock, Victor et al. 2006).

a. Liver

Osteoporosis is frequently associated with chronic liver diseases (CLD), especially with late stages, with established liver cirrhosis that may originate from alcoholic or non-alcoholic steatosis (Guanabens and Pares 2010). Mechanisms underlying this association are far from being completely understood and may involve fatty acid metabolism.

Two recent studies demonstrate that livers of rats fed with high levels of saturated fat present all features of evolved steatosis, with increased inflammation, oxidative stress and fibrosis (Carmiel-Haggai, Cederbaum et al. 2005; Zou, Li et al. 2006). This observation was later confirmed in humans, as lipid intake has been associated with hepatic steatosis and hepatic inflammation (steatohepatitis) (Vilar, Oliveira et al. 2008).

At a cellular level, conditioned media from palmitate treated primary hepatocytes has been shown to contain unidentified factors promoting the proliferation of Hepatic Stellate Cells (HSC) and inducing their expression of profibrogenic genes including TGF-β and tissue inhibitor of metalloproteinases 1 (TIMP1) (Wobser, Dorn et al. 2009). In addition, FFA-treated hepatocytes were shown to have an increased expression of the pro-inflammatory cytokines TNF-α (Feldstein, Werneburg et al. 2004) whereas conditioned media from palmitate treated primary hepatocytes has been shown to trigger increased expression of monocyte chimoattractant protein 1 (MCP1) by HSCs (Wobser, Dorn et al. 2009). These increases in liver inflammation and oxidative stress may, in addition to their contribution to the liver degradation, contribute to modulate systemic redox and inflammation status supporting increased bone resorption.

In the same way, an overexpression of the special fibronectin isoform containing the oncofetal domain, a protein is known to inhibit bone formation both *in vivo* and *in vitro*, was correlated with liver alterations (Kawelke, Bentmann et al. 2008). In contrast, production of the osteoblast-stimulating factor IGF-1 production by liver (Cemborain, Castilla-Cortazar et al. 1998) (Gallego-Rojo, Gonzalez-Calvin et al. 1998) was found to be reduced during steatosis establisment. Therefore, taken together, all these modifications may contribute to unbalance bone remodelling processes as observed in chronic liver diseases.

In the literature, osteoporosis prevalence seems to correlate with the severity of the liver cirrhosis rather than the etiology (Giouleme, Vyzantiadis et al. 2006). Therefore, it is of major interest to notice that the severity of the disease and its subsequent impact on bone may

depend on fatty acid type. Indeed, feeding mice with trans FAs (partially hydrogenated vegetable oil) has revealed an even more deleterious effect of these fatty acids on liver behaviour as compared to SFAs (Tetri, Basaranoglu et al. 2008; Obara, Fukushima et al. 2010). In contrast, n3 PUFAs have been shown to have beneficial properties on establishment and evolution of liver steatosis. As a matter of fact, ω-3 PUFAs or DHA dietary treatment of leptin-deficient obese mice result in reduced hepatic lipid content. (Sekiya, Yahagi et al. 2003; Kim, Lee et al. 2008). The beneficial effect of n3 PUFAs is thought to be caused by their action as suppressors of the sterol regulatory element binding protein 1 (SREBP1) which result in a downregulation of the lipogenic gene expressions and subsequent decrease of synthetic rate and amount of storage triglycerides in the liver. In another model of mice hepatic steatosis, EPA-mediated improved liver parameters were also linked to downregulation of SREBP1 and antioxidant, anti-inflammatory and anti-fibrotic actions (Kajikawa, Harada et al. 2009) (Hao, Wong et al. 2010). This reduction in disease burden, oxidative stress and inflammation was confirmed by different clinical trials both in adults and children (Capanni, Calella et al. 2006) (Zhu, Liu et al. 2008; Sofi, Giangrandi et al. 2010) (Tanaka, Sano et al. 2008) (Nobili, Bedogni et al. 2010). However all these studies were done on relatively small samples and appropriately powered trials are required to definitely validate the clinical benefit of ω-3 PUFAs on liver steatosis. In parallel to what was observed with PUFAs ω-3, MUFAs and CLA were both shown to exhibit beneficial effect on chronic liver diseases development. Olive oil (rich in MUFAs) fed rats resulted in milder liver fat content as compared to SFA fed rats in the methionine-choline deficient diet model of hepatic steatosis (Hussein, Grosovski et al. 2007) whereas CLA protected fa/fa rats against the accumulation of fat in their liver with a reduced expression of the TNF-α mRNA (Nagao, Inoue et al. 2005). Overall, available data clearly suggest a potential beneficial impact of ω-3 PUFAs, MUFAs and CLA on liver-induced bone alterations. Mechanisms may involve prevention of both, liver steatosis establishment and pro-inflammatory context driving its evolution to bone loss-associated cirrhosis state.

In another hand, alcohol-induced hepatic steatosis is histologically indistinguishable from high fat diet-induced liver steatosis and share at least two mechanistic pathways, oxidative stress and pro-inflammatory cytokines. (Lieber 2000; Tilg and Diehl 2000) Nevertheless, despite these similarities the role of fatty acid composition in the development of the liver injury dramatically differs. In fact, in the presence of alcohol SFAs prevented whereas PUFAs promoted alcohol-related liver injury (Nanji, Mendenhall et al. 1989; Ronis, Korourian et al. 2004). Surprisingly, the mechanism(s) by which saturated fats protect against alcohol-induced

liver injury involve reduction of proinflammatory mediators, including TNF-α, cyclooxygenase-2 and NF-κB, and reduction of oxidative stress. (Nanji, Zakim et al. 1997; Nanji, Jokelainen et al. 2001) Since long chain SFAs promote oxidative stress and activate inflammatory pathways in absence of alcohol, it seems likely that the presence of alcohol alters metabolism of specific fatty acids within tissues. Therefore, despite the global beneficial effect of ω-3 on bone metabolism, the design of nutritional strategies to prevent from liver-associated bone alteration must take into account the cause of chronic liver diseases.

b. Kidneys

Kidneys and bone work in concert to regulate the homeostasis of body electrolytes. Then, it is not surprising that secondary bone defects in patients with either acute or chronic renal disease (CKD) are frequent clinical features. As in liver, kidney steatosis is mostly asymptomatic but it may evolve and associates itself with more deleterious features including lipoapotosis, increase of pro-inflammatory molecules, oxidative stress and progressive organ fibrosis. If not taken care of, it may ultimately results in severe renal glomerulosclerosis (Kume, Uzu et al. 2007) and associated bone defects. Depending of their amount and structure, lipids in general and fatty acids in particular are key actors in the establishment of the ectopic fat accumulation and its above-mentioned evolution.

Mechanisms linking fatty acids to CKD and potent associated bone altered architecture are quite similar to those described for chronic liver diseases. Glomerulosclerosis and albuminuria observed in mice fed a high saturated fat diets (Kume, Uzu et al. 2007; Tanaka, Kume et al. 2010) was associated with an increase in renal pro-inflammatory markers (via a macrophage interstitial infiltration), oxidative stress markers and interstitial fibrosis (Okamura, Pennathur et al. 2009; Li, Wang et al. 2010). Investigating more in depth the cellular mechanisms, two studies demonstrated that the palmitate induce the production of the pro-inflammatory molecule MCP-1 from both mesangial cells (Tanaka, Kume et al. 2010) and proximal tubular cells (Soumura, Kume et al. 2010).

At the opposite of SFAs, ω-3 PUFAs (Barcelli, Weiss et al. 1990) (Chin, Fu et al. 2010) and t10,c12-CLA (Drury, Warford-Woolgar et al. 2009) exert renal protective effects. In both cases, renal anti-inflammatory and anti-fibrotic properties have been established in animal models (Cappelli, Di Liberato et al. 1997) (Ogborn, Nitschmann et al. 2003) (Lu, Bankovic-Calic et al. 2003). The ω-3 PUFA renal anti-inflammatory properties may come from their ability to blunt the palmitate-induced increased in MCP-1 production by mesangial

cells (Soumura, Kume et al. 2010). Besides, mice fed a mixture of DHA and EPA have reduced SREBP1 renal expression which has been shown *in vitro* to decrease the production of pro-fibrotic factors by mesangial cells (Chin, Fu et al. 2010). Finally, anyhow fatty acids impact kidney function greatly correlates with bone tissues behaviour; however, the underlying molecular mechanisms remain to be further deciphered.

c. Muscles

Skeletal muscle is a dynamic tissue whose primary role is the development of forces. In this regard, muscle mass and strength are key factors that contribute to bone quality such as density, strength, and microarchitecture by regulating bone mechanical stimulation (Crepaldi, Romanato et al. 2007). There are several arguments suggesting that muscle strength is altered in a context of diet induced obesity. Even if obese persons usually have more muscle mass and strength (Visser, Langlois et al. 1998), the contribution of muscle mass to strength seems to be inversely linked to the amount of body fat (Payette, Hanusaik et al. 1998). A potent explanation lye on the well established association between diet induced accumulation of fat in the muscle and insulin resistance (Silveira, Fiamoncini et al. 2008) (Kraegen and Cooney 2008). In addition to its obvious role in glucose homeostasis, insulin signalling contributes to the initiation of protein synthesis (Kimball, Horetsky et al. 1998) (Balage, Sinaud et al. 2001) (Prod'homme, Balage et al. 2005), a key mechanism involved in the regulation of muscle mass in response to external growth stimuli. This insulin-induced increase in muscle protein synthesis is inhibited in the context of diet induced obesity (Anderson, Gilge et al. 2008). In addition, fatty acid infusions have been shown to dysregulate the AKT/mTOR pathway, thus abrogating muscle adaptation (Belfort, Mandarino et al. 2005; Pedrini, Kranebitter et al. 2005) and leading to an attenuated load-induced muscle hypertrophy in mice fed a high saturated fat diet (Sitnick, Bodine et al. 2009). Then, it is very tempting to speculate that, by favouring insulin resistance in muscle, fatty acid accumulation leads to a decrease in muscle strength that in return contribute to reduce bone mechanical stimulation and subsequent bone loss.

D - Lipids, inflammation and bone

1. Inflammation controls bone health

Bone health results in a highly regulated balance between formation by osteoblasts and resorption by osteoclasts. During aging or under pathological circumstances as rheumatoid arthritis, inflammatory bowel disease or chronic obstructive pulmonary disease, that all share an inflammatory state disorder, bone homeostasis is dramatically impaired. It results in bone loss that could lead to osteoporosis, fractures and pain.

a. Inflammation is associated with bone loss

Type I osteoporosis is linked with estrogen withdrawal observed after menopause in women. It as been shown that estrogen deficiency stimulates the production of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 (Ralston, Russell et al. 1990; Pacifici, Brown et al. 1991; Pfeilschifter, Koditz et al. 2002; Rachon, Mysliwska et al. 2002). On the contrary, *in vitro* and *in vivo* studies mentioned that estrogen treatment tends to down-regulate their production (Pfeilschifter, Koditz et al. 2002). The role of pro-inflammatory cytokines in osteoporosis after estrogen withdrawal is confirmed by genetically modified mice models. Actually, mice lacking the IL-1 type 1 receptor or IL-6 genes did not significantly present trabecular bone loss after ovariectomy (Poli, Balena et al. 1994; Lorenzo, Naprta et al. 1998) . However, constitutive activation of inflammatory pathways as observed in transgenic mice overexpressing TNF-α, IL1 or IL-6, is sufficient to trigger systemic bone loss (Schett, Redlich et al. 2003; Aoki, Ichimura et al. 2005; De Benedetti, Rucci et al. 2006).

Type II osteoporosis, also called senile osteoporosis, occurs after the age of 70 years in women twice as frequently as in men. Ageing is associated with a global inflammatory state as revealed by an increase of circulating cytokine levels (Lencel and Magne). Actually, several studies reviewed in (Bruunsgaard, Pedersen et al. 2001; Krabbe, Pedersen et al. 2004) have reported that IL-6 serum level is increased in elderly populations. It seems that it is not always the case for TNF-α as some studies find an increase or decrease level with advanced aging; these differences can be explained by the health status or the age of the subjects. However, it is to note that this increase in the circulating inflammatory mediators is low compared with the one observed during an acute inflammatory response and is rather a chronic low-grade inflammatory state. These data support the idea that a local or a systemic inflammatory state can lead to osteopenia and osteoporosis.

b. Inflammation controls bone cells activity

Osteoclasts are giant multinucleated cells derivated from haematopoietic precursors. Their differentiation is largely dependent on the OPG/RANKL/RANK system besides the macrophage stimulating factor M-CSF. Once differentiated, osteoclasts are responsible for bone resorption (Asagiri and Takayanagi 2007; Edwards and Mundy 2011). There are two different ways by which pro-inflammatory cytokines modulate osteoclasts differentiation and activity: the first one implicates the regulation of OPG/RANKL/M-CSF secretion by stromal cells and the second one is the direct activity of cytokines on osteoclasts physiology. TNF-α is the major cytokine secreted by activated T cells, monocytes and macrophages. When the stromal/osteoblastic cells are stimulated by TNF-α, they produce more M-CSF and RANKL that will activate osteoclasts (Hofbauer, Lacey et al. 1999; Kwan Tat, Padrines et al. 2004; Kitaura, Zhou et al. 2005). IL-1 also promotes the RANKL expression by marrow stromal cells and osteoblasts (Hofbauer, Lacey et al. 1999) and it appears that it plays direct osteoclastogenic activity and also mediates the effect of TNF-α (Wei, Kitaura et al. 2005). When the intensity of the TNF-α stimulation is more pronounced, it can directly stimulate osteoclast differentiation independently of the presence of stromal cells (Azuma, Kaji et al. 2000; Fuller, Murphy et al. 2002; Kitaura, Sands et al. 2004). However, there is a strong consensus of the synergistic action of cytokines and RANKL on osteoclast differentiation and activity (Lam, Takeshita et al. 2000; Komine, Kukita et al. 2001; Zou, Hakim et al. 2001). Finally, a direct control of osteoclastogenesis can be performed by the T cells themselves through the secretion of RANKL. This could, in part, explain the bone loss induced by estrogen deficiency (Weitzmann and Pacifici 2006; Weitzmann and Pacifici 2007; Quinn and Saleh 2009).

Pro-inflammatory cytokines are also implicated in the control of osteoblast differentiation and activity. *In vitro* experiments have shown that TNF-α and IL1β inhibit the mRNA expression of the key transcription factor Runx2 in foetal cavarial cells, MC3T3E1 and human mesenchymal stem cells (Gilbert, He et al. 2002; Ding, Ghali et al. 2009; Lacey, Simmons et al. 2009). TNF-α also controls Runx2 protein level by promoting its degradation by the ubiquitin-proteasome pathway (Kaneki, Guo et al. 2006). Moreover, TNF-α also down-regulates the transcription of another transcription factor implicated in osteoblast differentiation: osterix (Lu, Gilbert et al. 2006). These effects result in a lower expression of target genes as the osteocalcin or the collagen1 implicated in osteoblast differentiation and activity, and a lower mineralization capacity in response to differentiation inducers (Gilbert, He et al. 2002; Nanes 2003; Ding, Ghali et al. 2009; Yamazaki, Fukushima et al. 2009). *In*

vivo evidences for the role of TNF-α in the control of osteoblastic differentiation is supported by the increase of BMD in TNF-α and TNF-αR type I (p55) knock out mice that present a higher bone formation (Li, Li et al. 2007). Also, IL-6 over-expressing mice have a decreased osteoblasts actvity (De Benedetti, Rucci et al. 2006). Thus, inflammatory cytokines exert deleterious effects on bone homeostasis by presenting opposite effects on osteoblasts/osteoclasts activity.

2. Dietary lipids regulate inflammatory cytokines production: role in bone tissue

A major way by witch lipids control the bone homeostasis is by their ability to regulate the pro-inflammatory cytokines profile. Actually, by modulating the systemic or local secretion of pro/anti-inflammatory cytokines, they thus participate in bone cell proliferation, differentiation and activity.

a. Human studies

In a clinical study reported by Griel et al., 6 weeks consumption of a high-PUFA diet enriched in α-linolenic acid (ω-3) resulted in a significant decrease in serum TNF-α compared to the control diet or a high-PUFA diet enriched in linoleic acid (ω-6) (Zhao, Etherton et al. 2007). Interestingly, the N-telopeptides (NTx) serum level, a bone resorption marker, was significantly lower following the consumption of the ω-3 rich diet, compared to the other diets, suggesting the existence of a positive correlation between the level of circulating TNF-α and the bone resorbing activity.

Also, another human study realised with astronauts during and after their spaceflights revealed that a short-duration flight resulted in an NF-κB signalling activation measured by p65 protein expression in peripheral blood mononuclear cells. This indicates that space flight is associated with a systemic inflammatory state (Zwart, Pierson et al.). However, a high consumption of fish (which is a rich source of ω-3 fatty acids) during the flight was associated with a reduced BMD loss and a reduced N-telopeptide excretion during bed rest. These human studies establish a link between lipid consumption, the inflammatory state and bone health.

b. Animal studies

In order to analyse the impact of dietary lipids on the age related evolution of bone, Wauquier et al. realized a lifelong lipid supplementation in a murine model of senile

osteoporosis SAMP8 (Wauquier, Barquissau et al. 2011). In this model, the 12 month old SAMP8 that received a 10 month diet of sunflower oil enriched diet (high ratio in ω-6/ω-3) presented a lower BMD that the control strain SAMR1, validating the age related bone loss model. However, the diet supplementation with borage oil (rich in γ–linolenic acid) or fish oil (rich in long chains ω-3 fatty acids) restored the femoral trabecular BMD at the level of the SAMR1 fed with the sunflower diet. This was associated with a negative and a positive modulation of mRNA expression markers of osteoclastogenesis and osteoblastogenesis respectively in the femurs. It is to note that IL-6, IL-6R and IFNgR1 inflammatory markers mRNA expression were significantly lowered by the fish oil diet consumption. In the same way, Uchida et al. reported that in OVX mice, a fish oil diet supplementation resulted in a higher femoral BMD and a lower TNF-α serum concentration than those in the safflower oil diet group (Uchida, Chiba et al. 2010). In another study realized with 12 month old mice, a 6 month corn oil diet (rich in ω-6 fatty acids) resulted in a loss of trabecular bone volume associated with a higher mRNA expression level of cytokines TNF-α, IL-1β and IL-6 in the femur (Halade, El Jamali et al. 2011).

Mammals cannot naturally produce ω-3 fatty acids from the naturally more abundant ω-6 fatty acids; thus, Kang et al. have developed a fat-1 transgenic mice model allowing the conversion of dietary ω-6 to ω-3 fatty acids (Kang, Wang et al. 2004). In this model, the trabecular and cortical bone mass were higher in the femur of fat-1 mice compared to the WT mice and the fat-1 mice were protected against ovariectomy induced bone loss (Bordin, Priante et al. 2003). Also, the TNF-α and IL-1β serum levels were constitutively lower in fat-1 mice (Rahman, Halade et al. 2009). Moreover, ex-vivo experiments have shown that bone marrow cells derived from OVX-fat-1 mice produce less TNF-α, IL-1β and IL-6 in response to LPS than cells derived from OVX-WT mice (Rahman, Bhattacharya et al. 2009). This effect was in part dependent on a lower activation of the NF-κB signalling.

c. Ex vivo studies

Several *ex vivo* studies have investigated the impact of dietary lipids on the inflammatory state in the context of mice osteoporosis. Sun et al. have shown that OVX mice consuming a 5% fish-oil diet rich in ω-3 fatty acids have a significantly higher femoral BMD than the corn oil diet OVX mice (Sun, Krishnan et al. 2003). More importantly, TNF-α and IFN-γ secretion from spleen lymphocytes collected at death of the animals and stimulated in culture with concanavalin A were lower in the OVX fish oil compared with the OVX corn oil

group. A same decrease in TNF-α and IL-6 was observed in splenocytes and bone marrow cells obtained from 6 month fish oil and Conjugated linoleic Acid (CLA) fed mice (Halade, Rahman et al.). Using a preventive and a curative protocol based on 12 weeks feeding with an ω-3 PUFA enriched diet post-OVX in mice, Zeitlin et al. have demonstrated that in both experiments, ω-3 PUFAs decreased the secretion of non-induced IL-6 and TNF-α from cultured BMC and the IL-1β in bone marrow plasma (Zeitlin, Segev et al. 2003). This ant-inflammatory activity of ω-3 PUFAs was corroborated by the significant increase in the secretion of IL-10, an anti-inflammatory cytokine witch present a critical role in preventing inflammatory and autoimmune pathologies (Moore, de Waal Malefyt et al. 2001; Hawrylowicz and O'Garra 2005; O'Garra, Barrat et al. 2008).

Also, in an aging female mice model of bone loss, after 6 month of dietary treatment, the 18-month-old fish oil-fed mice presented a higher femoral BMD in comparison to the corn oil-fed mice (Bhattacharya, Rahman et al. 2007). The osteoclast differentiation of bone marrow cells isolated from the mice and treated with M-CSF and RANKL was less effective in the fish oil-fed mice group. Moreover, concanavalin A stimulated splenocytes secreted less TNF-α and IL-6. It is to note that a 8 week dietary treatment with a low Eicosapentaenoic/Docosahexaenoic (EPA/DHA) ratio oil also negatively controlled the inflammatory cytokines TNF-α, IL-1β and IL-6 in LPS-activated murine resident peritoneal macrophage (Bhattacharya, Sun et al. 2007). On the contrary, the feeding of a high fat diet containing 10% corn oil (rich in ω-6) during 6 month in 12 month aged mice, resulted in a reduced BMD in femurs and tibias and an increase in proinflammatory cytokines secretion (TNF-α and IL-6) in *ex vivo* bone marrow and splenocytes cultures compared to the standard lab chow diet (Halade, Rahman et al. 2010).

d. In vitro studies

As cytokines are critical local factors playing a key role in bone remodelling, *in vitro* studies have investigated the role of lipids on bone cells cytokine expression and on immune T cells known to relay the inflammatory signal.

Priante et al. first reported that the ω-6 arachidonic acid (AA) induced the gene expression, in a dose and time-dependent manner, of TNF-α, IL-1α, IL-1β and M-CSF in an osteoblast cell line MG-63 (Priante, Bordin et al. 2002). This increased expression can be suppressed in the presence of oleic acid (OA) and EPA. A same increase in response of AA was observed for IL-6 gene expression, and in the same way, OA and EPA repressed the

effect (Bordin, Priante et al. 2003). In both studies, the action of AA was mediated by PKC activation. Moreover, a 20 μM AA dose largely increase the secretion of RANKL and decrease the secretion of OPG in MC3T3-E1 pre-osteoblasts, thus, resulting in a reduced OPG/RANKL ratio favouring osteoclast differenciation (Coetzee, Haag et al. 2007). These results indicate that AA induces pro-inflammatory signals and the secretion of signalling molecules (RANKL/OPG), both known to have pro-osteoclastic activities.

In an effort to better understand the mechanisms by witch ω-3 PUFAs exert their protective bone functions, Rahman et al. have studied the action of EPA and DHA on osteoclast formation and RANKL signalling in osteoclastic precursors RAW267.4 (Rahman, Bhattacharya et al. 2008). They have first shown that EPA and DHA dose-dependently (50 and 100 μM) decrease the TNF-α secretion in response to RANKL with a greater effect for DHA. This action was associated with an inhibition of the NF-κB pathway measured by a decrease in p65 expression and DNA binding activity. Moreover, these two ω-3 fatty-acids down-regulated the gene expression of osteoclast differenciation markers as TRAP, MMP-9, CTR or cathepsin K and their bone resorbing activity on dentine slices. Similar results on osteoclast differentiation were obtained with bone-marrow derived macrophages instead of the RAW267.4 cell line (Sun, Krishnan et al. 2003). Also, 9cis,11trans and 10trans, 12cis CLA were able to inhibit osteoclast formation and activity by more than 70% from human CD14+ monocyte at 50μM (Platt and El-Sohemy 2009). However, none of the CLA inhibited TNF-α gene expression in these cells.

RANKL is a key component of the interaction between the bone and the immune system. Actually, activated lymphocytes can secret RANKL and thus participate in osteoclast differentiation (Walsh, Kim et al. 2006; Leibbrandt and Penninger 2008). Graham et al. have thus studied the impact of oxidized lipids on T lymphocytes RANKL production (Graham, Parhami et al. 2009). They have shown that minimally oxidized LDL that are highly inflammatory, largely increase the RANKL secretion in both unstimulated and activated T lymphocytes. Using specific inhibitors, they have also demonstrated that this effect was mediated by the NF-κB signalling activation. Finally, they have realized an *in vivo* study in which they have fed mice with a control or a high-fat diet for 11 months. They have nicely shown that compared to the control diet, the high fat diet resulted in a significant decrease in femoral BMC, an increase in the RANKL transcript in T lymphocytes and an increase in RANKL serum level. These results points out an interesting way by which lipids could control the immune system responses and thus the bone homeostasis.

All these human studies, animal and cellular experiments clearly demonstrates that dietary ω-3 fatty acids could help preventing bone loss by, at least in part, down-regulating the production of pro-inflammatory cytokines. Inversely, the ω-6 fatty acids tend to have opposite effects and thus present deleterious bone effects.

3. Dietary lipids are sources of pro/anti-inflammatory eicosanoids: role in bone tissue

Eicosanoids represent a key link between dietary lipids and inflammation. Actually, they are derived from dietary PUFAs and are, besides cytokines, a family of pro/anti-inflammatory mediators (Basu; Ricciotti and FitzGerald 2011). Their actions have been associated with risk factors and progression of many diseases such as arthritis, atherosclerosis or cancers. Their complex role in the bone will be reported below.

a. Fatty acids are precursors of eicosanoids

Eicosanoids are derived from cell membranes phospholipids which composition is modulated by dietary PUFA intake (Sardesai 1992; Calder and Yaqoob 2009). Following desaturases and elongases actions, the essential fatty acids Linoleic Acid (ω3) and Alpha Linoleic-Acid (ω6) will be transformed in Arachidonic Acid (AA: 20C-ω6), Dihomogammalinoleic Acid (DGLA: 20C-ω6) and Eicosapentaenoic Acid (EPA: 20C-ω3). Then, cyclooxygenases (COX), lipoxygenases (LOX) and non-enzymatic oxidations will generate oxygenated derivatives eicosanoids: prostaglandins, leukotrienes, thromboxanes, isoprostanes, lipoxins and E-resolvins (Poulsen, Moughan et al. 2007; Kruger, Coetzee et al. 2010). Besides eicosanoids, docosanoids are derivates from Docosahexaenoic Acid (DHA: 22C-ω3). Basically, eicosanoids derived from AA presents pro-inflammatory activities and the one derived from EPA are anti-inflammatory (Calder and Yaqoob 2009; Galli and Calder 2009). Thus, the consumption of ω3 PUFAs will influence the membrane composition by replacing the AA by EPA and DHA, then the eicosanoids production and modulate the inflammatory state (Calder 2006).

b. Role of prostaglandin E2 (PGE2) in bone

Prostaglandines are key lipid mediators that have been linked with bone remodelling associated with inflammation or related diseases (Hikiji, Takato et al. 2008; Blackwell, Raisz et al. 2010). PGE2 seems to be the main PG responsible for bone homeostasis regulation with

complex activities on bone formation and resorption. PGE2 is derived from AA and several studies have shown that modulating the ratio of ω3/ω6 dietary fatty acids could regulate the PGE2 synthesis and bone status. Actually, Watkins et al. first demonstrated that feeding chicks with menhaden/safflower oil compared with soybean oil, resulted in increased *ex vivo* PGE2 biosynthesis in bone organ cultures, higher ALP serum activity and increased fractional labelled trabecular surface and tissue level bone formation rates (Watkins, Shen et al. 1996). Other experiments conduced in rats and quails confirmed the positive correlation between an ω3 fatty acids enriched diet, the decrease in bone PGE2 production and the positive modulation of bone health as measured by serum biomarkers or BMD (Watkins, Li et al. 2000; Liu, Veit et al. 2003; Liu, Veit et al. 2004; Shen, Yeh et al. 2006; Shen, Yeh et al. 2007).

The PGE2 bone pro-resorbing activity has been largely studied *in vitro* as it exerts direct effect on osteoclast precursors. Actually PGE2 enhances OC differentiation from mouse bone marrow derived macrophages, murine spleen cells cultures or Raw264.7 precursors stimulated with RANKL with or whithout M-CSF (Wani, Fuller et al. 1999; Li, Okada et al. 2000; Kobayashi, Mizoguchi et al. 2005; Kobayashi, Take et al. 2005; Tsujisawa, Inoue et al. 2005). These effects are in part, mediated by the EP2 and EP4 PGE2 receptors (Minamizaki, Yoshiko et al. 2009). Besides these direct effects on OC, PGE2 also regulates their differentiation by modulating RANKL and OPG secretion by OB: it can both stimulate RANKL and inhibit OPG production in stromal cells (Vidal, Brandstrom et al. 1998; Li, Okada et al. 2000). Many studies have been conduced in MC3T3-E1 osteoblasts cultured in the presence of PUFAs. They have shown that AA increases the expression of COX-2 enzyme, the PGE2 synthesis, RANK-L expression and decreases OPG one. On the contrary, EPA, DHA and GLA present opposite effects (Coetzee, Haag et al. 2007; Poulsen, Moughan et al. 2007). Finally, PGE2 also regulates cytokines expression known to induce osteoclastogenesis as IL-6 or IL-1β (Amano, Naganuma et al. 1996; Kozawa, Suzuki et al. 1998; Millet, McCarthy et al. 1998; Park, Kang et al. 2004).

However, PGE2 has also some bone forming activities. *In vitro* experiments realized with osteoblasts precursors, demonstrated that PGE2 increases mineralization in primary rat calvaria cells and MC3T3-E1 (Flanagan and Chambers 1992; Nagata, Kaho et al. 1994; Wu, Zeng et al. 2007; Minamizaki, Yoshiko et al. 2009). This PGE2 activity is mediated by its EP4 and EP2 receptors (Suda, Tanaka et al. 1996; Li, Okada et al. 2000; Alander and Raisz 2006; Minamizaki, Yoshiko et al. 2009). Also, bone marrow stromal cell cultures derived

from COX2 deficient mice were impaired in their differentiation process and presented a lower expression level of Runx2 and osterix (Zhang, Schwarz et al. 2002). These effects were reversed with PGE2 cell treatment. Under physiological conditions, *in vivo* studies revealed that COX-2 loss in mouse (deletion of the *ptgs2* gene) has only a little positive effect on bone (Chen, Rho et al. 2003; Myers, Bhattacharya et al. 2006). Also, EP2/EP4 PGE2 receptors absence results in weak bone biomechanical strength properties (Akhter, Cullen et al. 2001; Akhter, Cullen et al. 2006) and EP4 is critical for bone fracture healing (Li, Healy et al. 2005).

In animals, like PTH, PGE2 presents opposite actions on bone depending on the way it is administrated and the doses used. In rats, while continuous delivery of PGE2 is bone deleterious, intermittent administration resulted from both cancellous and cortical bone gain (Tian, Zhang et al. 2007; Tian, Zhang et al. 2008). These results were confirmed in mice and it was also shown that PGE2 presents dose dependant activities: the authors concluded that intermittent exposure to high doses of PGE2 may be anabolic but is balanced by catabolic effects resulting in less bone benefits than with low doses (Gao, Xu et al. 2009).

c. Role of other eicosanoids in bone

Arachidonic Acid is the precursor of other prostaglandins as PGF2α or leukotrienes that mainly present pro-inflammatory activities. Their role on the control of osteoblast and osteoclast functions and bone activities under physiological and pathological conditions have been nicely reviewed in: (Hikiji, Takato et al. 2008). Recently, some studies have focused on an EPA oxygenated product, ResolvinE1 (RvE1). Hasturk et al. demonstrated that RvE1 protects local inflammation and osteoclast-mediated bone destruction in periodontis (Hasturk, Kantarci et al. 2006; Hasturk, Kantarci et al. 2007). As PGE2 or LTB4 present direct actions on bone cells besides their pro-inflammatory activities, Herrera et al. made the hypothesis of a direct role of RvE1 on osteoclast function, independently of its anti-inflammatory and pro resolution activities (Herrera, Ohira et al. 2008; Seki, Tani et al. 2009; Ji, Xu et al. 2011). In bone marrow cultures treated with RANK-L and M-CSF, RvE1 decreases OC growth, differentiation and resorption activity without affecting their death and apoptosis. It is interesting to note that a daily EPA supplementation in mice resulted in an increase of RvE1 in bone marrow (Poulsen, Gotlinger et al. 2008). We can thus speculate that *in vivo* action of EPA is directly mediated by RvE1 through modulation of osteoclast activity.

Inflammation has so far been associated with bone damages. By regulating pro/anti-inflammatory cytokine and other mediator secretions that would control bone cells

differentiation and activity, dietary lipids and their metabolites are key players of bone health linked to inflammatory processes.

E - Oxidative stress, fatty acids and bone

Redox status is a key protagonist in bone remodeling processes. It results from the balance between reactive oxygen species (ROS) production and antioxidant defenses, both potently being under the influence of fatty acid metabolism. In fact, fatty acid may either act as pro-oxidant compounds when undergoing peroxidation or as antioxidant factors to limit oxidative damage to bone.

1. Oxidative stress and bone

During the last decade, clinical and *in vivo* studies have widely demonstrated that oxidative stress are implicated in the pathogenesis of cancer, cardiovascular disease, atherosclerosis, diabetes mellitus, and age-related diseases including osteoporosis (Basu, Michaelsson et al. 2001; Valko, Leibfritz et al. 2007; Sendur, Turan et al. 2009). Indeed, several investigations have revealed a negative correlation between oxidative stress and BMD, while antioxidants have been found to prevent from excessive bone resorption (He, Andersson et al. 2010) (Kim, Ke et al. 2011). Oxidative stress is an inescapable consequence of life in an oxygen-rich environment and results from a redox imbalance that leads to reactive oxygen species (ROS) accumulation. ROS mainly originate from enzymatic complexes including mitochondria respiratory chain, NADPH oxydases, NO synthase and xanthine oxydase (Riley 1994). They are produced in response to different stimuli, such as inflammatory cytokines, growth factors, environmental toxins, chemotherapeutics, UV light or ionizing radiation. Interestingly, they are also generated during fatty acid oxidation (Manolagas 2010). Mechanisms of antioxidant defenses are able to eliminate these active oxygen derivatives: some of them are endogens (enzymatic system) such as superoxide dismutase, catalase and peroxidases, and others are provided by food such as vitamine C and E.

ROS activity depends on concentration and may be either beneficial or harmful. At moderate concentrations, nitric oxide (NO), superoxide anion, and related ROS play an important role as regulatory mediators in signaling processes (Droge 2002). Their production contributes to the innate immune response as well as the initiation and amplification of antigenic signal (Reth 2002).

Besides, ROS are known physiological regulators of bone remodeling. Indeed, *in vitro* and *in vivo* studies have shown that ROS increase osteoclast resorption and decrease

osteoblast bone formation by influencing the activity and survival of these cells. In fact, ROS such as H_2O_2 act on osteoclastogenesis and osteoblastogenesis through modulation of intracellular redox-sensitive signaling cascades by causing reversible oxidations of proteins on cystein residues (Wauquier, Leotoing et al. 2009). These cascades include ERKs, jun Nterminal kinases (JNKs), p38 MAPK, phosphatidyl inositol 3 kinase (PI3K)/Akt, activator protein-1, p53, tyrosine phosphatases, proteases, molecular adaptators and chaperones, the wnt/β-catenin signaling pathway, as well as transcription factors such as nuclear factor κB (NF-κB) (Janssen-Heininger, Mossman et al. 2008; Manolagas 2010). In addition, ROS stimulate osteoblastic production of Receptor Activator NF-κb Ligand (RANKL) thus driving an increased osteoblast-induced osteoclast differentiation (Bai, Lu et al. 2005).

2. Oxidized lipids and bone

At high concentrations, free radicals, radical-derived and non-radical reactive species damage all cellular constituents; however, lipids are particularly targeted upon oxidative attack (Beckman and Ames 1998).

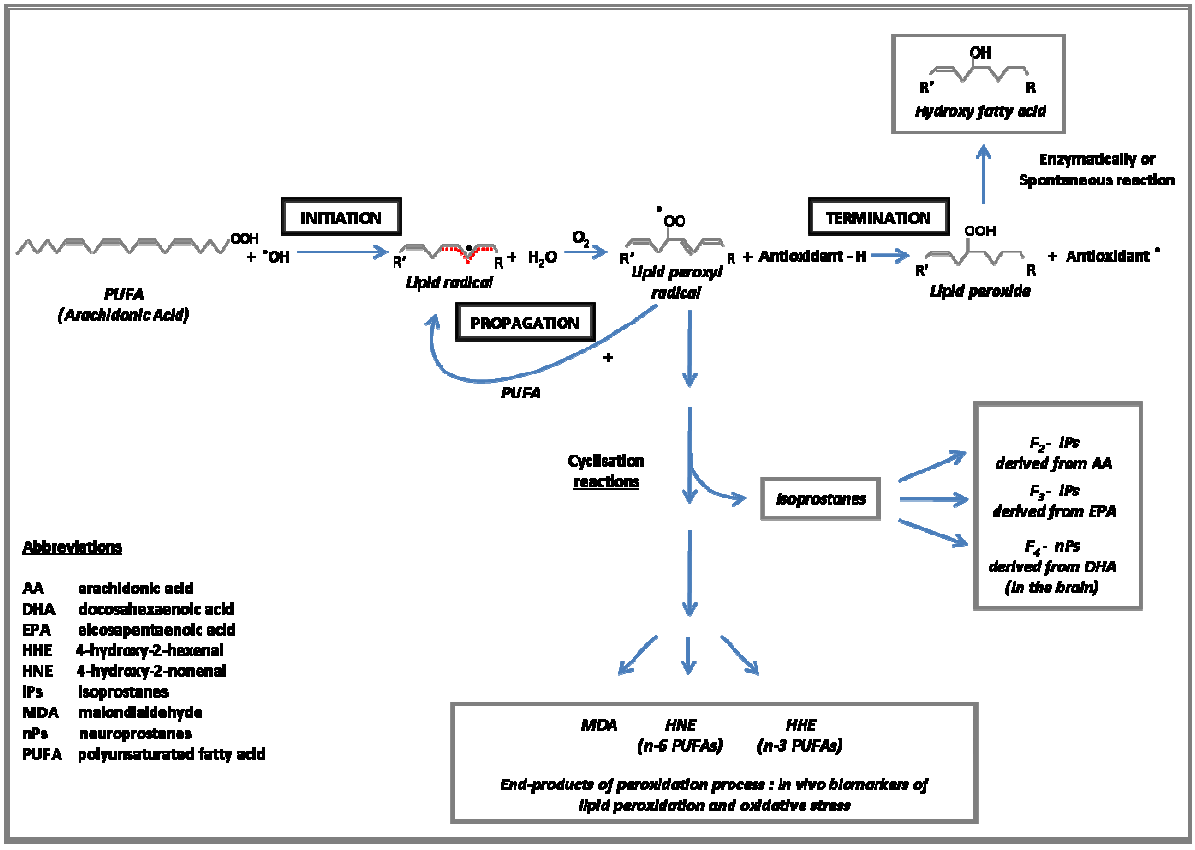
a. Fatty acids oxidation

Although oxidized lipids may either derive from an enzymatic or a non-enzymatic process, ROS always seem involved. For instance, ROS have been proved to enhance the activity of Alox-15, a lipoxygenase that converts polyunsaturated fatty acids to oxidized fatty acids such as 9-hydroxy-10, 12-octadecadienoic acid (Galli, Passeri et al. 2011). Regarding cyclooxygenases, this part was previously described in the inflammation section.

Fatty acids, especially PUFAs, can be both substrates and vectors of non-enzymatic oxidation leading to an amplification of the peroxidation process. DHA, EPA and AA are very prone to this phenomenon due to their unstable chemical structure with several double bonds (Melhus, Michaelsson et al. 1999) (Halliwell and Gutteridge 1984) (Morrow and Roberts 1996). A vicious cycle may then ensue, driving the formation and accumulation of lipid oxidation products that undergo fragmentation and produce a broad range of reactive carbonyl intermediates (Figure 3). Later, these unsaturated aldehydes deplete glutathione and further increase oxidative stress (Negre-Salvayre, Auge et al. 2010).

Besides, isoprostanes are formed cyclic eicosanoids produced *in vivo* during peroxidation of free or esterified PUFAs in membrane phospholipids. In regard of this mechanism, a multitude of isomers can be generated depending on the site of radical attack. They are not

formed in equal quantity in tissues and may partially account for specific known activity of fatty acids (Michel, Bonnefont-Rousselot et al. 2008).



Non-enzymatic PUFA peroxidation
PUFA peroxidation takes place in 3 steps : Initiation, propagation and termination. Many reaction intermediates can be generated according to the site of free radical attack. MDA, HNE and HHE are used *in vivo* to measure peroxidation

Figure 3 : Non-enzymatic PUFA peroxidation

b. Effects on bone

Bone tends to accumulate high quantities of lipid oxidation products that in long term, lead to dysfunction and aging of the tissue (Negre-Salvayre, Auge et al. 2010). Muthusami et al. showed that levels of lipid peroxidation, together with ROS production, were increased in a rat model of oestrogen defiency-related bone loss, when compared to control rats (Muthusami, Ramachandran et al. 2005). In parallel, a recent work from Xiao et al. (Xiao, Cui et al. 2010) demonstrated that mice fed a high fat diet (HFD) exhibited a decrease in intestinal calcium absorption together with a reduced BMD of whole body due redox imbalance in duodenum. Furthermore, a growing body of evidence suggests that specific oxidized lipids may be the common factors underlying the pathogenesis of both atherosclerotic calcification

and osteoporosis (Parhami, Tintut et al. 2001; Schulz, Arfai et al. 2004; Marcovitz, Tran et al. 2005; Farhat, Cauley et al. 2006).

Underlying mechanisms remain unclear, however, extensive investigations have revealed a few hypothesizes. The non-charged structure of peroxidized fatty acids allows them to easily migrate through hydrophobic membranes and hydrophilic cytosolic media and to damage distant targets. As described above, the 9-hydroxy-10, 12-octadecadienoic acid derives from PUFAs via the Alox-15 lipoxygenase activity. This end product is a high affinity ligand of the adipogenic transcription factor peroxisome proliferator-activated receptor gamma that may contribute to favor adipogenesis at the expense of osteoblastogenesis, thus leading to lesser bone formation (Galli, Passeri et al. 2011). Along with this hypothesize, pharmacologic inhibition of Alox15 has been shown to improve bone mass in mice (Klein, Allard et al. 2004).

Oxidized lipids were shown *in vitro* to inhibit osteoblast differentiation of the MC3T3-E1 cells mostly by preventing the alkaline phosphatase induction and calcium uptake. As described previously, RANKL is a key cytokine in the osteoblast-induced osteoclastogenesis; however RANKL may also be produced by activated T-lymphocytes. Very recently, Graham and al. (Graham, Parhami et al. 2009) demonstrated that minimally oxidized LDL (MM-LDL) increased RANKL transcripts and RANKL secretion *ex-vivo* in human T lymphocytes. They further correlated these data in mice with enhanced osteoclastic activity and subsequent bone loss.

In parallel, Brooks et. al. (Brooks, Musiek et al. 2011) reported that a specific oxidative metabolites of EPA, 15-A_{3t}-IsoP, was a potent inhibitor of NF-κB activity during macrophage transformation in RAW264.7 through modulation of IκK function. In addition, treatment with 15-A_{3t}-IsoP decreased oxidized LDL loading of the cells. In contrast, when using 15-A_{2t}-IsoP, an isoprotane formed by oxidation of arachidonic acid, oxidative stress was increased in the same cellular model (Musiek, Gao et al. 2005); thus suggesting that not only the site of oxidation but also the fatty acid origin may be essential for determining the biological activities of these compounds. Interestingly, RAW264.7 is a well acknowledged cellular model of both macrophage and osteoclast precursor (Yang, Wang et al. 2004). Consequently, one may speculate that these data could be transposed to osteoclastic bone cells, thus supporting the undoubted influence of oxidized lipids on oxidative stress and related bone remodeling.

3. Direct modulation of oxidative stress

Although fatty acids and more generally lipids may affect physiologic processes via their oxidation status, they may directly act on oxidant environment. Indeed, arachidonic acid significantly increases inducible Nitric Oxide synthase (iNOS) expression in human osteoblast-like after 3 hours exposure, thus favouring oxidative stress and inflammation context. However, in presence of ω-3 PUFAs, these effects were abrogated (Priante, Musacchio et al. 2005) suggesting a mechanism dependent on fatty acids type. NO has been demonstrated to plays an important role in the regulation of bone cell function, with effects on both the osteoblast and osteoclast lineage. In particular, NO derived from the iNOS pathway has been shown to regulate the effects of pro-inflammatory cytokines on bone and to be essential for the stimulatory activity of IL-1 on bone resorption both *in vitro* and *in vivo* (van't Hof and Ralston 2001).

In another hand, lipoic acid (LA) is known to be a powerful thiol antioxidant which has the distinction of being effective in its reduced and oxidized form. It reduces oxidative stress by scavenging free radicals and preventing lipid peroxidation and protein damage through regeneration of vitamins C and E, and induced increased intracellular glutathione. LA is not, *per se*, a fatty acid, but a derivative of octanoic acid, a saturated fatty acid. α-LA has been described *in vitro* and *in vivo* to inhibit osteoclastogenesis through suppression of NF-κB signaling. In fact, proteomic analysis identified that RANKL is significantly up-regulated both in ARNm and protein in bone marrow stromal cells. Nevertheless, despite an increasing ratio RANKL/OPG, α-LA abolished osteoclast formation through suppression of RANKL induced-ROS elevation, thus inhibiting NF-κB activation in osteoclast precursor cells. Moreover, while IKK activation is not affected, α-LA reduces DNA binding of NF-κB (Koh, Lee et al. 2005; Kim, Chang et al. 2006). Besides, recent *in vivo* study has shown that LA supplementation (0,1%) to HFD during 12 weeks in mouse model is associated to decrease ROS generation and oxidative damage compared to HFD-group, and attenuate the HFD-induced inhibition of bone formation (Xiao, Cui et al. 2010).

In parallel, CLnA isomers could exert antioxidant activities in response to oxidative stress through reduction lipid peroxidation and improvement of antioxidant enzyme levels. Recent study has showed that punicic acid and α-eleostearic acid, mainly found respectively in pomegranate and china wood oil, exhibit protective effects against lipid peroxidation induced by arsenite in brain and in liver in rat model. However, according to its concentration, punicic acid seems have both pro-oxidant and antioxidant effect (Saha and Ghosh 2011). Few hypothesis are proposed to explain this antioxidant property; among them addition of free

radical to one of the conjugated double bonds of CLnA might have taken place, resulting in the formation of conjugated dienes that could have possibly acted as antioxidants. Moreover, it has been showed that CLA decrease of iNOS ARNm expression in Raw264.7 (Yu, Adams et al. 2002). These data suggest that CLA could have *in vivo* antioxidant activity in bone cell lineage. Taken together, these results strongly support the importance of both lipids and oxidized lipids on oxidative stress and related bone remodeling.

F - Fatty acid receptors

Metabolites deriving from free fatty acids might not be the only ones responsible for mediating effects of fatty acids on bone remodeling. For the last decade, different receptors including nuclear and membrane-bound receptors have been extensively described for their involment in transducing a specific signal when interacting with free fatty acids. The mainly documented receptors belong to the PPARs, TLRs and GPRs receptor families.

1. PPARs

PPARs are nuclear receptors with high affinity for fatty acids and act as transcription factors to regulate peroxysome proliferation and subsequent lipid metabolism. Besides its role as well known target for antidiabetic drug therapies (Berger, Bailey et al. 1996), PPARγ has been extensively studied for its crucial role in mensemchymal cells commitment and adipocyte differentiation. Interestingly, bone cells such as osteoblasts share a common precursor with adipocyte and several groups have speculated that PPARs stimulation by fatty acids may control the switch between adipocyte, myoblast and osteoblast differentiation. Diascro et coll. were the first to demonstrate that a mixture of palmitic, oleic, and linoleic acids, activate the PPARs and induce adipocyte-like differentiation of both ROS17/2.8 and SaOS-2/B10 pre-osteoblast cell lines suggesting that fatty acids can initiate the switch from osteoblasts to adipocyte-like cells (Diascro, Vogel et al. 1998). Molecular mechanisms responsible for this unbalance were later described. At a cellular level, a fatty acid rich environment was proven to stimulate PPARγ activity while drastically reducing Runx2 expression and function, a transcription factor responsible for inducing osteoblastogenesis, in MC3T3-E1 and primary osteoblast precursors (Maurin, Chavassieux et al. 2005) (Jeon, Kim et al. 2003). Taken together, these data strongly support that our food habits in terms of fatty acids intake may have a dramatic impact on bone metabolism through PPARs activation.

As bone remodeling results from the balance between osteoblast and osteoclast activities, PPARs influence on osteoclastogenesis was investigated as well. Using non-discriminately activator of all PPAR isoforms, Chan et al. demonstrated an overall inhibition of the formation of multinucleated osteoclasts from PBMC cultures suggesting a detrimental role of PPARs in osteoclastogenesis (Chan, Gartland et al. 2007). These data parallel with the inhibition of TNF-α-induced osteoclastogenesis upon PPARγ activation (Hounoki, Sugiyama et al. 2008). Nevertheless, the role of PPARs remains controversial. As a matter of fact,

antisense strategies for PPARδ/β were shown to abrogate the increase in pit area induced by carbaprostacyclin in mature primary rabbit osteoclasts suggesting pro-resorbing activity of PPARδ/β stimulation (Mano, Kimura et al. 2000). Thus, these data lead to the hypothesis that PPARs effects on bone may depend on PPAR type. In another hand, given the potent inhibitory effect of PPARγ on both osteoblast and osteoclast, one might finally wonder what could be the global impact of these fatty acid receptors on bone physiology. Recently, the role of PPARγ was further investigated using heterozygous PPARγ deficient mice (Viccica, Francucci et al. 2010). This model exhibits enhanced bone formation with increased osteoblastogenesis and reduced marrow adiposity. In another hand, Wan et al. uncovered a pro-osteoclastogenic effect of PPARγ by using a Tie2Cre/flox mouse model in which PPARγ is deleted in osteoclasts but not in osteoblasts suggesting that observed inhibitory effects of PPARγ agonist on osteoclast differentiation might be due to non specific effects (Wan, Chong et al. 2007). To conclude, PPARs studies make more complex the potent impact of fatty on bone through receptor signaling. This complexity is even strengthened by the fact that PPARs ligands are not restrited to fatty acids binding but can be extended to fatty acids metabolites such as prostaglandins and their degradation products (Still, Grabowski et al. 2008).

2. TLRs

In drosophilia Toll receptors contribute to host defense and body development. In mammalians homologues of Toll, Toll-like receptors (TLRs) are known to play a crucial role in the immune response. Nevertheless TLRs were recently acknowledged for binding saturated fatty acid and activate different intracellular signaling pathways (Hwang 2001). These interactions have been mainly described in the establishment of metabolic syndrome (Shi, Kokoeva et al. 2006). Indeed, mice model invalidated for TLR2 and 4 were shown to be protected against induced inflammation, increased adiposity and insulin resistance upon enriched saturated fatty acids diet (Himes and Smith; Coenen, Gruen et al. 2009).

Interestingly, in bone tissues, Johson et al. reported that mice deficient for TLR4 exhibit increased bone size with increased bone mineral content and density (Johnson, Riggs et al. 2004). Therefore, one might hypothesize that TLR4 could represent an original target in the design of nutritional strategies for the treatment of osteoporosis. The underlying mechanisms remain to be elucidated, however, a recent study by Oh et al. reveals that saturated fatty acids, binding TLR4 at the cell surface of osteoclasts, caused an increased of MIP-1a expression thus leading to increased activation of NF-κB (Oh, Sul et al. 2009). This transcription factor is essential for osteoclast differentiation and survival suggesting that

elevated levels of SFAs in bone marrow plasma may enhance osteoclast behavior and contribute to altered bone structure during obesity. These data are correlated with the role described for TLR2 and TLR4 in Raw264.7 cells. In the presence of FFA mixture containing equimolar amounts of arachidonic, lauric, linoleic, oleic, and myristic acids, these monocytes/osteoclasts precursor cells present increased pro-inflammatory signals through TLR2/TLR4/JNK pathways (Nguyen, Favelyukis et al. 2007). Given the contribution of inflammation in osteoclast differentiation and function, this study enlightens potent molecular mechanisms to explain the bone phenotype observed in TLR4-/- mice.

In addition, cellular responses to TLRs activation by SFAs were found to be modulated depending on the type of fatty acid. DHA (an ω-3 PUFA) abrogated the pro-inflammatory effects of lauric acid-induced TLR2/4 activation in Raw264.7 cells (Lee, Zhao et al. 2004). Nevertheless, these demonstrations are still debated. Indeed, in contrast to Oh's group, Cornish et al. showed an inhibition of osteoclast differentiation upon SFAs treatment (Cornish, MacGibbon et al. 2008). Then, despite a few discrepancies, these different studies they have contributed to reveal a key new role for TLRs in the fat and bone metabolism interactions and give new insight in the design of new nutritional strategies in bone loss prevention.

3. GPRs

While searching for novel human galanin receptor (GALR) subtypes, Sawzdargo et al. discovered in 1997 a cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1 (Sawzdargo, George et al. 1997). These four genes GPR40, 41, 42 and 43 were first considered as orphan receptors. In 2003, two teams demonstrated that GPR40, 41 and 43 were able to interact with free fatty acids and to transduce a specific signal across the cell membrane. Using Ca(2+) mobilization probes and cellular models transfected with either GPR40, 41 or 43, Brown et al. showed that short chain fatty acids activated GPR41 and GPR43 while Briscoe demonstrated that GPR40 activation was restricted to medium and long chain fatty acids (Briscoe, Tadayyon et al. 2003; Brown, Goldsworthy et al. 2003). More recently, CLAs were added to the list of LCFAs ligands of GPR40 by Stewart and Schmidt's groups (Schmidt, Liebscher et al. 2011).

Regarding GPR120, this receptor was firstly described in 2003 by Fredriksson et al. (Fredriksson, Hoglund et al. 2003) while its ability to interact with fatty acids was established in 2005 (Hirasawa, Tsumaya et al. 2005) (Katsuma, Hatae et al. 2005).

The roles of these fatty acids receptors were mainly documented in pancreatic tissues for their involvement in β-cells functions (Covington, Briscoe et al. 2006). Indeed, free fatty acids were found to regulate insulin secretion from pancreatic beta cells through GPR40 (Itoh, Kawamata et al. 2003). Using GPR40 deficient mice, Latour et al. lately showed that GPR40 stimulation contribute to fatty acid potentiation of glucose induced insulin secretion (Latour, Alquier et al. 2007). More recently, oxidative stress was proven as a key protagonist in the control of insulin secretion by palmitate through GPR40 stimulation (Graciano, Santos et al. 2011).

As expected regarding their role in pancreatic islets functions, GPRs expression was also evidenced in other tissues related to food metabolism for their role in nutra-sensing and fat storage. As a matter of fact, GPR40 and 120 were shown to mediate fatty acid sensing in rat taste bud as well as taste preference (Cartoni, Yasumatsu et al. 2010) (Matsumura, Mizushige et al. 2007). More interestingly, GPR120 but not GPR40 were found to modulate the apoptotic process of enteroendocrine cells (Katsuma, Hatae et al. 2005) while GPRs for short-chain fatty acids are believed to mediate suppression of colon cancer development (Tang, Chen et al. 2011).

In the same way, adipose tissues were also investigated. Using adipocytes culture models, GPR41 and 43 were shown to be key molecular targets mediating leptin production and lipolysis regulation upon short-chain fatty acid stimulation, respectively (Xiong, Miyamoto et al. 2004; Ge, Li et al. 2008). In parallel, Gotoh et al. demonstrated that GPR120 expression increases during adipocyte differentiation and, using RNA interference protocols, established that GPR120 stimulation controls adipogenic processes (Gotoh, Hong et al. 2007).

Besides their roles in fat metabolism, literature data evidenced the expression of these G-coupled fatty acids receptors in a wide range of tissues. Recent studies demonstrated that spatial memory and hippocampal long-term potentiation of rodents and cognitive function of humans are improved by a dietary supplementation with arachidonic and/or docosahexaenoic acids, which are possible ligands for GPR40 (Ma, Tao et al. 2007). Since, a growing body of evidence has enlightened the involvement of GPR40 stimulation by fatty acids in neuronal development. For instance, DHA was shown to enhance neuronal differentiation of rat neural stem cells transfected with GPR40 gene (Ma, Zhang et al. 2010). The same year, Kaplamadzh et al. demonstrated in adult primate BMSC, that DHA led to upregulation of neuronal markers such as beta III-tubulin, NF-M and Map2 through GPR40 signaling (Kaplamadzhiev, Hisha et al. 2010).

In another hand, G-coupled fatty acids receptors are expressed by immune cells such as leukocytes and monocytes (Nilsson, Kotarsky et al. 2003) (Oh, Talukdar et al. 2010). As bone tissues share common precursor cells with both the hematopoietic and the mesenchymal lineage, GPRs expression were investigated in bone cells. In 2008, Cornish et al. revealed the expression of GPR120 in both osteoclast and osteoblast and hypothesized that part of saturated fatty acid influence on osteoclastogenesis might occur through GPR120 stimulation (Cornish, MacGibbon et al. 2008). However, as described above, effects of fatty acids on bone remains quite controversy and according to the literature neither GPR120 nor GPR40 allow distinguishing among long chain fatty acids and their associated effects. Taken together these data suggest that GPRs stimulation by fatty acid cannot explain by themselves the global effects of fatty acids on bone. In our hand we hypothesized that GPR40 counteracts bone loss associated with detrimental properties of lipids and demonstrated that GPR40 is a key modulator of bone remodeling (ASBMR 2011). Our data correlate with a recent study published by Oh et al. Using the monocytic RAW264.7 cell line and primary intraperitoneal macrophages they demonstrated that DHA exerts anti-inflammatory effects through GPR120 stimulation (Oh, Talukdar et al. 2010). As Raw264.7 cells present the ability to differentiate into osteoclasts, their data strengthen our hypothesis and support our observations.

G – Conclusions

According to the statistics, obesity occurrence has reached an alarming rate. Due to its high morbitity, and consequently its high social and economic cost, this “overweight” pathology has become one of the main targets of health systems across industrialized countries.

In contrast to what has been previously thought, obesity is rather associated with bone tissue alteration which in return leads to higher disability and mortality, most notably in the elderly population. However, as described in the manuscript fat and bone are not systematically antonymic and should rather be seen as ”best enemies”. Indeed, while high fat diets, as any over excesses, may account for poor bone health, quality of the fatty acids within the diet may exhibit a duality of action regarding their impact on bone remodeling; PUFAs ω-3 being rather protecting while SFAs or PUFAs ω-6 being potentially harmful. In addition, location may also be crucial for determining fat impact on bone as waist circumference is thought to be more closely related to poor bone health as compared to global body overweight.

Therefore, with regards to this duality, an appropriate fatty acids modulation in daily food intake may succeed where drug based therapies have failed and thus representing a relevant opportunity in the design of nutritional approaches to attenuate excessive bone loss. In this context, public health systems have strongly supported communication plans aiming at informing and sensitizing population about diet recommendations resulting from the research trends in nutrition field. However, despite a few consistent lines, the “take home message” from the literature is quite confusing and somehow remains controversial as results may differ depending on the design of the studies. Indeed, as described in the manuscript, the fatty acids modes of action are rather complex due to the several processes involved that may counteract each other such as eicosanoid production and receptor activation. In addition, the potent beneficial effect may differ upon the targeted organ and “what’s good for” bone may not be the case for another tissue when taking into account all the fatty acid-induced signals.

In another hand, according to the public health system recommendations, food industry has made a few efforts to reduce saturated and trans fatty acids in its production. Nevertheless, these efforts represent an obvious conflict of interest and although industrials have widely claimed for “healthy foods”, fats still remain the “gold ingredient” to improve products sensory of “cheap products” and therefore to boost product sales and industry

financial reports. In the same way, high quality nutritional products remain quite expensive and challenge everyone savings thus limiting their access for the global population.

Finally, although promising, nutritional approaches should not be considered as drug-based strategies. Indeed, “this is not a matter of taking only ω-3 pills to get better”. In fact, nutritional concepts require balanced daily intakes. As mentioned above, linoleic and linolenic both are essential fatty acids which ratio should be kept between 3:1 to 5:1, in order to optimize beneficial health impact. In addition, while clinical studies tend to draw general concepts for food recommendations, evidences show that a same diet may exhibit different physiological impacts. Indeed, a growing body of data has enlightened the concept of metabolic profiles making the way for the nutrigenomic (Wittwer, Rubio-Aliaga et al. 2011). In this context, it becomes obvious that appropriate nutritional strategies will now require more than general guidelines.

References

Adachi, J. D., S. Adami, et al. (2010). "Impact of prevalent fractures on quality of life: baseline results from the global longitudinal study of osteoporosis in women." Mayo Clin Proc **85**(9): 806-13.

Ahn, I. S., B. H. Choi, et al. (2006). "Isomer-specific effect of conjugated linoleic acid on inflammatory adipokines associated with fat accumulation in 3T3-L1 adipocytes." J Med Food **9**(3): 307-12.

Akhter, M. P., D. M. Cullen, et al. (2001). "Bone biomechanical properties in prostaglandin EP1 and EP2 knockout mice." Bone **29**(2): 121-5.

Akhter, M. P., D. M. Cullen, et al. (2006). "Bone biomechanical properties in EP4 knockout mice." Calcif Tissue Int **78**(6): 357-62.

Akune, T., N. Ogata, et al. (2002). "Insulin receptor substrate-2 maintains predominance of anabolic function over catabolic function of osteoblasts." J Cell Biol **159**(1): 147-56.

Alam, A. S., B. S. Moonga, et al. (1993). "Amylin inhibits bone resorption by a direct effect on the motility of rat osteoclasts." Exp Physiol **78**(2): 183-96.

Alander, C. B. and L. G. Raisz (2006). "Effects of selective prostaglandins E2 receptor agonists on cultured calvarial murine osteoblastic cells." Prostaglandins Other Lipid Mediat **81**(3-4): 178-83.

Albertazzi, P. and K. Coupland (2002). "Polyunsaturated fatty acids. Is there a role in postmenopausal osteoporosis prevention?" Maturitas **42**(1): 13-22.

Alfin Slater, R. B. and B. Bernick (1958). "Changes in Tissue Lipids and Tissue Histology Resulting from Essential Fatty Acid Deficiency in Rats." Am J Clin Nutr **6**(6): 613-624.

Ali, T., D. Lam, et al. (2009). "Osteoporosis in inflammatory bowel disease." Am J Med **122**(7): 599-604.

Amano, S., K. Naganuma, et al. (1996). "Prostaglandin E2 stimulates osteoclast formation via endogenous IL-1 beta expressed through protein kinase A." J Immunol **156**(5): 1931-6.

Ammann, P., R. Rizzoli, et al. (1997). "Transgenic mice expressing soluble tumor necrosis factor-receptor are protected against bone loss caused by estrogen deficiency." J Clin Invest **99**(7): 1699-703.

Anderson, S. R., D. A. Gilge, et al. (2008). "Diet-induced obesity alters protein synthesis: tissue-specific effects in fasted versus fed mice." Metabolism **57**(3): 347-54.

Aoki, Y., S. Ichimura, et al. (2005). "Overexpression of the human interleukin 1a gene causes osteopenia in mice." J Rheumatol **32**(2): 320-4.

Appleton, K. M., W. D. Fraser, et al. (2011). "Supplementation with a low-moderate dose of n-3 long-chain PUFA has no short-term effect on bone resorption in human adults." Br J Nutr **105**(8): 1145-9.

Arikan, D. C., A. Coskun, et al. (2011). "Plasma Selenium, Zinc, Copper and Lipid Levels in Postmenopausal Turkish Women and Their Relation with Osteoporosis." Biol Trace Elem Res.

Asagiri, M. and H. Takayanagi (2007). "The molecular understanding of osteoclast differentiation." Bone **40**(2): 251-64.

Azevedo-Martins, A. K., A. P. Monteiro, et al. (2006). "Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells." Toxicol In Vitro **20**(7): 1106-13.

Azuma, Y., K. Kaji, et al. (2000). "Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts." J Biol Chem **275**(7): 4858-64.

Bai, X. C., D. Lu, et al. (2005). "Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast." J Biol Chem **280**(17): 17497-506.
Balage, M., S. Sinaud, et al. (2001). "Amino acids and insulin are both required to regulate assembly of the eIF4E. eIF4G complex in rat skeletal muscle." Am J Physiol Endocrinol Metab **281**(3): E565-74.
Banni, S. P., A. Petroni, et al. (2004). "Conjugated Linoleic Acids (CLA) as Precursors of a Distinct Family of PUFA." Lipids **39**(11): 1143-1146.
Banu, J., A. Bhattacharya, et al. (2010). "Endogenously produced n-3 fatty acids protect against ovariectomy induced bone loss in fat-1 transgenic mice." J Bone Miner Metab **28**(6): 617-26.
Barcelli, U. O., M. Weiss, et al. (1990). "High linoleic acid diets ameliorate diabetic nephropathy in rats." Am J Kidney Dis **16**(3): 244-51.
Bassey, E. J., J. J. Littlewood, et al. (2000). "Lack of effect of supplementation with essential fatty acids on bone mineral density in healthy pre- and postmenopausal women: two randomized controlled trials of Efacal v. calcium alone." Br J Nutr **83**(6): 629-35.
Basu, S. (2010). "Bioactive eicosanoids: role of prostaglandin F(2alpha) and F-isoprostanes in inflammation and oxidative stress related pathology." Mol Cells **30**(5): 383-91.
Basu, S., K. Michaelsson, et al. (2001). "Association between oxidative stress and bone mineral density." Biochem Biophys Res Commun **288**(1): 275-9.
Beckman, K. B. and B. N. Ames (1998). "The free radical theory of aging matures." Physiol Rev **78**(2): 547-81.
Beeharry, N., J. E. Lowe, et al. (2003). "Linoleic acid and antioxidants protect against DNA damage and apoptosis induced by palmitic acid." Mutat Res **530**(1-2): 27-33.
Belfort, R., L. Mandarino, et al. (2005). "Dose-response effect of elevated plasma free fatty acid on insulin signaling." Diabetes **54**(6): 1640-8.
Bellenger, J., S. Bellenger, et al. (2011). "High Pancreatic n-3 Fatty Acids Prevent STZ-Induced Diabetes in Fat-1 Mice: Inflammatory Pathway Inhibition." Diabetes.
Berger, J., P. Bailey, et al. (1996). "Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in db/db mice." Endocrinology **137**(10): 4189-95.
Berner, H. S., S. P. Lyngstadaas, et al. (2004). "Adiponectin and its receptors are expressed in bone-forming cells." Bone **35**(4): 842-9.
Bernstein, C. N., M. Sargent, et al. (2005). "Serum osteoprotegerin is increased in Crohn's disease: a population-based case control study." Inflamm Bowel Dis **11**(4): 325-30.
Bhattacharya, A., M. Rahman, et al. (2007). "Effect of fish oil on bone mineral density in aging C57BL/6 female mice." J Nutr Biochem **18**(6): 372-9.
Bhattacharya, A., D. Sun, et al. (2007). "Different ratios of eicosapentaenoic and docosahexaenoic omega-3 fatty acids in commercial fish oils differentially alter pro-inflammatory cytokines in peritoneal macrophages from C57BL/6 female mice." J Nutr Biochem **18**(1): 23-30.
Bianchi, M. L. (2010). "Inflammatory bowel diseases, celiac disease, and bone." Arch Biochem Biophys **503**(1): 54-65.
Biran, L. A., W. Bartley, et al. (1964). "Studies on essential fatty acid deficiency. Effect of the deficiency on the lipids in various rat tissues and the influence of dietary supplementation with essential fatty acids on deficient rats." Biochem J **93**(3): 492-8.
Blackwell, K. A., L. G. Raisz, et al. (2010). "Prostaglandins in bone: bad cop, good cop?" Trends Endocrinol Metab **21**(5): 294-301.
Blanaru, J. L., J. R. Kohut, et al. (2004). "Dose response of bone mass to dietary arachidonic acid in piglets fed cow milk-based formula." Am J Clin Nutr **79**(1): 139-47.

Blum, M., S. S. Harris, et al. (2003). "Leptin, body composition and bone mineral density in premenopausal women." Calcif Tissue Int **73**(1): 27-32.
Bollag, R. J., Q. Zhong, et al. (2001). "Glucose-dependent insulinotropic peptide is an integrative hormone with osteotropic effects." Mol Cell Endocrinol **177**(1-2): 35-41.
Bollag, R. J., Q. Zhong, et al. (2000). "Osteoblast-derived cells express functional glucose-dependent insulinotropic peptide receptors." Endocrinology **141**(3): 1228-35.
Bordin, L., G. Priante, et al. (2003). "Arachidonic acid-induced IL-6 expression is mediated by PKC alpha activation in osteoblastic cells." Biochemistry **42**(15): 4485-91.
Borland, V. G. and C. M. Jackson (1931). "Effects of a fat-free diet on the structure of the kidney in rats." Arch Pathol **11**: 687-708.
Bouillon, R., M. Bex, et al. (1995). "Influence of age, sex, and insulin on osteoblast function: osteoblast dysfunction in diabetes mellitus." J Clin Endocrinol Metab **80**(4): 1194-202.
Briaud, I., C. L. Kelpe, et al. (2002). "Differential effects of hyperlipidemia on insulin secretion in islets of langerhans from hyperglycemic versus normoglycemic rats." Diabetes **51**(3): 662-8.
Briscoe, C. P., M. Tadayyon, et al. (2003). "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids." J Biol Chem **278**(13): 11303-11.
Bronsky, J. and R. Prusa (2004). "Amylin fasting plasma levels are decreased in patients with osteoporosis." Osteoporos Int **15**(3): 243-7.
Brooks, J. D., E. S. Musiek, et al. (2011). "The Fatty Acid Oxidation Product 15-A(3t) - Isoprostane is a Potent Inhibitor Of NFkappaB Transcription and Macrophage Transformation." J Neurochem.
Brown, A. J., S. M. Goldsworthy, et al. (2003). "The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids." J Biol Chem **278**(13): 11312-9.
Brownlee, M., H. Vlassara, et al. (1984). "Nonenzymatic glycosylation and the pathogenesis of diabetic complications." Ann Intern Med **101**(4): 527-37.
Bruunsgaard, H., M. Pedersen, et al. (2001). "Aging and proinflammatory cytokines." Curr Opin Hematol **8**(3): 131-6.
Burghardt, P. R., E. S. Kemmerer, et al. (2010). "Dietary n-3:n-6 fatty acid ratios differentially influence hormonal signature in a rodent model of metabolic syndrome relative to healthy controls." Nutr Metab (Lond) **7**: 53.
Busch, A. K., D. Cordery, et al. (2002). "Expression profiling of palmitate- and oleate-regulated genes provides novel insights into the effects of chronic lipid exposure on pancreatic beta-cell function." Diabetes **51**(4): 977-87.
Calder, P. C. (2001). "Polyunsaturated fatty acids, inflammation, and immunity." Lipids **36**(9): 1007-24.
Calder, P. C. (2006). "n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases." Am J Clin Nutr **83**(6 Suppl): 1505S-1519S.
Calder, P. C. (2006). "Polyunsaturated fatty acids and inflammation." Prostaglandins Leukot Essent Fatty Acids **75**(3): 197-202.
Calder, P. C. and P. Yaqoob (2009). "Omega-3 polyunsaturated fatty acids and human health outcomes." Biofactors **35**(3): 266-72.
Capanni, M., F. Calella, et al. (2006). "Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study." Aliment Pharmacol Ther **23**(8): 1143-51.
Cappelli, P., L. Di Liberato, et al. (1997). "N-3 polyunsaturated fatty acid supplementation in chronic progressive renal disease." J Nephrol **10**(3): 157-62.
Carmiel-Haggai, M., A. I. Cederbaum, et al. (2005). "A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats." Faseb J **19**(1): 136-8.

Carnielli, V. P., I. H. Luijendijk, et al. (1996). "Structural position and amount of palmitic acid in infant formulas: effects on fat, fatty acid, and mineral balance." J Pediatr Gastroenterol Nutr **23**(5): 553-60.
Cartoni, C., K. Yasumatsu, et al. (2010). "Taste preference for fatty acids is mediated by GPR40 and GPR120." J Neurosci **30**(25): 8376-82.
Cartwright-Shamoon, J. M., J. A. Dodge, et al. (1995). "A complex biochemical modulation of intestinal ion transport in rats fed on high-fat diets." J Pediatr Gastroenterol Nutr **20**(1): 36-43.
Cemborain, A., I. Castilla-Cortazar, et al. (1998). "Osteopenia in rats with liver cirrhosis: beneficial effects of IGF-I treatment." J Hepatol **28**(1): 122-31.
Chan, B. Y., A. Gartland, et al. (2007). "PPAR agonists modulate human osteoclast formation and activity in vitro." Bone **40**(1): 149-59.
Chan, C. B., D. De Leo, et al. (2001). "Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action." Diabetes **50**(6): 1302-10.
Chen, J. R., O. P. Lazarenko, et al. (2010). "Obesity reduces bone density associated with activation of PPARgamma and suppression of Wnt/beta-catenin in rapidly growing male rats." PLoS One **5**(10): e13704.
Chen, Q., J. Y. Rho, et al. (2003). "Congenital lack of COX-2 affects mechanical and geometric properties of bone in mice." Calcif Tissue Int **73**(4): 387-92.
Chin, H. J., Y. Y. Fu, et al. (2010). "Omacor, n-3 polyunsaturated fatty acid, attenuated albuminuria and renal dysfunction with decrease of SREBP-1 expression and triglyceride amount in the kidney of type II diabetic animals." Nephrol Dial Transplant **25**(5): 1450-7.
Claassen, N., H. C. Potgieter, et al. (1995). "Supplemented gamma-linolenic acid and eicosapentaenoic acid influence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyproline, and bone calcium content." Bone **16**(4 Suppl): 385S-392S.
Cnop, M. (2008). "Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes." Biochem Soc Trans **36**(Pt 3): 348-52.
Coenen, K. R., M. L. Gruen, et al. (2009). "Impact of macrophage toll-like receptor 4 deficiency on macrophage infiltration into adipose tissue and the artery wall in mice." Diabetologia **52**(2): 318-28.
Coetzee, M., M. Haag, et al. (2007). "Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E(2) on cell proliferation and morphology of MG-63 and MC3T3-E1 osteoblast-like cells." Prostaglandins Leukot Essent Fatty Acids **76**(1): 35-45.
Coetzer, H., N. Claassen, et al. (1994). "Calcium transport by isolated brush border and basolateral membrane vesicles: role of essential fatty acid supplementation." Prostaglandins Leukot Essent Fatty Acids **50**(5): 257-66.
Cohen, S. L. and W. E. Ward (2005). "Flaxseed oil and bone development in growing male and female mice." J Toxicol Environ Health A **68**(21): 1861-70.
Cornish, J., K. E. Callon, et al. (2002). "Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo." J Endocrinol **175**(2): 405-15.
Cornish, J., K. E. Callon, et al. (2007). "Preptin, another peptide product of the pancreatic beta-cell, is osteogenic in vitro and in vivo." Am J Physiol Endocrinol Metab **292**(1): E117-22.
Cornish, J., K. E. Callon, et al. (1995). "Amylin stimulates osteoblast proliferation and increases mineralized bone volume in adult mice." Biochem Biophys Res Commun **207**(1): 133-9.

Cornish, J., K. E. Callon, et al. (1998). "Systemic administration of amylin increases bone mass, linear growth, and adiposity in adult male mice." Am J Physiol **275**(4 Pt 1): E694-9.
Cornish, J., K. E. Callon, et al. (1999). "Comparison of the effects of calcitonin gene-related peptide and amylin on osteoblasts." J Bone Miner Res **14**(8): 1302-9.
Cornish, J., K. E. Callon, et al. (1998). "Dissociation of the effects of amylin on osteoblast proliferation and bone resorption." Am J Physiol **274**(5 Pt 1): E827-33.
Cornish, J., K. E. Callon, et al. (1996). "Insulin increases histomorphometric indices of bone formation In vivo." Calcif Tissue Int **59**(6): 492-5.
Cornish, J., A. MacGibbon, et al. (2008). "Modulation of osteoclastogenesis by fatty acids." Endocrinology **149**(11): 5688-95.
Corwin, R. L. (2003). "Effects of dietary fats on bone health in advanced age." Prostaglandins Leukot Essent Fatty Acids **68**(6): 379-86.
Corwin, R. L., T. J. Hartman, et al. (2006). "Dietary saturated fat intake is inversely associated with bone density in humans: analysis of NHANES III." J Nutr **136**(1): 159-65.
Covington, D. K., C. A. Briscoe, et al. (2006). "The G-protein-coupled receptor 40 family (GPR40-GPR43) and its role in nutrient sensing." Biochem Soc Trans **34**(Pt 5): 770-3.
Crepaldi, G., G. Romanato, et al. (2007). "Osteoporosis and body composition." J Endocrinol Invest **30**(6 Suppl): 42-7.
Dacquin, R., R. A. Davey, et al. (2004). "Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo." J Cell Biol **164**(4): 509-14.
Dam, T. T., S. Harrison, et al. (2010). "Bone mineral density and fractures in older men with chronic obstructive pulmonary disease or asthma." Osteoporos Int **21**(8): 1341-9.
De Benedetti, F., N. Rucci, et al. (2006). "Impaired skeletal development in interleukin-6-transgenic mice: a model for the impact of chronic inflammation on the growing skeletal system." Arthritis Rheum **54**(11): 3551-63.
De Laet, C., J. A. Kanis, et al. (2005). "Body mass index as a predictor of fracture risk: a meta-analysis." Osteoporos Int **16**(11): 1330-8.
Delhanty, P. J., B. C. van der Eerden, et al. (2006). "Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a." J Endocrinol **188**(1): 37-47.
Delhanty, P. J., A. van Kerkwijk, et al. (2010). "Unsaturated fatty acids prevent desensitization of the human growth hormone secretagogue receptor by blocking its internalization." Am J Physiol Endocrinol Metab **299**(3): E497-505.
Deng, F., J. Ling, et al. (2008). "Stimulation of intramembranous bone repair by ghrelin." Exp Physiol.
Dhayal, S., H. J. Welters, et al. (2008). "Structural requirements for the cytoprotective actions of mono-unsaturated fatty acids in the pancreatic beta-cell line, BRIN-BD11." Br J Pharmacol **153**(8): 1718-27.
Diascro, D. D., Jr., R. L. Vogel, et al. (1998). "High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells." J Bone Miner Res **13**(1): 96-106.
Ding, J., O. Ghali, et al. (2009). "TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells." Life Sci **84**(15-16): 499-504.
Ding, K. H., X. M. Shi, et al. (2008). "Impact of glucose-dependent insulinotropic peptide on age-induced bone loss." J Bone Miner Res **23**(4): 536-43.

Dodin, S., A. Lemay, et al. (2005). "The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: a randomized, double-blind, wheat germ placebo-controlled clinical trial." J Clin Endocrinol Metab **90**(3): 1390-7.
Droge, W. (2002). "Free radicals in the physiological control of cell function." Physiol Rev **82**(1): 47-95.
Drury, B., L. J. Warford-Woolgar, et al. (2009). "Dietary trans-10, cis-12 conjugated linoleic acid reduces early glomerular enlargement and elevated renal cyclooxygenase-2 levels in young obese fa/fa zucker rats." J Nutr **139**(2): 285-90.
Ducy, P., M. Amling, et al. (2000). "Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass." Cell **100**(2): 197-207.
Duque, G. (2008). "Bone and fat connection in aging bone." Curr Opin Rheumatol **20**(4): 429-34.
Ealey, K. N., J. Kaludjerovic, et al. (2008). "Adiponectin is a negative regulator of bone mineral and bone strength in growing mice." Exp Biol Med (Maywood) **233**(12): 1546-53.
Ebal, E., H. Cavalie, et al. (2007). "Effect of a lipid-enriched diet on body composition and some regulatory hormones of food intake in growing rats." Ann Endocrinol (Paris) **68**(5): 366-71.
Edwards, J. R. and G. R. Mundy (2011). "Advances in osteoclast biology: old findings and new insights from mouse models." Nat Rev Rheumatol **7**(4): 235-43.
Eitel, K., H. Staiger, et al. (2002). "Different role of saturated and unsaturated fatty acids in beta-cell apoptosis." Biochem Biophys Res Commun **299**(5): 853-6.
El-Assaad, W., J. Buteau, et al. (2003). "Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death." Endocrinology **144**(9): 4154-63.
Elefteriou, F., J. D. Ahn, et al. (2005). "Leptin regulation of bone resorption by the sympathetic nervous system and CART." Nature **434**(7032): 514-20.
Elks, M. L. (1993). "Chronic perifusion of rat islets with palmitate suppresses glucose-stimulated insulin release." Endocrinology **133**(1): 208-14.
Ellrichmann, M., M. Kapelle, et al. (2008). "Orlistat inhibition of intestinal lipase acutely increases appetite and attenuates postprandial glucagon-like peptide-1-(7-36)-amide-1, cholecystokinin, and peptide YY concentrations." J Clin Endocrinol Metab **93**(10): 3995-8.
Enc, F. Y., T. Ones, et al. (2009). "Orlistat accelerates gastric emptying and attenuates GIP release in healthy subjects." Am J Physiol Gastrointest Liver Physiol **296**(3): G482-9.
Farhat, G. N., J. A. Cauley, et al. (2006). "Volumetric BMD and vascular calcification in middle-aged women: the Study of Women's Health Across the Nation." J Bone Miner Res **21**(12): 1839-46.
Farina, E. K., D. P. Kiel, et al. (2011). "Protective effects of fish intake and interactive effects of long-chain polyunsaturated fatty acid intakes on hip bone mineral density in older adults: the Framingham Osteoporosis Study." Am J Clin Nutr **93**(5): 1142-51.
Feldstein, A. E., N. W. Werneburg, et al. (2004). "Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway." Hepatology **40**(1): 185-94.
Felson, D. T., Y. Zhang, et al. (1993). "Effects of weight and body mass index on bone mineral density in men and women: the Framingham study." J Bone Miner Res **8**(5): 567-73.
Fernandes, G., R. Lawrence, et al. (2003). "Protective role of n-3 lipids and soy protein in osteoporosis." Prostaglandins Leukot Essent Fatty Acids **68**(6): 361-72.

Flanagan, A. M. and T. J. Chambers (1992). "Stimulation of bone nodule formation in vitro by prostaglandins E1 and E2." Endocrinology **130**(1): 443-8.
Fredriksson, R., P. J. Hoglund, et al. (2003). "Seven evolutionarily conserved human rhodopsin G protein-coupled receptors lacking close relatives." FEBS Lett **554**(3): 381-8.
Fujiwara, K., F. Maekawa, et al. (2005). "Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release." Am J Physiol Endocrinol Metab **289**(4): E670-7.
Fukushima, N., R. Hanada, et al. (2005). "Ghrelin directly regulates bone formation." J Bone Miner Res **20**(5): 790-8.
Fuller, K., C. Murphy, et al. (2002). "TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL." Endocrinology **143**(3): 1108-18.
Fulzele, K., D. J. DiGirolamo, et al. (2007). "Disruption of the insulin-like growth factor type 1 receptor in osteoblasts enhances insulin signaling and action." J Biol Chem **282**(35): 25649-58.
Furstova, V., T. Kopska, et al. (2008). "Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic beta-cell line NES2Y." Life Sci **82**(13-14): 684-91.
Gallego-Rojo, F. J., J. L. Gonzalez-Calvin, et al. (1998). "Bone mineral density, serum insulin-like growth factor I, and bone turnover markers in viral cirrhosis." Hepatology **28**(3): 695-9.
Galli, C. and P. C. Calder (2009). "Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review." Ann Nutr Metab **55**(1-3): 123-39.
Galli, C., G. Passeri, et al. (2011). "FoxOs, Wnts and oxidative stress-induced bone loss: new players in the periodontitis arena?" J Periodontal Res **46**(4): 397-406.
Gan, W. Q., S. F. Man, et al. (2004). "Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis." Thorax **59**(7): 574-80.
Gao, Q., M. Xu, et al. (2009). "Effects of prostaglandin E2 on bone in mice in vivo." Prostaglandins Other Lipid Mediat **89**(1-2): 20-5.
Ge, H., X. Li, et al. (2008). "Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids." Endocrinology **149**(9): 4519-26.
Gebauer, S. K., T. L. Psota, et al. (2007). "The diversity of health effects of individual trans fatty acid isomers." Lipids **42**(9): 787-99.
Genaro, P. S., G. A. Pereira, et al. (2010). "Influence of body composition on bone mass in postmenopausal osteoporotic women." Arch Gerontol Geriatr **51**(3): 295-8.
Giaccari, A., G. Sorice, et al. (2009). "Glucose toxicity: the leading actor in the pathogenesis and clinical history of type 2 diabetes - mechanisms and potentials for treatment." Nutr Metab Cardiovasc Dis **19**(5): 365-77.
Gilbert, L., X. He, et al. (2002). "Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha." J Biol Chem **277**(4): 2695-701.
Gillingham, L. G., S. Harris-Janz, et al. (2011). "Dietary Monounsaturated Fatty Acids Are Protective Against Metabolic Syndrome and Cardiovascular Disease Risk Factors."
Gilman, J. and K. D. Cashman (2007). "The effect of marine oil-derived n-3 fatty acids on transepithelial calcium transport in Caco-2 cell models of healthy and inflamed intestines." Br J Nutr **97**(2): 281-8.

Gimble, J. M., C. E. Robinson, et al. (1996). "The function of adipocytes in the bone marrow stroma: an update." Bone **19**(5): 421-8.
Gimble, J. M., S. Zvonic, et al. (2006). "Playing with bone and fat." J Cell Biochem **98**(2): 251-66.
Giouleme, O. I., T. A. Vyzantiadis, et al. (2006). "Pathogenesis of osteoporosis in liver cirrhosis." Hepatogastroenterology **53**(72): 938-43.
Gniuli, D., L. Dalla Libera, et al. (2008). "High saturated-fat diet induces apoptosis in rat enterocytes and blunts GIP and insulin-secretive response to oral glucose load." Int J Obes (Lond) **32**(5): 871-4.
Gordeladze, J. O., C. A. Drevon, et al. (2002). "Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling." J Cell Biochem **85**(4): 825-36.
Gotoh, C., Y. H. Hong, et al. (2007). "The regulation of adipogenesis through GPR120." Biochem Biophys Res Commun **354**(2): 591-7.
Graat-Verboom, L., E. F. Wouters, et al. (2009). "Current status of research on osteoporosis in COPD: a systematic review." Eur Respir J **34**(1): 209-18.
Graciano, M. F., L. R. Santos, et al. (2011). "NAD(P)H oxidase participates in the palmitate-induced superoxide production and insulin secretion by rat pancreatic islets." J Cell Physiol **226**(4): 1110-7.
Graham, L. S., F. Parhami, et al. (2009). "Oxidized lipids enhance RANKL production by T lymphocytes: implications for lipid-induced bone loss." Clin Immunol **133**(2): 265-75.
Green, K. H., S. C. Wong, et al. (2004). "The effect of dietary n-3 long-chain polyunsaturated fatty acids on femur mineral density and biomarkers of bone metabolism in healthy, diabetic and dietary-restricted growing rats." Prostaglandins Leukot Essent Fatty Acids **71**(2): 121-30.
Griel, A. E., P. M. Kris-Etherton, et al. (2007). "An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans." Nutr J **6**: 2.
Guanabens, N. and A. Pares (2010). "Liver and bone." Arch Biochem Biophys **503**(1): 84-94.
Guardado-Mendoza, R., A. M. Davalli, et al. (2009). "Pancreatic islet amyloidosis, beta-cell apoptosis, and alpha-cell proliferation are determinants of islet remodeling in type-2 diabetic baboons." Proc Natl Acad Sci U S A **106**(33): 13992-7.
Gudbrandsen, O. A., E. Rodriguez, et al. (2009). "Trans-10, cis-12-conjugated linoleic acid reduces the hepatic triacylglycerol content and the leptin mRNA level in adipose tissue in obese Zucker fa/fa rats." Br J Nutr **102**(6): 803-15.
Guesnet, P., J. M. Alessandri, et al. (2005). "Les rôles physiologiques majeurs exercés par les acides gras polyinsaturés (AGPI)."
Guidobono, F., F. Pagani, et al. (2006). "Different skeletal regional response to continuous brain infusion of leptin in the rat." Peptides **27**(6): 1426-33.
Guilherme, A., J. V. Virbasius, et al. (2008). "Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes." Nat Rev Mol Cell Biol **9**(5): 367-77.
Haag, M., O. N. Magada, et al. (2003). "Omega-3 fatty acids modulate ATPases involved in duodenal Ca absorption." Prostaglandins Leukot Essent Fatty Acids **68**(6): 423-9.
Halade, G. V., A. El Jamali, et al. (2011). "Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice." Exp Gerontol **46**(1): 43-52.
Halade, G. V., M. M. Rahman, et al. (2010). "High fat diet-induced animal model of age-associated obesity and osteoporosis." J Nutr Biochem **21**(12): 1162-9.
Halade, G. V., M. M. Rahman, et al. (2011). "Combination of conjugated linoleic acid with fish oil prevents age-associated bone marrow adiposity in C57Bl/6J mice." J Nutr Biochem **22**(5): 459-69.

Halliwell, B. and J. M. Gutteridge (1984). "Free radicals, lipid peroxidation, and cell damage." Lancet **2**(8411): 1095.
Hamrick, M. W., C. Pennington, et al. (2004). "Leptin deficiency produces contrasting phenotypes in bones of the limb and spine." Bone **34**(3): 376-83.
Handjieva-Darlenska, T. and N. Boyadjieva (2009). "The effect of high-fat diet on plasma ghrelin and leptin levels in rats." J Physiol Biochem **65**(2): 157-64.
Hao, W., O. Y. Wong, et al. (2010). "omega-3 fatty acids suppress inflammatory cytokine production by macrophages and hepatocytes." J Pediatr Surg **45**(12): 2412-8.
Hara, T., A. Hirasawa, et al. (2009). "Novel selective ligands for free fatty acid receptors GPR120 and GPR40." Naunyn Schmiedebergs Arch Pharmacol **380**(3): 247-55.
Hasturk, H., A. Kantarci, et al. (2007). "Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo." J Immunol **179**(10): 7021-9.
Hasturk, H., A. Kantarci, et al. (2006). "RvE1 protects from local inflammation and osteoclast- mediated bone destruction in periodontitis." Faseb J **20**(2): 401-3.
Hawrylowicz, C. M. and A. O'Garra (2005). "Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma." Nat Rev Immunol **5**(4): 271-83.
He, X., G. Andersson, et al. (2010). "Resveratrol prevents RANKL-induced osteoclast differentiation of murine osteoclast progenitor RAW 264.7 cells through inhibition of ROS production." Biochem Biophys Res Commun **401**(3): 356-62.
Herrera, B. S., T. Ohira, et al. (2008). "An endogenous regulator of inflammation, resolvin E1, modulates osteoclast differentiation and bone resorption." Br J Pharmacol **155**(8): 1214-23.
Hikiji, H., T. Takato, et al. (2008). "The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease." Prog Lipid Res **47**(2): 107-26.
Himes, R. W. and C. W. Smith "Tlr2 is critical for diet-induced metabolic syndrome in a murine model." Faseb J **24**(3): 731-9.
Hirasawa, A., K. Tsumaya, et al. (2005). "Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120." Nat Med **11**(1): 90-4.
Hofbauer, L. C., D. L. Lacey, et al. (1999). "Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells." Bone **25**(3): 255-9.
Hogstrom, M., P. Nordstrom, et al. (2007). "n-3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the NO2 Study." Am J Clin Nutr **85**(3): 803-7.
Horcajada-Molteni, M. N., B. Chanteranne, et al. (2001). "Amylin and bone metabolism in streptozotocin-induced diabetic rats." J Bone Miner Res **16**(5): 958-65.
Horcajada-Molteni, M. N., M. J. Davicco, et al. (2000). "Amylin inhibits ovariectomy-induced bone loss in rats." J Endocrinol **165**(3): 663-8.
Hounoki, H., E. Sugiyama, et al. (2008). "Activation of peroxisome proliferator-activated receptor gamma inhibits TNF-alpha-mediated osteoclast differentiation in human peripheral monocytes in part via suppression of monocyte chemoattractant protein-1 expression." Bone **42**(4): 765-74.
Hsu, Y. H., S. A. Venners, et al. (2006). "Relation of body composition, fat mass, and serum lipids to osteoporotic fractures and bone mineral density in Chinese men and women." Am J Clin Nutr **83**(1): 146-54.
Huda, M. S., B. H. Durham, et al. (2007). "Lack of an acute effect of ghrelin on markers of bone turnover in healthy controls and post-gastrectomy subjects." Bone **41**(3): 406-13.
Hunter, J. E. (2006). "Dietary trans fatty acids: review of recent human studies and food industry responses." Lipids **41**(11): 967-92.

Hussein, O., M. Grosovski, et al. (2007). "Monounsaturated fat decreases hepatic lipid content in non-alcoholic fatty liver disease in rats." World J Gastroenterol **13**(3): 361-8.
Hwang, D. (2001). "Modulation of the expression of cyclooxygenase-2 by fatty acids mediated through toll-like receptor 4-derived signaling pathways." Faseb J **15**(14): 2556-64.
Irwin, R., H. V. Lin, et al. (2006). "Normal bone density obtained in the absence of insulin receptor expression in bone." Endocrinology **147**(12): 5760-7.
Islander, U., C. Jochems, et al. (2011). "Estrogens in rheumatoid arthritis; the immune system and bone." Mol Cell Endocrinol **335**(1): 14-29.
Itoh, Y., Y. Kawamata, et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-6.
James, W. P. (2008). "WHO recognition of the global obesity epidemic." Int J Obes (Lond) **32 Suppl 7**: S120-6.
Jeon, M. J., J. A. Kim, et al. (2003). "Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts." J Biol Chem **278**(26): 23270-7.
Jeong, I. K., S. W. Cho, et al. (2010). "Lipid profiles and bone mineral density in pre- and postmenopausal women in Korea." Calcif Tissue Int **87**(6): 507-12.
Janssen-Heininger, Y. M., B. T. Mossman, et al. (2008). "Redox-based regulation of signal transduction: principles, pitfalls, and promises." Free Radic Biol Med **45**(1): 1-17.
Ji, R. R., Z. Z. Xu, et al. (2011). "Emerging roles of resolvins in the resolution of inflammation and pain." Trends Neurosci.
Johnson, G. B., B. L. Riggs, et al. (2004). "A genetic basis for the "Adonis" phenotype of low adiposity and strong bones." Faseb J **18**(11): 1282-4.
Jorgensen, N. R. and P. Schwarz (2008). "Osteoporosis in chronic obstructive pulmonary disease patients." Curr Opin Pulm Med **14**(2): 122-7.
Joseph, J. W., V. Koshkin, et al. (2002). "Uncoupling protein 2 knockout mice have enhanced insulin secretory capacity after a high-fat diet." Diabetes **51**(11): 3211-9.
Kajikawa, S., T. Harada, et al. (2009). "Suppression of hepatic fat accumulation by highly purified eicosapentaenoic acid prevents the progression of d-galactosamine-induced hepatitis in mice fed with a high-fat/high-sucrose diet." Biochim Biophys Acta **1791**(4): 281-8.
Kalupahana, N. S., K. Claycombe, et al. (2010). "Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation." J Nutr **140**(11): 1915-22.
Kanazawa, I., T. Yamaguchi, et al. (2007). "Adiponectin and AMP kinase activator stimulate proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells." BMC Cell Biol **8**: 51.
Kaneki, H., R. Guo, et al. (2006). "Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts." J Biol Chem **281**(7): 4326-33.
Kang, J. X., J. Wang, et al. (2004). "Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids." Nature **427**(6974): 504.
Kaplamadzhiev, D. B., H. Hisha, et al. (2010). "Bone marrow-derived stromal cells can express neuronal markers by DHA/GPR40 signaling." Biosci Trends **4**(3): 119-29.
Katsuma, S., N. Hatae, et al. (2005). "Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1." J Biol Chem **280**(20): 19507-15.
Kawai, T., H. Hirose, et al. (2001). "Chronic effects of different fatty acids and leptin in INS-1 cells." Diabetes Res Clin Pract **51**(1): 1-8.

Kawelke, N., A. Bentmann, et al. (2008). "Isoform of fibronectin mediates bone loss in patients with primary biliary cirrhosis by suppressing bone formation." J Bone Miner Res **23**(8): 1278-86.
Kelpe, C. L., P. C. Moore, et al. (2003). "Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis." J Biol Chem **278**(32): 30015-21.
Kennedy, A., K. Martinez, et al. (2009). "Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications." J Nutr **139**(1): 1-4.
Khosla, S. (2001). "Minireview: the OPG/RANKL/RANK system." Endocrinology **142**(12): 5050-5.
Kim, H. J., E. J. Chang, et al. (2006). "Antioxidant alpha-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-kappaB DNA binding and prevents in vivo bone resorption induced by receptor activator of nuclear factor-kappaB ligand and tumor necrosis factor-alpha." Free Radic Biol Med **40**(9): 1483-93.
Kim, H. J., K. T. Lee, et al. (2008). "Dietary docosahexaenoic acid-rich diacylglycerols ameliorate hepatic steatosis and alter hepatic gene expressions in C57BL/6J-Lep(ob/ob) mice." Mol Nutr Food Res **52**(8): 965-73.
Kim, W. K., K. Ke, et al. (2011). "Curcumin protects against ovariectomy-induced bone loss and decreases osteoclastogenesis." J Cell Biochem.
Kim, Y. and J. Z. Ilich (2011). "Implications of dietary alpha-linolenic acid in bone health." Nutrition.
Kimball, S. R., R. L. Horetsky, et al. (1998). "Signal transduction pathways involved in the regulation of protein synthesis by insulin in L6 myoblasts." Am J Physiol **274**(1 Pt 1): C221-8.
Kimble, R. B., A. B. Matayoshi, et al. (1995). "Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period." Endocrinology **136**(7): 3054-61.
Kitaura, H., M. S. Sands, et al. (2004). "Marrow stromal cells and osteoclast precursors differentially contribute to TNF-alpha-induced osteoclastogenesis in vivo." J Immunol **173**(8): 4838-46.
Kitaura, H., P. Zhou, et al. (2005). "M-CSF mediates TNF-induced inflammatory osteolysis." J Clin Invest **115**(12): 3418-27.
Klein, R. F., J. Allard, et al. (2004). "Regulation of bone mass in mice by the lipoxygenase gene Alox15." Science **303**(5655): 229-32.
Kobayashi, Y., T. Mizoguchi, et al. (2005). "Prostaglandin E2 enhances osteoclastic differentiation of precursor cells through protein kinase A-dependent phosphorylation of TAK1." J Biol Chem **280**(12): 11395-403.
Kobayashi, Y., I. Take, et al. (2005). "Prostaglandin E2 receptors EP2 and EP4 are down-regulated during differentiation of mouse osteoclasts from their precursors." J Biol Chem **280**(25): 24035-42.
Koh, J. M., Y. S. Lee, et al. (2005). "Alpha-lipoic acid suppresses osteoclastogenesis despite increasing the receptor activator of nuclear factor kappaB ligand/osteoprotegerin ratio in human bone marrow stromal cells." J Endocrinol **185**(3): 401-13.
Komine, M., A. Kukita, et al. (2001). "Tumor necrosis factor-alpha cooperates with receptor activator of nuclear factor kappaB ligand in generation of osteoclasts in stromal cell-depleted rat bone marrow cell culture." Bone **28**(5): 474-83.
Koo, W. W., M. Hammami, et al. (2003). "Reduced bone mineralization in infants fed palm olein-containing formula: a randomized, double-blinded, prospective trial." Pediatrics **111**(5 Pt 1): 1017-23.

Kozawa, O., A. Suzuki, et al. (1998). "Interleukin-6 synthesis induced by prostaglandin E2: cross-talk regulation by protein kinase C." Bone **22**(4): 355-60.
Krabbe, K. S., M. Pedersen, et al. (2004). "Inflammatory mediators in the elderly." Exp Gerontol **39**(5): 687-99.
Kraegen, E. W. and G. J. Cooney (2008). "Free fatty acids and skeletal muscle insulin resistance." Curr Opin Lipidol **19**(3): 235-41.
Krarup, T., J. J. Holst, et al. (1985). "Responses and molecular heterogeneity of IR-GIP after intraduodenal glucose and fat." Am J Physiol **249**(2 Pt 1): E195-200.
Kriel, M., A. Sayers, et al. (2010). "IL-6 may modulate the skeletal response to glucocorticoids during exacerbations of inflammatory bowel disease." Calcif Tissue Int **86**(5): 375-81.
Kruger, M. C., M. Coetzee, et al. (2010). "Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone." Prog Lipid Res **49**(4): 438-49.
Kruger, M. C., H. Coetzer, et al. (1995). "Eicosapentaenoic acid and docosahexaenoic acid suppementation increases calcium balance." Nutr Res **15**(2): 211-219.
Kruger, M. C. and D. F. Horrobin (1997). "Calcium metabolism, osteoporosis and essential fatty acids: a review." Prog Lipid Res **36**(2-3): 131-51.
Kruger, M. C. and L. M. Schollum (2005). "Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability?" Prostaglandins Leukot Essent Fatty Acids **73**(5): 327-34.
Kume, S., T. Uzu, et al. (2007). "Role of altered renal lipid metabolism in the development of renal injury induced by a high-fat diet." J Am Soc Nephrol **18**(10): 2715-23.
Kwan Tat, S., M. Padrines, et al. (2004). "IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology." Cytokine Growth Factor Rev **15**(1): 49-60.
Lacey, D. C., P. J. Simmons, et al. (2009). "Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation." Osteoarthritis Cartilage **17**(6): 735-42.
Lam, J., S. Takeshita, et al. (2000). "TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand." J Clin Invest **106**(12): 1481-8.
Lameloise, N., P. Muzzin, et al. (2001). "Uncoupling protein 2: a possible link between fatty acid excess and impaired glucose-induced insulin secretion?" Diabetes **50**(4): 803-9.
Lamghari, M., L. Tavares, et al. (2006). "Leptin effect on RANKL and OPG expression in MC3T3-E1 osteoblasts." J Cell Biochem **98**(5): 1123-9.
Laneuville, O., D. K. Breuer, et al. (1995). "Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)- octadecatrienoic acids from alpha-linolenic acid." J Biol Chem **270**(33): 19330-6.
Latour, M. G., T. Alquier, et al. (2007). "GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo." Diabetes **56**(4): 1087-94.
Lau, B. Y., W. E. Ward, et al. (2009). "Femur EPA and DHA are correlated with femur biomechanical strength in young fat-1 mice." J Nutr Biochem **20**(6): 453-61.
Laybutt, D. R., A. M. Preston, et al. (2007). "Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes." Diabetologia **50**(4): 752-63.
Lee, J. Y., L. Zhao, et al. (2004). "Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1." J Biol Chem **279**(17): 16971-9.
Legrand, P. (2007). "Les acides gras: structures, fonctions, apports nutritionnels conseillés." Cah Nutr Diét **42**(Hors série 1): 1S7-1S12.

Leibbrandt, A. and J. M. Penninger (2008). "RANK/RANKL: regulators of immune responses and bone physiology." Ann N Y Acad Sci **1143**: 123-50.
Lencel, P. and D. Magne (2011). "Inflammaging: the driving force in osteoporosis?" Med Hypotheses **76**(3): 317-21.
Lenchik, L., T. C. Register, et al. (2003). "Adiponectin as a novel determinant of bone mineral density and visceral fat." Bone **33**(4): 646-51.
Leveille, S. G., A. Z. LaCroix, et al. (1997). "Dietary vitamin C and bone mineral density in postmenopausal women in Washington State, USA." J Epidemiol Community Health **51**(5): 479-85.
Li, F., C. H. Wang, et al. (2010). "Elevated tissue factor expression contributes to exacerbated diabetic nephropathy in mice lacking eNOS fed a high fat diet." J Thromb Haemost **8**(10): 2122-32.
Li, M., D. R. Healy, et al. (2005). "Osteopenia and impaired fracture healing in aged EP4 receptor knockout mice." Bone **37**(1): 46-54.
Li, X., Y. Okada, et al. (2000). "Knockout of the murine prostaglandin EP2 receptor impairs osteoclastogenesis in vitro." Endocrinology **141**(6): 2054-61.
Li, Y., A. Li, et al. (2007). "Endogenous TNFalpha lowers maximum peak bone mass and inhibits osteoblastic Smad activation through NF-kappaB." J Bone Miner Res **22**(5): 646-55.
Li, Y., M. F. Seifert, et al. (1999). "Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids." J Bone Miner Res **14**(7): 1153-62.
Lieber, C. S. (2000). "Alcoholic liver disease: new insights in pathogenesis lead to new treatments." J Hepatol **32**(1 Suppl): 113-28.
Liu, D., H. P. Veit, et al. (2004). "Effects of long-term dietary lipids on mature bone mineral content, collagen, crosslinks, and prostaglandin E2 production in Japanese quail." Poult Sci **83**(11): 1876-83.
Liu, D., H. P. Veit, et al. (2003). "Long-term supplementation of various dietary lipids alters bone mineral content, mechanical properties and histological characteristics of Japanese quail." Poult Sci **82**(5): 831-9.
Lorenzo, J. A., A. Naprta, et al. (1998). "Mice lacking the type I interleukin-1 receptor do not lose bone mass after ovariectomy." Endocrinology **139**(6): 3022-5.
Lu, J., N. Bankovic-Calic, et al. (2003). "Detrimental effects of a high fat diet in early renal injury are ameliorated by fish oil in Han:SPRD-cy rats." J Nutr **133**(1): 180-6.
Lu, X., L. Gilbert, et al. (2006). "Transcriptional regulation of the osterix (Osx, Sp7) promoter by tumor necrosis factor identifies disparate effects of mitogen-activated protein kinase and NF kappa B pathways." J Biol Chem **281**(10): 6297-306.
Lucia, V. D., S. C. Fitzpatrick-Wong, et al. (2003). "Dietary arachidonic acid suppresses bone turnover in contrast to low dosage exogenous prostaglandin E2 that elevates bone formation in the piglet." Prostaglandins Leukot Essent Fatty Acids **68**: 407-413.
Lukas, R., J. C. Gigliotti, et al. (2011). "Consumption of different sources of omega-3 polyunsaturated fatty acids by growing female rats affects long bone mass and microarchitecture." Bone **49**(3): 455-62.
Lundman, P., S. Boquist, et al. (2007). "A high-fat meal is accompanied by increased plasma interleukin-6 concentrations." Nutr Metab Cardiovasc Dis **17**(3): 195-202.
Luo, X. H., L. J. Guo, et al. (2006). "Adiponectin stimulates RANKL and inhibits OPG expression in human osteoblasts through the MAPK signaling pathway." J Bone Miner Res **21**(10): 1648-56.
Luo, X. H., L. J. Guo, et al. (2005). "Adiponectin stimulates human osteoblasts proliferation and differentiation via the MAPK signaling pathway." Exp Cell Res **309**(1): 99-109.

Lupi, R., F. Dotta, et al. (2002). "Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated." Diabetes **51**(5): 1437-42.
Ma, D., B. Tao, et al. (2007). "Expression of free fatty acid receptor GPR40 in the central nervous system of adult monkeys." Neurosci Res **58**(4): 394-401.
Ma, D., M. Zhang, et al. (2010). "DHA promotes the neuronal differentiation of rat neural stem cells transfected with GPR40 gene." Brain Res.
Ma, Z. and G. T. Westermark (2002). "Effects of free fatty acid on polymerization of islet amyloid polypeptide (IAPP) in vitro and on amyloid fibril formation in cultivated isolated islets of transgenic mice overexpressing human IAPP." Mol Med **8**(12): 863-8.
Maccarinelli, G., V. Sibilia, et al. (2005). "Ghrelin regulates proliferation and differentiation of osteoblastic cells." J Endocrinol **184**(1): 249-56.
Macdonald, H. M., S. A. New, et al. (2004). "Nutritional associations with bone loss during the menopausal transition: evidence of a beneficial effect of calcium, alcohol, and fruit and vegetable nutrients and of a detrimental effect of fatty acids." Am J Clin Nutr **79**(1): 155-65.
Maedler, K., J. Oberholzer, et al. (2003). "Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function." Diabetes **52**(3): 726-33.
Maedler, K., G. A. Spinas, et al. (2001). "Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function." Diabetes **50**(1): 69-76.
Maestre, I., J. Jordan, et al. (2003). "Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1." Endocrinology **144**(1): 335-45.
Mano, H., C. Kimura, et al. (2000). "Cloning and function of rabbit peroxisome proliferator-activated receptor delta/beta in mature osteoclasts." J Biol Chem **275**(11): 8126-32.
Manolagas, S. C. (2010). "From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis." Endocr Rev **31**(3): 266-300.
Marcovitz, P. A., H. H. Tran, et al. (2005). "Usefulness of bone mineral density to predict significant coronary artery disease." Am J Cardiol **96**(8): 1059-63.
Martin, A., V. David, et al. (2007). "Opposite effects of leptin on bone metabolism: a dose-dependent balance related to energy intake and insulin-like growth factor-I pathway." Endocrinology **148**(7): 3419-25.
Martin, A., R. de Vittoris, et al. (2005). "Leptin modulates both resorption and formation while preventing disuse-induced bone loss in tail-suspended female rats." Endocrinology **146**(8): 3652-9.
Martinez-Ramirez, M. J., S. Palma, et al. (2007). "Dietary fat intake and the risk of osteoporotic fractures in the elderly." Eur J Clin Nutr **61**(9): 1114-20.
Mathias, S., L. A. Pena, et al. (1998). "Signal transduction of stress via ceramide." Biochem J **335 (Pt 3)**: 465-80.
Matsumura, S., T. Mizushige, et al. (2007). "GPR expression in the rat taste bud relating to fatty acid sensing." Biomed Res **28**(1): 49-55.
Maurin, A. C., P. M. Chavassieux, et al. (2005). "Expression of PPARgamma and beta/delta in human primary osteoblastic cells: influence of polyunsaturated fatty acids." Calcif Tissue Int **76**(5): 385-92.
McCabe, L. R. (2007). "Understanding the pathology and mechanisms of type I diabetic bone loss." J Cell Biochem **102**(6): 1343-57.

McGavock, J. M., R. G. Victor, et al. (2006). "Adiposity of the heart, revisited." Ann Intern Med **144**(7): 517-24.
Melhus, H., K. Michaelsson, et al. (1999). "Smoking, antioxidant vitamins, and the risk of hip fracture." J Bone Miner Res **14**(1): 129-35.
Merrill, A. R., P. Proulx, et al. (1986). "Effects of exogenous fatty acids on calcium uptake by brush-border membrane vesicles from rabbit small intestine." Biochim Biophys Acta **855**(3): 337-44.
Metz, S. A. (1988). "Exogenous arachidonic acid promotes insulin release from intact or permeabilized rat islets by dual mechanisms. Putative activation of Ca2+ mobilization and protein kinase C." Diabetes **37**(11): 1453-69.
Micha, R. (2010). "Saturated Fat and Cardiometabolic Risk Factors, Coronary Heart Disease, Stroke, and Diabetes: a Fresh Look at the Evidence." Lipids **45**: 893-905.
Michel, F., D. Bonnefont-Rousselot, et al. (2008). "[Biomarkers of lipid peroxidation: analytical aspects]." Ann Biol Clin (Paris) **66**(6): 605-20.
Millet, I., T. L. McCarthy, et al. (1998). "Regulation of interleukin-6 production by prostaglandin E2 in fetal rat osteoblasts: role of protein kinase A signaling pathway." J Bone Miner Res **13**(7): 1092-100.
Minamizaki, T., Y. Yoshiko, et al. (2009). "EP2 and EP4 receptors differentially mediate MAPK pathways underlying anabolic actions of prostaglandin E2 on bone formation in rat calvaria cell cultures." Bone **44**(6): 1177-85.
Miyawaki, K., Y. Yamada, et al. (1999). "Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice." Proc Natl Acad Sci U S A **96**(26): 14843-7.
Moesgaard, S. G., B. Ahren, et al. (2004). "Effects of high-fat feeding and fasting on ghrelin expression in the mouse stomach." Regul Pept **120**(1-3): 261-7.
Mollard, R. C., H. R. Kovacs, et al. (2005). "Low levels of dietary arachidonic and docosahexaenoic acids improve bone mass in neonatal piglets, but higher levels provide no benefit." J Nutr **135**(3): 505-12.
Moore, K. W., R. de Waal Malefyt, et al. (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.
Moore, P. C., M. A. Ugas, et al. (2004). "Evidence against the involvement of oxidative stress in fatty acid inhibition of insulin secretion." Diabetes **53**(10): 2610-6.
Morrow, J. D. and L. J. Roberts, 2nd (1996). "The isoprostanes. Current knowledge and directions for future research." Biochem Pharmacol **51**(1): 1-9.
Moschen, A. R., A. Kaser, et al. (2005). "The RANKL/OPG system is activated in inflammatory bowel disease and relates to the state of bone loss." Gut **54**(4): 479-87.
Mundy, G. R. (2007). "Osteoporosis and inflammation." Nutr Rev **65**(12 Pt 2): S147-51.
Munoz-Torres, M., E. Jodar, et al. (1996). "Bone mineral density measured by dual X-ray absorptiometry in Spanish patients with insulin-dependent diabetes mellitus." Calcif Tissue Int **58**(5): 316-9.
Musiek, E. S., L. Gao, et al. (2005). "Cyclopentenone isoprostanes inhibit the inflammatory response in macrophages." J Biol Chem **280**(42): 35562-70.
Muthusami, S., I. Ramachandran, et al. (2005). "Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats." Clin Chim Acta **360**(1-2): 81-6.
Myers, L. K., S. D. Bhattacharya, et al. (2006). "The isozyme-specific effects of cyclooxygenase-deficiency on bone in mice." Bone **39**(5): 1048-52.
Nagao, K., N. Inoue, et al. (2005). "Dietary conjugated linoleic acid alleviates nonalcoholic fatty liver disease in Zucker (fa/fa) rats." J Nutr **135**(1): 9-13.

Nagasumi, K., R. Esaki, et al. (2009). "Overexpression of GPR40 in pancreatic beta-cells augments glucose-stimulated insulin secretion and improves glucose tolerance in normal and diabetic mice." Diabetes **58**(5): 1067-76.
Nagata, T., K. Kaho, et al. (1994). "Effect of prostaglandin E2 on mineralization of bone nodules formed by fetal rat calvarial cells." Calcif Tissue Int **55**(6): 451-7.
Nakamura, M. T., H. P. Cho, et al. (2001). "Metabolism and functions of highly unsaturated fatty acids: an update." Lipids **36**(9): 961-4.
Nanes, M. S. (2003). "Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology." Gene **321**: 1-15.
Nanji, A. A., K. Jokelainen, et al. (2001). "Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines." Am J Physiol Gastrointest Liver Physiol **281**(6): G1348-56.
Nanji, A. A., C. L. Mendenhall, et al. (1989). "Beef fat prevents alcoholic liver disease in the rat." Alcohol Clin Exp Res **13**(1): 15-9.
Nanji, A. A., D. Zakim, et al. (1997). "Dietary saturated fatty acids down-regulate cyclooxygenase-2 and tumor necrosis factor alfa and reverse fibrosis in alcohol-induced liver disease in the rat." Hepatology **26**(6): 1538-45.
Naot, D. and J. Cornish (2008). "The role of peptides and receptors of the calcitonin family in the regulation of bone metabolism." Bone **43**(5): 813-8.
Napoli, N., C. Pedone, et al. (2010). "Adiponectin and bone mass density: The InCHIANTI study." Bone **47**(6): 1001-5.
Nass, R., S. S. Pezzoli, et al. (2008). "Effects of an oral ghrelin mimetic on body composition and clinical outcomes in healthy older adults: a randomized trial." Ann Intern Med **149**(9): 601-11.
Negre-Salvayre, A., N. Auge, et al. "Pathological aspects of lipid peroxidation." Free Radic Res **44**(10): 1125-71.
Nguyen, M. T., S. Favelyukis, et al. (2007). "A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways." J Biol Chem **282**(48): 35279-92.
Nilsson, N. E., K. Kotarsky, et al. (2003). "Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids." Biochem Biophys Res Commun **303**(4): 1047-52.
Nobili, V., G. Bedogni, et al. (2010). "Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial." Arch Dis Child **96**(4): 350-3.
Nolan, C. J., M. S. Madiraju, et al. (2006). "Fatty acid signaling in the beta-cell and insulin secretion." Diabetes **55 Suppl 2**: S16-23.
Noto, A., P. Zahradka, et al. (2007). "Dietary conjugated linoleic acid preserves pancreatic function and reduces inflammatory markers in obese, insulin-resistant rats." Metabolism **56**(1): 142-51.
Nuche-Berenguer, B., P. Moreno, et al. (2009). "Effect of GLP-1 treatment on bone turnover in normal, type 2 diabetic, and insulin-resistant states." Calcif Tissue Int **84**(6): 453-61.
O'Garra, A., F. J. Barrat, et al. (2008). "Strategies for use of IL-10 or its antagonists in human disease." Immunol Rev **223**: 114-31.
Obara, N., K. Fukushima, et al. (2010). "Possible involvement and the mechanisms of excess trans-fatty acid consumption in severe NAFLD in mice." J Hepatol **53**(2): 326-34.
Ogata, N., D. Chikazu, et al. (2000). "Insulin receptor substrate-1 in osteoblast is indispensable for maintaining bone turnover." J Clin Invest **105**(7): 935-43.

Ogborn, M. R., E. Nitschmann, et al. (2003). "Dietary conjugated linoleic acid reduces PGE2 release and interstitial injury in rat polycystic kidney disease." Kidney Int **64**(4): 1214-21.
Oh, D. Y., S. Talukdar, et al. (2010). "GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects." Cell **142**(5): 687-98.
Oh, S. R., O. J. Sul, et al. (2009). "Saturated fatty acids enhance osteoclast survival." J Lipid Res.
Okamura, D. M., S. Pennathur, et al. (2009). "CD36 regulates oxidative stress and inflammation in hypercholesterolemic CKD." J Am Soc Nephrol **20**(3): 495-505.
Olofsson, C. S., S. Collins, et al. (2007). "Long-term exposure to glucose and lipids inhibits glucose-induced insulin secretion downstream of granule fusion with plasma membrane." Diabetes **56**(7): 1888-97.
Ono, K., H. Kaneko, et al. (2005). "Biphasic effect of prostaglandin E2 on osteoclast formation in spleen cell cultures: role of the EP2 receptor." J Bone Miner Res **20**(1): 23-9.
Orchard, T. S., J. A. Cauley, et al. (2010). "Fatty acid consumption and risk of fracture in the Women's Health Initiative." Am J Clin Nutr **92**(6): 1452-60.
Oster, R. T., J. M. Tishinsky, et al. (2010). "Docosahexaenoic acid increases cellular adiponectin mRNA and secreted adiponectin protein, as well as PPARgamma mRNA, in 3T3-L1 adipocytes." Appl Physiol Nutr Metab **35**(6): 783-9.
Ouchi, N., S. Kihara, et al. (2000). "Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway." Circulation **102**(11): 1296-301.
Pacifici, R., C. Brown, et al. (1991). "Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells." Proc Natl Acad Sci U S A **88**(12): 5134-8.
Paganelli, M., C. Albanese, et al. (2007). "Inflammation is the main determinant of low bone mineral density in pediatric inflammatory bowel disease." Inflamm Bowel Dis **13**(4): 416-23.
Papadimitriou, A., A. J. King, et al. (2007). "Anti-apoptotic effects of arachidonic acid and prostaglandin E2 in pancreatic beta-cells." Cell Physiol Biochem **20**(5): 607-16.
Parhami, F., Y. Tintut, et al. (2001). "Atherogenic high-fat diet reduces bone mineralization in mice." J Bone Miner Res **16**(1): 182-8.
Park, Y. G., S. K. Kang, et al. (2004). "PGE2 induces IL-1beta gene expression in mouse osteoblasts through a cAMP-PKA signaling pathway." Int Immunopharmacol **4**(6): 779-89.
Parker, H. E., A. M. Habib, et al. (2009). "Nutrient-dependent secretion of glucose-dependent insulinotropic polypeptide from primary murine K cells." Diabetologia **52**(2): 289-98.
Patsch, J. M., F. W. Kiefer, et al. (2011). "Increased bone resorption and impaired bone microarchitecture in short-term and extended high-fat diet-induced obesity." Metabolism **60**(2): 243-9.
Payette, H., N. Hanusaik, et al. (1998). "Muscle strength and functional mobility in relation to lean body mass in free-living frail elderly women." Eur J Clin Nutr **52**(1): 45-53.
Pedrini, M. T., M. Kranebitter, et al. (2005). "Human triglyceride-rich lipoproteins impair glucose metabolism and insulin signalling in L6 skeletal muscle cells independently of non-esterified fatty acid levels." Diabetologia **48**(4): 756-66.
Peng, X. D., H. Xie, et al. (2008). "Relationships between serum adiponectin, leptin, resistin, visfatin levels and bone mineral density, and bone biochemical markers in Chinese men." Clin Chim Acta **387**(1-2): 31-5.

Perez-Matute, P., A. Marti, et al. (2007). "Conjugated linoleic acid inhibits glucose metabolism, leptin and adiponectin secretion in primary cultured rat adipocytes." Mol Cell Endocrinol **268**(1-2): 50-8.
Perez-Matute, P., J. A. Martinez, et al. (2007). "Linoleic acid decreases leptin and adiponectin secretion from primary rat adipocytes in the presence of insulin." Lipids **42**(10): 913-20.
Perez de Heredia, F., J. Sanchez, et al. (2009). "Adiponectin is associated with serum and adipose tissue fatty acid composition in rats." J Endocrinol Invest **32**(8): 659-65.
Pfeilschifter, J., R. Koditz, et al. (2002). "Changes in proinflammatory cytokine activity after menopause." Endocr Rev **23**(1): 90-119.
Pieber, T. R., J. Roitelman, et al. (1994). "Direct plasma radioimmunoassay for rat amylin-(1-37): concentrations with acquired and genetic obesity." Am J Physiol **267**(1 Pt 1): E156-64.
Piro, S., M. Anello, et al. (2002). "Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress." Metabolism **51**(10): 1340-7.
Platt, I. and A. El-Sohemy (2009). "Effects of 9cis,11trans and 10trans,12cis CLA on osteoclast formation and activity from human CD14+ monocytes." Lipids Health Dis **8**: 15.
Poirier, H., J. S. Shapiro, et al. (2006). "Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue." Diabetes **55**(6): 1634-41.
Poitout, V., D. Hagman, et al. (2006). "Regulation of the insulin gene by glucose and fatty acids." J Nutr **136**(4): 873-6.
Poitout, V. and R. P. Robertson (2008). "Glucolipotoxicity: fuel excess and beta-cell dysfunction." Endocr Rev **29**(3): 351-66.
Poli, V., R. Balena, et al. (1994). "Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion." Embo J **13**(5): 1189-96.
Poulsen, R. C., K. H. Gotlinger, et al. (2008). "Identification of inflammatory and proresolving lipid mediators in bone marrow and their lipidomic profiles with ovariectomy and omega-3 intake." Am J Hematol **83**(6): 437-45.
Poulsen, R. C. and M. C. Kruger (2006). "Detrimental effect of eicosapentaenoic acid supplementation on bone following ovariectomy in rats." Prostaglandins Leukot Essent Fatty Acids **75**(6): 419-27.
Poulsen, R. C., P. J. Moughan, et al. (2007). "Long-chain polyunsaturated fatty acids and the regulation of bone metabolism." Exp Biol Med (Maywood) **232**(10): 1275-88.
Prentki, M., E. Joly, et al. (2002). "Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes." Diabetes **51 Suppl 3**: S405-13.
Priante, G., L. Bordin, et al. (2002). "Fatty acids and cytokine mRNA expression in human osteoblastic cells: a specific effect of arachidonic acid." Clin Sci (Lond) **102**(4): 403-9.
Priante, G., E. Musacchio, et al. (2005). "Specific effect of arachidonic acid on inducible nitric oxide synthase mRNA expression in human osteoblastic cells." Clin Sci (Lond) **109**(2): 177-82.
Prod'homme, M., M. Balage, et al. (2005). "Differential effects of insulin and dietary amino acids on muscle protein synthesis in adult and old rats." J Physiol **563**(Pt 1): 235-48.
Puel, C., A. Quintin, et al. (2004). "Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat." Br J Nutr **92**(1): 119-27.

Pun, K. K., P. Lau, et al. (1989). "The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line." J Bone Miner Res **4**(6): 853-62.
Qi, D., K. Cai, et al. (2010). "Fatty acids induce amylin expression and secretion by pancreatic beta-cells." Am J Physiol Endocrinol Metab **298**(1): E99-E107.
Quinn, J. M. and H. Saleh (2009). "Modulation of osteoclast function in bone by the immune system." Mol Cell Endocrinol **310**(1-2): 40-51.
Rachon, D., J. Mysliwska, et al. (2002). "Effects of oestrogen deprivation on interleukin-6 production by peripheral blood mononuclear cells of postmenopausal women." J Endocrinol **172**(2): 387-95.
Rahman, M., G. V. Halade, et al. (2009). "The fat-1 transgene in mice increases antioxidant potential, reduces pro-inflammatory cytokine levels, and enhances PPAR-gamma and SIRT-1 expression on a calorie restricted diet." Oxid Med Cell Longev **2**(5): 307-16.
Rahman, M. M., A. Bhattacharya, et al. (2007). "Conjugated linoleic acid protects against age-associated bone loss in C57BL/6 female mice." J Nutr Biochem **18**(7): 467-74.
Rahman, M. M., A. Bhattacharya, et al. (2009). "Endogenous n-3 fatty acids protect ovariectomy induced bone loss by attenuating osteoclastogenesis." J Cell Mol Med **13**(8B): 1833-44.
Rahman, M. M., A. Bhattacharya, et al. (2008). "Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid." J Cell Physiol **214**(1): 201-9.
Rahman, M. M., G. V. Halade, et al. (2011). "t10c12-CLA maintains higher bone mineral density during aging by modulating osteoclastogenesis and bone marrow adiposity." J Cell Physiol **226**(9): 2406-14.
Raisz, L. G., C. B. Alander, et al. (1989). "Effects of prostaglandin E3 and eicosapentaenoic acid on rat bone in organ culture." Prostaglandins **37**(5): 615-25.
Ralston, S. H., R. G. Russell, et al. (1990). "Estrogen inhibits release of tumor necrosis factor from peripheral blood mononuclear cells in postmenopausal women." J Bone Miner Res **5**(9): 983-8.
Reda, T. K., A. Geliebter, et al. (2002). "Amylin, food intake, and obesity." Obes Res **10**(10): 1087-91.
Reid, D. M. (2008). "Relationships between fat and bone." Osteoporos Int **19**: 595-606.
Reid, I. R. (2002). "Relationships among body mass, its components, and bone." Bone **31**(5): 547-55.
Reid, I. R., R. Ames, et al. (1992). "Determinants of total body and regional bone mineral density in normal postmenopausal women--a key role for fat mass." J Clin Endocrinol Metab **75**(1): 45-51.
Reid, I. R., L. D. Plank, et al. (1992). "Fat mass is an important determinant of whole body bone density in premenopausal women but not in men." J Clin Endocrinol Metab **75**(3): 779-82.
Reseland, J. E., F. Haugen, et al. (2001). "Reduction of leptin gene expression by dietary polyunsaturated fatty acids." J Lipid Res **42**(5): 743-50.
Reth, M. (2002). "Hydrogen peroxide as second messenger in lymphocyte activation." Nat Immunol **3**(12): 1129-34.
Reynolds, C. M. and H. M. Roche (2010). "Conjugated linoleic acid and inflammatory cell signalling." Prostaglandins Leukot Essent Fatty Acids **82**(4-6): 199-204.
Rezq, A. A., F. A. Labib, et al. (2010). "Effect of some dietary oils and fats on serum lipid profile, calcium absorption and bone mineralization in mice." Pak J Nutr **9**(7): 643-650.

Ricciotti, E. and G. A. FitzGerald (2011). "Prostaglandins and inflammation." Arterioscler Thromb Vasc Biol **31**(5): 986-1000.
Riley, P. A. (1994). "Free radicals in biology: oxidative stress and the effects of ionizing radiation." Int J Radiat Biol **65**(1): 27-33.
Riserus, U. (2008). "Fatty acids and insulin sensitivity." Curr Opin Clin Nutr Metab Care **11**(2): 100-5.
Rizzoli, R., J. Y. Reginster, et al. (2011). "Adverse reactions and drug-drug interactions in the management of women with postmenopausal osteoporosis." Calcif Tissue Int **89**(2): 91-104.
Robertson, M. D., R. A. Henderson, et al. (2004). "Plasma ghrelin response following a period of acute overfeeding in normal weight men." Int J Obes Relat Metab Disord **28**(6): 727-33.
Rocca, A. S., J. LaGreca, et al. (2001). "Monounsaturated fatty acid diets improve glycemic tolerance through increased secretion of glucagon-like peptide-1." Endocrinology **142**(3): 1148-55.
Roduit, R., C. Nolan, et al. (2004). "A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli." Diabetes **53**(4): 1007-19.
Ronis, M. J., S. Korourian, et al. (2004). "Dietary saturated fat reduces alcoholic hepatotoxicity in rats by altering fatty acid metabolism and membrane composition." J Nutr **134**(4): 904-12.
Rosen, C. J. (2009). "Bone, Fat, and Body Composition: Evolving Concepts in the Pathogenesis of Osteoporosis." Am J Med **122**: 409-414.
Rosen, C. J. and M. L. Bouxsein (2006). "Mechanisms of disease: is osteoporosis the obesity of bone?" Nat Clin Pract Rheumatol **2**(1): 35-43.
Roux, C. (2011). "Osteoporosis in inflammatory joint diseases." Osteoporos Int **22**(2): 421-33.
Ruderman, N. B., J. M. Cacicedo, et al. (2003). "Malonyl-CoA and AMP-activated protein kinase (AMPK): possible links between insulin resistance in muscle and early endothelial cell damage in diabetes." Biochem Soc Trans **31**(Pt 1): 202-6.
Sabit, R., C. E. Bolton, et al. (2007). "Arterial stiffness and osteoporosis in chronic obstructive pulmonary disease." Am J Respir Crit Care Med **175**(12): 1259-65.
Saha, S. S. and M. Ghosh (2011). "Antioxidant effect of vegetable oils containing conjugated linolenic acid isomers against induced tissue lipid peroxidation and inflammation in rat model." Chem Biol Interact **190**(2-3): 109-20.
Sakaguchi, K., I. Morita, et al. (1994). "Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats." Prostaglandins Leukot Essent Fatty Acids **50**(2): 81-4.
Salari, P., A. Rezaie, et al. (2008). "A systematic review of the impact of n-3 fatty acids in bone health and osteoporosis." Med Sci Monit **14**(3): RA37-44.
Saleh, N. K. and H. A. Saleh (2011). "Olive oil effectively mitigates ovariectomy-induced osteoporosis in rats." BMC Complement Altern Med **11**: 10.
Samuel, V. T., K. F. Petersen, et al. (2010). "Lipid-induced insulin resistance: unravelling the mechanism." Lancet **375**(9733): 2267-77.
Sanz, C., P. Vazquez, et al. (2010). "Signaling and biological effects of glucagon-like peptide 1 on the differentiation of mesenchymal stem cells from human bone marrow." Am J Physiol Endocrinol Metab **298**(3): E634-43.
Saravanan, N., A. Haseeb, et al. (2005). "Differential effects of dietary saturated and trans-fatty acids on expression of genes associated with insulin sensitivity in rat adipose tissue." Eur J Endocrinol **153**(1): 159-65.
Sardesai, V. M. (1992). "The essential fatty acids." Nutr Clin Pract **7**(4): 179-86.

Sawzdargo, M., S. R. George, et al. (1997). "A cluster of four novel human G protein-coupled receptor genes occurring in close proximity to CD22 gene on chromosome 19q13.1." Biochem Biophys Res Commun **239**(2): 543-7.
Schaeffler, A., P. Gross, et al. (2009). "Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity." Immunology **126**(2): 233-45.
Schett, G., K. Redlich, et al. (2003). "Osteoprotegerin protects against generalized bone loss in tumor necrosis factor-transgenic mice." Arthritis Rheum **48**(7): 2042-51.
Schlemmer, C. K., H. Coetzer, et al. (1999). "Oestrogen and essential fatty acid supplementation corrects bone loss due to ovariectomy in the female Sprague Dawley rat." Prostaglandins Leukot Essent Fatty Acids **61**(6): 381-90.
Schmidt, J., K. Liebscher, et al. (2011). "Conjugated linoleic acids mediate insulin release through islet G protein coupled receptor FFA1/GPR40." J Biol Chem.
Schulz, E., K. Arfai, et al. (2004). "Aortic calcification and the risk of osteoporosis and fractures." J Clin Endocrinol Metab **89**(9): 4246-53.
Seifert, M. F., A. Bruce, et al. (1997). "Role of dietary lipid and antioxidants in bone metabolism." Nutr Res **17**(7): 1209-1228.
Seki, H., Y. Tani, et al. (2009). "Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1." Prostaglandins Other Lipid Mediat **89**(3-4): 126-30.
Sekiya, M., N. Yahagi, et al. (2003). "Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression." Hepatology **38**(6): 1529-39.
Sendur, O. F., Y. Turan, et al. (2009). "Antioxidant status in patients with osteoporosis: a controlled study." Joint Bone Spine **76**(5): 514-8.
Sevenoaks, M. J. and R. A. Stockley (2006). "Chronic Obstructive Pulmonary Disease, inflammation and co-morbidity--a common inflammatory phenotype?" Respir Res **7**: 70.
Shen, C. L., J. K. Yeh, et al. (2007). "Improvement of bone quality in gonad-intact middle-aged male rats by long-chain n-3 polyunsaturated fatty acid." Calcif Tissue Int **80**(4): 286-93.
Shen, C. L., J. K. Yeh, et al. (2006). "Protective effect of dietary long-chain n-3 polyunsaturated fatty acids on bone loss in gonad-intact middle-aged male rats." Br J Nutr **95**(3): 462-8.
Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-25.
Shimabukuro, M., M. Higa, et al. (1998). "Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression." J Biol Chem **273**(49): 32487-90.
Shinoda, Y., M. Yamaguchi, et al. (2006). "Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways." J Cell Biochem **99**(1): 196-208.
Silveira, L. R., J. Fiamoncini, et al. (2008). "Updating the effects of fatty acids on skeletal muscle." J Cell Physiol **217**(1): 1-12.
Sirois, I., A. M. Cheung, et al. (2003). "Biomechanical bone strength and bone mass in young male and female rats fed a fish oil diet." Prostaglandins Leukot Essent Fatty Acids **68**(6): 415-21.
Sitnick, M., S. C. Bodine, et al. (2009). "Chronic high fat feeding attenuates load-induced hypertrophy in mice." J Physiol **587**(Pt 23): 5753-65.
Sofi, F., I. Giangrandi, et al. (2010). "Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study." Int J Food Sci Nutr **61**(8): 792-802.

Solinas, G., W. Naugler, et al. (2006). "Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates." Proc Natl Acad Sci U S A **103**(44): 16454-9.
Soumura, M., S. Kume, et al. (2010). "Oleate and eicosapentaenoic acid attenuate palmitate-induced inflammation and apoptosis in renal proximal tubular cell." Biochem Biophys Res Commun **402**(2): 265-71.
Starcke, S. and G. Vollmer (2006). "Is there an estrogenic component in the metabolic syndrome?" Genes Nutr **1**(3-4): 177-88.
Stein, D. T., V. Esser, et al. (1996). "Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat." J Clin Invest **97**(12): 2728-35.
Stein, D. T., B. E. Stevenson, et al. (1997). "The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation." J Clin Invest **100**(2): 398-403.
Steneberg, P., N. Rubins, et al. (2005). "The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse." Cell Metab **1**(4): 245-58.
Still, K., P. Grabowski, et al. (2008). "The peroxisome proliferator activator receptor alpha/delta agonists linoleic acid and bezafibrate upregulate osteoblast differentiation and induce periosteal bone formation in vivo." Calcif Tissue Int **83**(4): 285-92.
Suda, M., K. Tanaka, et al. (1996). "Prostaglandin E receptor subtypes in mouse osteoblastic cell line." Endocrinology **137**(5): 1698-705.
Suganami, T., K. Tanimoto-Koyama, et al. (2007). "Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages." Arterioscler Thromb Vasc Biol **27**(1): 84-91.
Sun, D., A. Krishnan, et al. (2003). "Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice." J Bone Miner Res **18**(7): 1206-16.
Sun, Y., S. Ahmed, et al. (2003). "Deletion of ghrelin impairs neither growth nor appetite." Mol Cell Biol **23**(22): 7973-81.
Takahashi, K., S. Yamaguchi, et al. (2008). "JNK- and IkappaB-dependent pathways regulate MCP-1 but not adiponectin release from artificially hypertrophied 3T3-L1 adipocytes preloaded with palmitate in vitro." Am J Physiol Endocrinol Metab **294**(5): E898-909.
Takeda, S., F. Elefteriou, et al. (2002). "Leptin regulates bone formation via the sympathetic nervous system." Cell **111**(3): 305-17.
Tanaka, N., K. Sano, et al. (2008). "Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis." J Clin Gastroenterol **42**(4): 413-8.
Tanaka, Y., S. Kume, et al. (2010). "Fenofibrate, a PPARalpha agonist, has renoprotective effects in mice by enhancing renal lipolysis." Kidney Int.
Tang, Y., Y. Chen, et al. (2011). "G-protein-coupled receptor for short-chain fatty acids suppresses colon cancer." Int J Cancer **128**(4): 847-56.
Tetri, L. H., M. Basaranoglu, et al. (2008). "Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent." Am J Physiol Gastrointest Liver Physiol **295**(5): G987-95.
Thomas, D. M., N. Udagawa, et al. (1998). "Insulin receptor expression in primary and cultured osteoclast-like cells." Bone **23**(3): 181-6.
Thomsen, C., O. Rasmussen, et al. (1999). "Differential effects of saturated and monounsaturated fatty acids on postprandial lipemia and incretin responses in healthy subjects." Am J Clin Nutr **69**(6): 1135-43.
Thrailkill, K. M., L. Liu, et al. (2005). "Bone formation is impaired in a model of type 1 diabetes." Diabetes **54**(10): 2875-81.

Tian, X. Y., Q. Zhang, et al. (2008). "Continuous PGE2 leads to net bone loss while intermittent PGE2 leads to net bone gain in lumbar vertebral bodies of adult female rats." Bone **42**(5): 914-20.
Tian, X. Y., Q. Zhang, et al. (2007). "Continuous infusion of PGE2 is catabolic with a negative bone balance on both cancellous and cortical bone in rats." J Musculoskelet Neuronal Interact **7**(4): 372-81.
Tilg, H. and A. M. Diehl (2000). "Cytokines in alcoholic and nonalcoholic steatohepatitis." N Engl J Med **343**(20): 1467-76.
Tishinsky, J. M., D. W. Ma, et al. (2010). "Eicosapentaenoic acid and rosiglitazone increase adiponectin in an additive and PPARgamma-dependent manner in human adipocytes." Obesity (Silver Spring) **19**(2): 262-8.
Todoric, J., M. Loffler, et al. (2006). "Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids." Diabetologia **49**(9): 2109-19.
Tschop, M., C. Weyer, et al. (2001). "Circulating ghrelin levels are decreased in human obesity." Diabetes **50**(4): 707-9.
Tsujisawa, T., H. Inoue, et al. (2005). "SC-19220, antagonist of prostaglandin E2 receptor EP1, inhibits osteoclastogenesis by RANKL." J Bone Miner Res **20**(1): 15-22.
Tsukiyama, K., Y. Yamada, et al. (2006). "Gastric inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion." Mol Endocrinol **20**(7): 1644-51.
Tsutsumi, R., C. Xie, et al. (2009). "PGE2 signaling through the EP4 receptor on fibroblasts upregulates RANKL and stimulates osteolysis." J Bone Miner Res **24**(10): 1753-62.
Uchida, R., H. Chiba, et al. (2010). "Combined effects of soy isoflavone and fish oil on ovariectomy-induced bone loss in mice." J Bone Miner Metab **29**(4): 404-13.
Ukropec, J., J. E. Reseland, et al. (2003). "The hypotriglyceridemic effect of dietary n-3 FA is associated with increased beta-oxidation and reduced leptin expression." Lipids **38**(10): 1023-9.
Unger, R. H. and L. Orci (2001). "Diseases of liporegulation: new perspective on obesity and related disorders." Faseb J **15**(2): 312-21.
Valko, M., D. Leibfritz, et al. (2007). "Free radicals and antioxidants in normal physiological functions and human disease." Int J Biochem Cell Biol **39**(1): 44-84.
van't Hof, R. J. and S. H. Ralston (2001). "Nitric oxide and bone." Immunology **103**(3): 255-61.
van Dielen, F. M., C. van't Veer, et al. (2001). "Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals." Int J Obes Relat Metab Disord **25**(12): 1759-66.
van Dijk, S. J., E. J. Feskens, et al. (2009). "A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome." Am J Clin Nutr **90**(6): 1656-64.
van Papendorp, D. H., H. Coetzer, et al. (1995). "Biochemical profile of osteoporotic patients on essential fatty acid supplementation." Nutr Res **15**(3): 325-334.
van Staa, T. P., C. Cooper, et al. (2003). "Inflammatory bowel disease and the risk of fracture." Gastroenterology **125**(6): 1591-7.
Vargas, S. J., A. Naprta, et al. (1996). "Interleukin-6 expression and histomorphometry of bones from mice deficient in receptors for interleukin-1 or tumor necrosis factor." J Bone Miner Res **11**(11): 1736-44.
Viccica, G., C. M. Francucci, et al. (2010). "The role of PPARgamma for the osteoblastic differentiation." J Endocrinol Invest **33**(7 Suppl): 9-12.

Vidal, N. O., H. Brandstrom, et al. (1998). "Osteoprotegerin mRNA is expressed in primary human osteoblast-like cells: down-regulation by glucocorticoids." J Endocrinol **159**(1): 191-5.
Vilar, L., C. P. Oliveira, et al. (2008). "High-fat diet: a trigger of non-alcoholic steatohepatitis? Preliminary findings in obese subjects." Nutrition **24**(11-12): 1097-102.
Visser, M., J. Langlois, et al. (1998). "High body fatness, but not low fat-free mass, predicts disability in older men and women: the Cardiovascular Health Study." Am J Clin Nutr **68**(3): 584-90.
Wahrburg, U. (2004). "What are the health effects of fat?" Eur J Nutr **43**(suppl 1): I/6-I/11.
Walsh, M. C., N. Kim, et al. (2006). "Osteoimmunology: interplay between the immune system and bone metabolism." Annu Rev Immunol **24**: 33-63.
Wan, Y., L. W. Chong, et al. (2007). "PPAR-gamma regulates osteoclastogenesis in mice." Nat Med **13**(12): 1496-503.
Wani, M. R., K. Fuller, et al. (1999). "Prostaglandin E2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion." Endocrinology **140**(4): 1927-35.
Watkins, B. A., Y. Li, et al. (2000). "Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats." J Nutr **130**(9): 2274-84.
Watkins, B. A., Y. Li, et al. (2003). "Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism." Prostaglandins Leukot Essent Fatty Acids **68**(6): 387-98.
Watkins, B. A., Y. Li, et al. (2001). "Omega-3 polyunsaturated fatty acids and skeletal health." Exp Biol Med (Maywood) **226**(6): 485-97.
Watkins, B. A., Y. Li, et al. (2001). "Nutraceutical fatty acids as biochemical and molecular modulators of skeletal biology." J Am Coll Nutr **20**(5 Suppl): 410S-416S; discussion 417S-420S.
Watkins, B. A., Y. Li, et al. (2006). "Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats." J Nutr Biochem **17**(4): 282-9.
Watkins, B. A., H. E. Lippman, et al. (2001). "Bioactive fatty acids: role in bone biology and bone cell function." Prog Lipid Res **40**(1-2): 125-48.
Watkins, B. A. and M. F. Seifert (2000). "Conjugated linoleic acid and bone biology." J Am Coll Nutr **19**(4): 478S-486S.
Watkins, B. A., C. L. Shen, et al. (1996). "Dietary (n-3) and (n-6) polyunsaturates and acetylsalicylic acid alter ex vivo PGE2 biosynthesis, tissue IGF-I levels, and bone morphometry in chicks." J Bone Miner Res **11**(9): 1321-32.
Wauquier, F., V. Barquissau, et al. (2011). "Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis." Bone.
Wauquier, F., L. Leotoing, et al. (2009). "Oxidative stress in bone remodelling and disease." Trends Mol Med **15**(10): 468-77.
Wei, D., J. Li, et al. (2010). "Cellular production of n-3 PUFAs and reduction of n-6-to-n-3 ratios in the pancreatic beta-cells and islets enhance insulin secretion and confer protection against cytokine-induced cell death." Diabetes **59**(2): 471-8.
Wei, S., H. Kitaura, et al. (2005). "IL-1 mediates TNF-induced osteoclastogenesis." J Clin Invest **115**(2): 282-90.

Weiler, H. A. and S. C. Fitzpatrick-Wong (2002). "Modulation of essential (n-6):(n-3) fatty acid ratios alters fatty acid status but not bone mass in piglets." J Nutr **132**(9): 2667-72.
Weisberg, S. P., D. Hunter, et al. (2006). "CCR2 modulates inflammatory and metabolic effects of high-fat feeding." J Clin Invest **116**(1): 115-24.
Weisberg, S. P., D. McCann, et al. (2003). "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest **112**(12): 1796-808.
Weiss, L. A., E. Barrett-Connor, et al. (2005). "Ratio of n-6 to n-3 fatty acids and bone mineral density in older adults: the Rancho Bernardo Study." Am J Clin Nutr **81**(4): 934-8.
Weiss, R. J., M. C. Wick, et al. (2010). "Increased fracture risk in patients with rheumatic disorders and other inflammatory diseases -- a case-control study with 53,108 patients with fracture." J Rheumatol **37**(11): 2247-50.
Weitzmann, M. N. and R. Pacifici (2006). "Estrogen deficiency and bone loss: an inflammatory tale." J Clin Invest **116**(5): 1186-94.
Weitzmann, M. N. and R. Pacifici (2007). "T cells: unexpected players in the bone loss induced by estrogen deficiency and in basal bone homeostasis." Ann N Y Acad Sci **1116**: 360-75.
Welters, H. J., M. Tadayyon, et al. (2004). "Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure." FEBS Lett **560**(1-3): 103-8.
Westermark, G., M. B. Arora, et al. (1995). "Amyloid formation in response to beta cell stress occurs in vitro, but not in vivo, in islets of transgenic mice expressing human islet amyloid polypeptide." Mol Med **1**(5): 542-53.
Westermark, G. T., A. Leckstrom, et al. (1998). "Increased release of IAPP in response to long-term high fat intake in mice." Horm Metab Res **30**(5): 256-8.
Williams, J. P., H. C. Blair, et al. (1997). "Regulation of osteoclastic bone resorption by glucose." Biochem Biophys Res Commun **235**(3): 646-51.
Wittwer, J., I. Rubio-Aliaga, et al. (2011). "Nutrigenomics in human intervention studies: current status, lessons learned and future perspectives." Mol Nutr Food Res **55**(3): 341-58.
Wobser, H., C. Dorn, et al. (2009). "Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells." Cell Res **19**(8): 996-1005.
Woodside, J. V. and D. Kromhout (2005). "Fatty acids and CHD." Proceedings of the nutrition society **64**: 554-564.
Wright, M. J., D. D. Proctor, et al. (2008). "Proton pump-inhibiting drugs, calcium homeostasis, and bone health." Nutr Rev **66**(2): 103-8.
Wu, X., L. H. Zeng, et al. (2007). "Activation of PKA and phosphorylation of sodium-dependent vitamin C transporter 2 by prostaglandin E2 promote osteoblast-like differentiation in MC3T3-E1 cells." Cell Death Differ **14**(10): 1792-801.
Xiao, Y., J. Cui, et al. (2010). "Expression of genes associated with bone resorption is increased and bone formation is decreased in mice fed a high-fat diet." Lipids **45**(4): 345-55.
Xiao, Y., J. Cui, et al. (2010). "Effects of duodenal redox status on calcium absorption and related genes expression in high-fat diet-fed mice." Nutrition **26**(11-12): 1188-94.
Xie, D., Q. Zhong, et al. (2007). "Glucose-dependent insulinotropic peptide-overexpressing transgenic mice have increased bone mass." Bone **40**(5): 1352-60.
Xiong, Y., N. Miyamoto, et al. (2004). "Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41." Proc Natl Acad Sci U S A **101**(4): 1045-50.

Yamada, C., Y. Yamada, et al. (2008). "The murine glucagon-like peptide-1 receptor is essential for control of bone resorption." Endocrinology **149**(2): 574-9.
Yamada, Y., H. Fushimi, et al. (1995). "Effect of eicosapentaenoic acid and docosahexaenoic acid on diabetic osteopenia." Diabetes Res Clin Pract **30**(1): 37-42.
Yamaguchi, N., T. Kukita, et al. (2008). "Adiponectin inhibits induction of TNF-alpha/RANKL-stimulated NFATc1 via the AMPK signaling." FEBS Lett **582**(3): 451-6.
Yamazaki, M., H. Fukushima, et al. (2009). "Tumor necrosis factor alpha represses bone morphogenetic protein (BMP) signaling by interfering with the DNA binding of Smads through the activation of NF-kappaB." J Biol Chem **284**(51): 35987-95.
Yang, C. R., J. H. Wang, et al. (2004). "Decoy receptor 3 (DcR3) induces osteoclast formation from monocyte/macrophage lineage precursor cells." Cell Death Differ **11 Suppl 1**: S97-107.
Yasuda, T., T. Masaki, et al. (2003). "Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats." Neurosci Lett **349**(2): 75-8.
Yu, L., D. Adams, et al. (2002). "Conjugated linoleic acid isomers differ in their free radical scavenging properties." J Agric Food Chem **50**(14): 4135-40.
Yuan, J., M. Akiyama, et al. (2010). "The effects of polyunsaturated fatty acids and their metabolites on osteoclastogenesis in vitro." Prostaglandins Other Lipid Mediat **92**(1-4): 85-90.
Zeitlin, L., E. Segev, et al. (2003). "Effects of long-term administration of N-3 polyunsaturated fatty acids (PUFA) and selective estrogen receptor modulator (SERM) derivatives in ovariectomized (OVX) mice." J Cell Biochem **90**(2): 347-60.
Zhang, X., E. M. Schwarz, et al. (2002). "Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair." J Clin Invest **109**(11): 1405-15.
Zhao, G., T. D. Etherton, et al. (2007). "Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects." Am J Clin Nutr **85**(2): 385-91.
Zhao, L. J., Y. J. Liu, et al. (2007). "Relationship of obesity with osteoporosis." J Clin Endocrinol Metab **92**(5): 1640-6.
Zhong, Q., T. Itokawa, et al. (2007). "Effects of glucose-dependent insulinotropic peptide on osteoclast function." Am J Physiol Endocrinol Metab **292**(2): E543-8.
Zhou, Y. P. and V. E. Grill (1994). "Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle." J Clin Invest **93**(2): 870-6.
Zhu, F. S., S. Liu, et al. (2008). "Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia." World J Gastroenterol **14**(41): 6395-400.
Zou, W., I. Hakim, et al. (2001). "Tumor necrosis factor-alpha mediates RANK ligand stimulation of osteoclast differentiation by an autocrine mechanism." J Cell Biochem **83**(1): 70-83.
Zou, Y., J. Li, et al. (2006). "High-fat emulsion-induced rat model of nonalcoholic steatohepatitis." Life Sci **79**(11): 1100-7.
Zwart, S. R., D. Pierson, et al. (2010). "Capacity of omega-3 fatty acids or eicosapentaenoic acid to counteract weightlessness-induced bone loss by inhibiting NF-kappaB activation: from cells to bed rest to astronauts." J Bone Miner Res **25**(5): 1049-57.

Article numéro 2:

Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis

Fabien Wauquier, Valentin Barquissau, Laurent Léotoing, Marie-Jeanne Davicco, Patrice Lebecque, Sylvie Mercier, Claire Philippe, Elisabeth Miot-Noirault, Jean-Michel Chardigny , Béatrice Morio, Yohann Wittrant, Véronique Coxam

Bone, 2011

L'analyse exhaustive de la littérature synthétisée dans l'article 1, nous a permis de cibler une certaine classe d'acide gras qui apparaissait la plus prometteuse du point de vue de son potentiel ostéoprotecteur : les AGPI.

Le processus inflammatoire semble clairement influencer le remodelage osseux. D'un coté, l'activation des cellules T tout comme la production de cytokines spécifiques, deux mécanismes observés au cours de l'inflammation ont le potentiel d'activer la résorption osseuse. De l'autre, le vieillissement de l'organisme est associé à l'apparition d'un état inflammatoire sub-chronique systémique qui pourrait donc contribuer à la perte osseuse. Enfin, la propriété la plus évidente des AGPI correspond à un effet anti-inflammatoire marqué qui pourrait s'expliquer, entre autre, par une modification de la voie de production des éicosanoïdes aboutissant à la synthèse de composés moins inflammatoires.

Dans ce contexte, notre hypothèse de travail était que les AGPI à propriétés anti-inflammatoires pouvaient éventuellement limiter l'inflammation chronique associée au vieillissement et ainsi contribuer à préserver le capital osseux. Nous avons utilisé la souris « Senescence Accelerated Mouse Prone 8 » (SamP8) qui est un modèle de *progeria* caractérisé par un développement précoce de pathologies liées à l'âge dont l'ostéoporose. Pour tester notre hypothèse, ces souris ont été nourries *ad libitum* tout au long de leur vie avec des régimes qui différaient essentiellement au niveau des proportions respectives des AGPI d'intérêt.

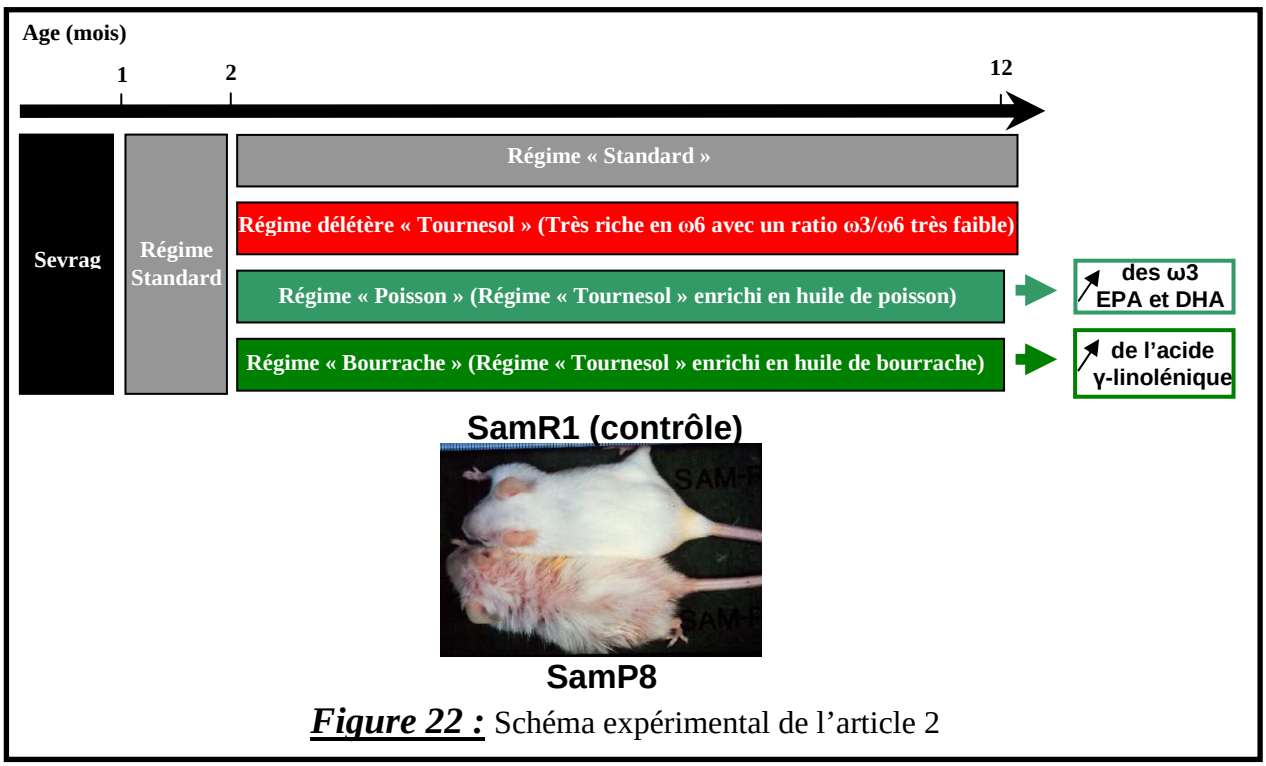


Figure 22 : Schéma expérimental de l'article 2

Dans ce modèle, l'administration d'un régime délétère à base d'huile de tournesol (ratio acide gras ω6 /ω3 très important) aggrave la perte osseuse en association avec une augmentation des marqueurs inflammatoires systémiques et osseux et avec une augmentation des marqueurs de la résorption osseuse. La supplémentation du régime tournesol avec de l'huile de bourrache ou de l'huile de poisson permet d'apporter des quantités importantes d'AGPI anti-inflammatoires (acide γ-linolénique pour l'huile de bourrache et ω3 pour l'huile de poisson). De manière intéressante, ces deux régimes supplémentés permettent, en plus de réduire les paramètres inflammatoires, de s'opposer à l'augmentation des marqueurs de résorption osseuse et de limiter la diminution de la densité minérale de l'os chez ces souris.

Cette étude met donc en évidence le potentiel santé de certains AGPI au regard de la préservation du capital osseux et suggère un rôle déterminant de leurs propriétés anti-inflammatoires systémiques.

Bone xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis

Fabien Wauquier [a,b,c], Valentin Barquissau [a,b,d], Laurent Léotoing [a,b,c], Marie-Jeanne Davicco [a,b,c], Patrice Lebecque [a,b,c], Sylvie Mercier [a,b,c], Claire Philippe [a,b,c], Elisabeth Miot-Noirault [e,f], Jean-Michel Chardigny [a,b,d], Béatrice Morio [a,b,d], Yohann Wittrant [a,b,c,*], Véronique Coxam [a,b,c]

[a] Clermont Université, Université d'Auvergne, Unité de Nutrition Humaine, BP 10448, F-63000 Clermont-Ferrand, France
[b] INRA, UMR 1019, UNH, CRNH Auvergne, F-63009 Clermont-Ferrand, France
[c] Equipe Alimentation, Squelette et Métabolismes, France
[d] Equipe Contrôle de l'Homéostasie Lipido-Energétique et Obésité, France
[e] Clermont Université, Université d'Auvergne, Imagerie moléculaire et thérapie vectorisée, BP 10448, F-63000 Clermont-Ferrand, France
[f] Inserm, U 990, F-63000 Clermont-Ferrand, France

ARTICLE INFO

Article history:
Received 4 April 2011
Revised 29 April 2011
Accepted 26 May 2011
Available online xxxx

Edited by: Clifford Rosen

Keywords:
Osteoporosis
Aging
Nutrition
Lipids
SAMP8
Inflammation

ABSTRACT

Fats are prevalent in western diets; they have known deleterious effects on muscle insulin resistance and may contribute to bone loss. However, relationships between fatty acids and locomotor system dysfunctions in elderly population remain controversial. The aim of this study was to analyze the impact of fatty acid quality on the age related evolution of the locomotor system and to understand which aging mechanisms are involved. In order to analyze age related complications, the SAMP8 mouse strain was chosen as a progeria model as compared to the SAMR1 control strain. Then, two months old mice were divided in different groups and subjected to the following diets : (1) standard "growth" diet – (2) "sunflower" diet (high ω6/ω3 ratio) – (3) "borage" diet (high γ-linolenic acid) – (4) "fish" diet (high in long chain ω3). Mice were fed ad libitum through the whole protocol. At 12 months old, the mice were sacrificed and tissues were harvested for bone studies, fat and muscle mass measures, inflammation parameters and bone cell marker expression. We demonstrated for the first time that borage and fish diets restored inflammation and bone parameters using an original model of senile osteoporosis that mimics clinical features of aging in humans. Therefore, our study strongly encourages nutritional approaches as relevant and promising strategies for preventing aged-related locomotor dysfunctions.

This article is part of a Special Issue entitled Bone and Fat Special Issue.

Introduction

All industrialized countries face a progressive increase in life expectancy due to declining mortality. Consequently, prevalence of chronic age-related metabolic complications has grown, including locomotor dysfunctions. Among those diseases, impaired capacities of movement due to both sarcopenia and osteoporosis, are especially disabling and represent a social and economic burden world-wide. With regards to the quality of life, osteoporosis has devastating psychological and socio-economic effects. Osteoporotic fractures can result in up to 20% excess mortality within one year. Additionally, up to 50% of patients will be disabled, half of them requiring long term nursing home care, while only one third fully recover from hip fracture [1].

Osteoporosis is characterized by an excessive bone fragility that leads to an increased risk of fractures. Today's treatments such as hormone replacement therapy (HRT) demonstrate clear benefits on post-menopausal osteoporotic women [2,3]. However a higher prevalence of thrombo-embolic accidents, uterine and breast cancers was reported for HRT [4]. Because research in nutrition over the past 30 years, has led to exciting progress supporting the hypothesis that, by modulating specific target functions in the body, diet can help to achieve optimal health by reducing the risk of disease, the present study aimed at investigating the role of lipid diets on bone health status.

Literature has widely linked lipid intake and inflammation status, a key protagonist involved in bone resorption [5,6]. As a matter of fact, inflammation favors bone degradation by stimulating osteoclast activity while inhibiting osteoblast bone formation leading to imbalance in bone remodeling and subsequent bone loss. Regarding inflammation, lipids exhibit a duality, with both pro- and anti-inflammatory effects depending on their structures and metabolism

* Corresponding author at: UMR 1019, Centre de Recherche INRA de Clermont-Ferrand/Theix, France. Fax: +33 9 57 05 99 12.
E-mail address: yohann.wittrant@clermont.inra.fr (Y. Wittrant).

8756-3282/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2011.05.030

[7,8]. In this light, a growing body of evidence has revealed that ω-6 increase bone loss while ω-3 are believed to protect bone health [5,6,9,10]. Nevertheless, this debate remains controversial and the mechanisms of action are poorly understood.

In this work, the senescence-accelerated prone mouse (SAMP8) was used to mimic senile osteoporosis rather than post-menopausal osteoporosis [11,12]. This model exhibits both sarcopenia and osteoporosis and matches clinical features observed in elderly. Therefore, this study investigated the effects of different diets enriched in lipids described for their effect on inflammation on bone status. After 10 months of supplementation, our results clearly demonstrate that diets enriched in borage and fish oils protected from bone loss by blocking inflammation and osteoclastogenesis.

Materials and methods

Ethics

All animal procedures were approved by the institution's animal welfare committee and were conducted in accordance with the National Research Council's guidelines for the care and use of laboratory animals. Animals were housed in the animal laboratory of the INRA Research Center for Human Nutrition (http://www1.clermont.inra.fr/unh/telechargementinternet/ienplaquette.pdf). Throughout the study, animals were housed in a controlled environment (12:12 h light–dark cycle, 20–22 °C, 50–60% relative humidity/5 mice per plastic cage with free access to water). Animals were delivered to our facility 1 month before study for acclimatization to our animal environment. At the end of the protocol, blood was withdrawn on anesthetized animals. Then, animals were sacrificed by IP injection of lethal dose of pentobarbital sodique (0.1 ml/g of body weight — CEVA Santé Animale, Libourne, France) and tissues were harvested, frozen and stocked prior to investigation.

Statistics

Experimental procedure was conducted with a total of 20 mice per group to allow data analysis on at least 12 mice per group at the end of the protocol. Data obtained were analyzed either by ANOVA Tukey's test (ExcelStat Pro software — Microsoft Office 2007). Groups with significant differences ($p<0.05$) are indicated with different letters. Correlation tests were performed using Pearson's parametric test (ExcelStat Pro software — Microsoft Office 2007).

Animals

One month old female Senescence accelerated mouse-Prone 8 (SamP8) and Senescence accelerated mouse-Resistant 1 (SamR1) derived from AKR/J strain were obtained from INRA-Dijon (UMR INRA-ENESAD Flaveur, Vision et Comportement du consommateur, 21065 Dijon Cedex, France). As an acclimatization period, they were provided with free access to a standard growth diet for a month. Subsequently, SamR1 and SamP8 animals were randomly divided in different groups and submitted to different diets ad libitum for 10 months (Fig. 1) after checking that diet enrichment did not modified significantly daily food intake. Besides, the experimental protocol was designed not to exceed 10 months to avoid a dramatic increase in SAMP8 mortality currently observed from 12 months of age [12].

Diets

Diets were purchased from INRA (Jouy-en-Josas, France) or Harlan (Ganat France). All diets were adjusted to be approximately isocaloric ($\Delta<5\%$). SamR1 mice were fed either a standard growth diet (Harlan Teklad Global 2019) or a sunflower oil based diet with a high ω6/ω3 Polyunsaturated Fatty Acid (PUFA) ratio (Tables 1 and 2). In parallel, SamP8 mice were fed either a standard growth diet (Harlan Teklad Global 2019), a sunflower oil based diet or an enriched sunflower oil based diet that modulates the fatty acid composition (Table 1). Borage and fish oils were used to test the effect of a γ-Linolenic acid (18:3 ω6; GLA) enrichment or to reduce the ω6/ω3 ratio by providing Eicosapentaenoic acid (20:5 ω3; EPA) and Docosohexaenoic (22:6 ω3; DHA) respectively.

Bone morphological analysis

Bone morphological analysis was done using an eXplore CT 120 scanner (GE Healthcare, Canada). Frozen femora, cleaned of soft tissues, were scanned. Acquisition consisted of 360 views acquired in 1° increments collected in one full gantry rotation, with a 20 ms exposure/view and X-ray tube settings being 100 kV and 50 mA. CT images were reconstructed using a modified conebeam algorithm with an isotropic voxel of $0.045\times0.045\times0.045\ mm^3$. CT scans were analyzed using MicroView® version 2.3 software (GE Healthcare, Canada). A hydroxyapatite calibration phantom (SB3, Gamex RMI, WI) was used to convert gray-scale levels to HA density values. Trabecular bone of the distal femur was selected for bone mineral density and bone volume fraction (BVF = Bone Volume/Total volume) analyses by fitting a cylindrical region ($r=0.7$ mm) of interest in the center of the femur, starting 0.1 mm proximally from the growth plate and extending a further 0.32 mm in the proximal direction. Bone Volume Fraction was calculated using a threshold of $150\ mg/cm^3$ to isolate bone tissue. Bone mineral density was estimated as the mean converted gray-scale level within the region of interest.

Tissue weight analysis

Mice total body weight was measured weekly throughout the study. Spleen, muscle tissues (including gastrocnemius and quadriceps)

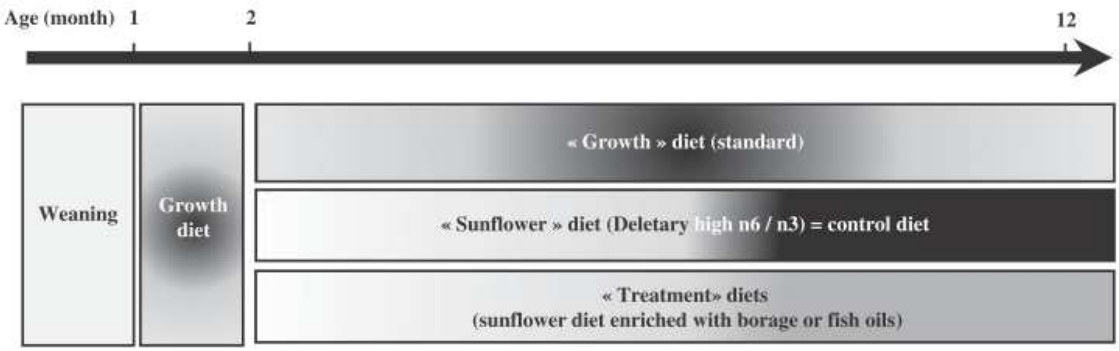


Fig. 1. Experimental protocol design.

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

Table 1
Diets formulations.

Ingredient (g/100 g diet)	Standard diet (Harlan 2019)	Sunflower based diet	Borage enriched diet	Fish enriched diet
Wheat starch	55.24	59.14	56.79	56.79
Casein	19.00	19.20	19.20	19.20
Sucrose	4.16	4.16	4.16	4.16
Fiber cellulose	3.60	3.50	3.50	3.50
DL méthionine	3.30	0.30	0.30	0.30
Choline bitartrate	0.20	0.20	0.20	0.20
Mineral mix	3.50	2.50	2.50	2.50
Vitamin mix	1.00	1.00	1.00	1.00
Sunflower oil	0.00	5.45	5.62	6.73
Canola oil	0.00	2.73	3.37	3.37
Borage oil	0.00	0.00	2.79	0.00
Fish oil	0.00	0.00	0.00	1.68
Oleisol oil	0.00	1.82	0.57	0.57
Soybean oil	10.00	0.00	0.00	0.00
Energy (cal)	420	428	440	440

From I.N.R.A. (Jouy en Josas, France); casein (Union des caséinerie, Surgères, France), cornstarch (Cerestar, Saint-Maur, France), cellulose (Durieux, Marne la Vallée, France), oil (Bailly, Aulnay sous Bois, France), vitamin mixture (Roche, Neuilly sur Seine, France), mineral mixture (Prolabo, Fontenay sous bois, France), DL-methionine and choline bitartrate (Jerafrance, Jeufosse, France).

and visceral adipose tissues were collected from mice and weighted immediately after sacrifice. Using scissors, approximately 90% of fat depots were removed around kidneys and gut.

Biochemical parameters

Venous blood sampling from twelve months old mice was collected in plain tubes, and was centrifuged. Serum was subsequently removed, aliquoted and stored at −80 °C. CRP level was measured by mouse C-Reactive Protein (Mouse) EIA Kit (ALPCO Diagnostics, Salem, USA) according to the manufacturer's instructions. CTX-1 level was measured by ELISA Kit for Mouse Cross Linked C-Telopeptide of Type 1 Collagen (Uscn Life Science Inc., Wuhan, China).

Table 2
Diets' fatty acid composition (% of total fatty acids).

Fatty acids	Standard diet (Harlan 2019)	Sunflower based diet	Borage enriched diet	Fish enriched diet
12:0	0.71	0.00	0.00	0.00
14:0	0.15	0.05	0.05	0.09
15:0	0.00	0.00	0.00	0.00
16:0	11.35	5.11	6.58	5.03
17:0	0.00	0.02	0.01	0.01
18:0	3.24	2.75	2.89	2.97
19:0	0.00	0.00	0.00	0.00
20:0	0.25	0.19	0.16	0.16
22:0	0.08	0.24	0.12	0.12
Total SFA	*15.78*	*8.36*	*9.81*	*8.38*
16:1	0.15	0.08	0.14	0.19
18:1 n−9	**23.4**	**46.89**	**36.84**	**37.77**
18:1 n−7	0.00	0.00	0.21	0.52
20:1	0.54	0.34	1.17	0.73
22:1	0.05	0.00	0.54	0.00
24:1	0.00	0.00	0.34	0.00
Total MUFA	*24.14*	*47.31*	*39.24*	*39.21*
18:2 n−6	**54.23**	**42.04**	**43.40**	**40.51**
18:3 n−6	**0.00**	**0.00**	**5.19**	**0.00**
20:4 n−6	0.00	0.00	0.00	0.31
Total PUFA ω-6	*54.23*	*42.04*	*48.59*	*40.82*
18:3 n−3	**5.93**	**2.29**	**2.35**	**2.38**
18:4 n−3	0.01	0.00	0.00	0.52
20:4 n−3	0.00	0.00	0.00	0.32
20:5 n−3	**0.00**	**0.00**	**0.00**	**6.35**
21:5 n−3	0.00	0.00	0.00	0.22
22:5 n−3	0.00	0.00	0.00	0.31
22:6 n−3	0.00	0.00	0.00	1.50
Total PUFA ω-3	*5.94*	*2.29*	*2.35*	*11.60*
Total PUFA	60.16	44.33	50.94	52.42
LA/ALA	9.14	18.35	18.46	17.02
n−6/n−3	9.13	18.35	20.67	3.52
Total percent	*100.00*	*100.00*	*100.00*	*100.00*

Bold script aimed at highlighting main lipid differences.

RNA extraction

For each experimental group, two sets of extractions were done with three tibias pooled in each. Frozen bones were ground in liquid nitrogen to obtain a fine powder. Subsequently, total RNA was isolated using RNeasy Mini Kit (Qiagen, France), following the manufacturer's instructions. Total RNA was quantified by measuring OD260, and integrity was checked by 1% agarose/formaldehyde gel electrophoresis.

Reverse transcription

1 μg of total RNA was converted to cDNA using the high capacity cDNA reverse transcription (RT) kit (Applied Biosystems). Ten microliters of total extract were incubated with 10 μl of 2× RT master mix. RT was performed as following (step 1: temperature 25 °C, time 10 min; step 2: temperature 37 °C, time 120 min; step 3: temperature 85 °C, time 5 min). The resulting cDNA was used immediately for TaqMan® low-density arrays (TLDAs) (Applied Biosystems 7900HT real-time PCR system), a real-time polymerase chain reaction (PCR) in a low-density array format, to assess simultaneously the expression of multiple genes.

Taqman low density arrays (TLDA)

Each card of the low-density array has 8 separate loading ports, each with 48 separate wells, for a total of 384 wells per card. Each 2-μl well contains specific, user-defined primers and probes, capable of detecting a single gene. 18S ribosomal RNA and actin served as the housekeeping genes. Each cDNA sample (100 μl) was added to an equal volume of 2× TaqMan® universal PCR master mix (Applied Biosystems). After gentle mixing and centrifugation, the mixture was transferred to a loading port on a TLDA card. The array was centrifuged twice for 1 min, each at 1200 rpm, to distribute the samples from the loading port to each well. The card was then sealed and PCR amplification performed using an Applied Biosystems Prism 7900HT sequence detection system (equipped with a TaqMan® low density array upgrade). Thermal cycler conditions were as follows: 2 min at 50 °C, 10 min at 94.5 °C, 30 s at 97 °C, and 1 min at 59.7 °C for 40 cycles. Expression values were calculated using the comparative threshold cycle (C_T) method as directed by the manufacturer (User Bulletin No. 2, Applied Biosystems). Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the relative expression of transcripts for each gene. C_T values for each gene and for 18S and actin both as housekeeping genes, were calculated. The ΔC_T value [$\Delta C_T = C_T$ (Sam R1 sunflower fed mice) − C_T(18S and actin)] was used as the calibrator. Assigning to the calibrator value of 1.00, the relative expression of each gene was calculated in arbitrary units (AU) with the formula: $\Delta\Delta C_T = \Delta C_T$(sample) − ΔC_T(calibrator).

Results

Sunflower diet exacerbates osteoporosis features in SAMP8 mice

Before analyzing the effects of nutritional interventions, we aimed at validating that our mouse model of progeria was associated with bone loss and mimicked senile osteoporosis. Then, we first studied the effect of the strain on bone health by comparing the bone mineral density (BMD) of SAMP8 to SAMR1 mice. Under growth diet, SAMP8 mice exhibited lower BMD of approximately 15% (Fig. 2A). However, this decrease was just below significance. In contrast, the effect was greater and became significant when mice were fed a sunflower diet

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

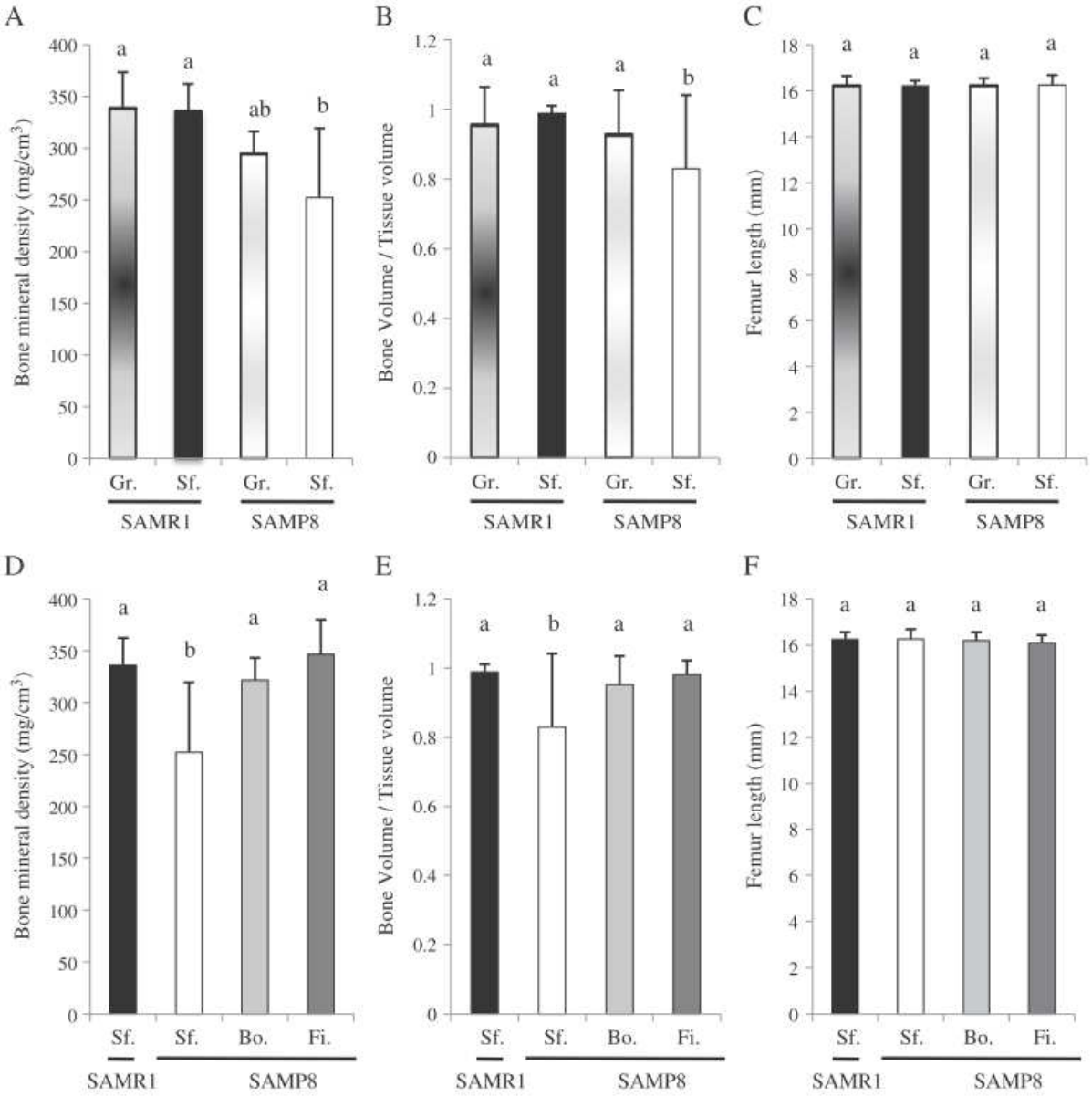


Fig. 2. Bone status: A, D: bone mineral density analysis. B, E: bone volume analysis. C, F: femur length analysis. (Gr: Growth diet; Sf: Sunflower diet; Bo: borage diet; Fi: fish diet). Significant different groups are represented by different letters as analyzed by ANOVA ($p<0.05$).

(Fig. 2A). Indeed, while no effect was observed on the resistant strain (SAMR1), sunflower "treatment" highlighted the decrease in bone mineral density in the progeria model. As a matter of fact, loss of BMD was almost doubled to reach 27% (Fig. 2A). This changing in BMD was associated with a reduction of bone volume (Fig. 2B) but not related to any alteration in bone growth (Fig. 2C), thus validating the establishment of an aged-related bone loss features. Therefore, we set the sunflower diet as control and aimed at elucidating whether addition of alternative fatty acids may rescue mice from sunflower-stimulated senile osteoporosis.

Nutritional treatments allow bone mineralization rescue without affecting bone growth

At two months old, mice started to be fed a "sunflower" diet enriched or not either with borage oil or fish oil. At 12 months old, mice were sacrificed and femorae were harvested for micro computed X-rays tomography analysis. Results reveal that nutritional treatments prevented SAMP8 mice from altered bone architecture by restoring both bone mineral density and bone volume back to values observed in SAMR1 mice (Fig. 2D and E). Since no effect was observed on femorae length, this rescue in bone phenotype could not be attributed to any growth effect (Fig. 2F). Then we questioned the origin of such a rescue.

"Fish diet"-induced bone rescue is correlated with fat mass gain

Bone growth and mineralization may be stimulated by mechanical loading. In order to elucidate whether body mass may be involved in the bone phenotype rescue we investigated the effects of diets on total body, adipose tissues and muscle weight gain. As revealed by Fig. 3A, fish diet induced a significant raise of 70% in total body weight while borage diet showed no impact despite the absence of difference in total food intake (Fig. 3B). Then, total body weight gain was tested for correlation with BMD values. Pearson analysis demonstrates that fish

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

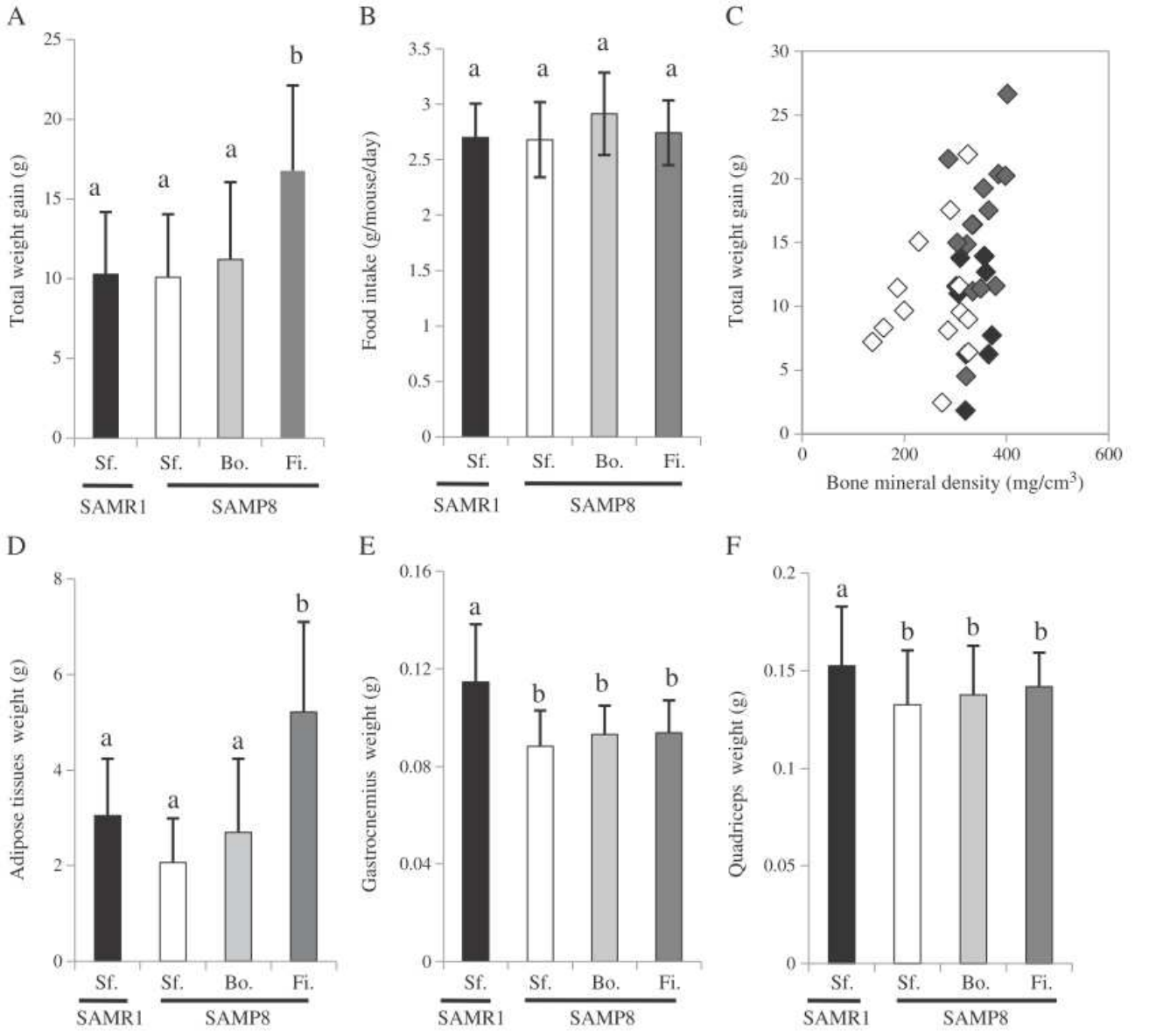


Fig. 3. Weight tissues: A: total body weight gain over the complete study. B: food intake. C: Pearson correlation between bone mineral density and total body weight gain. D, E and F: weight analysis for visceral adipose tissues (D), gastrocnemius muscles (E) and quadriceps muscles (F). (Sf: Sunflower diet; Bo: borage diet; Fi: fish diet). Significant different groups are represented by different letters as analyzed by ANOVA ($p<0.05$).

diet shows a significant correlation between BMD and body mass increase with a Pearson correlation value of +0.345 and a *p*-value of 0.037 (Fig. 3C). To determine the origin of fish diet-induced total body weight gain, measures of fat and lean tissues weight were performed when mice were sacrificed. The data obtained clearly show that only fat mass parallels with body weight gain with an increase of approximately 73% for fish diet (Fig. 3D). In contrast, muscle mass was altered in SAMP8 groups with a rough 20% and 10% weight loss for gastrocnemius and quadriceps respectively (Fig. 3E and F). None of the diets were able to counteract the muscle loss suggesting a strain effect rather than a nutritional effect.

Both, "fish" and "borage" diets restore inflammatory parameters in SAMP8 mice

In contrast to mechanical loading, inflammation is a major contributor to osteoclastogenesis and subsequent bone resorption. Then the hypothesis was tested whether effects of diets on bone may originate from inflammatory parameters modulation. As a relevant indicator of global inflammatory status, spleen weight was measured for the four groups. Data enlighten a significant higher organ weight under sunflower treatment in SAMP8 that may be prevented by borage and fish oils addition (Fig. 4A/see Supplementary data A for SAMP8 growth diet control). Therefore, it is a major interest to notice that for all groups this increase in spleen weight was negatively correlated with the BMD (Pearson value: −0.208/*p*-value: 0.044; Fig. 4B). In order to strengthen our results, we explored the blood level of inflammatory markers such as C reactive protein (CRP). As expected, data obtained linked with spleen weight measures. However, only fish diet significantly lowered CRP values back to SAMR1 values (Fig. 4C/see Supplementary data B for SAMP8 growth diet control).

As a matter of fact, when analyzing transcriptomic data from TLDA performed on bone tissue samples, fish diet significantly lowered

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

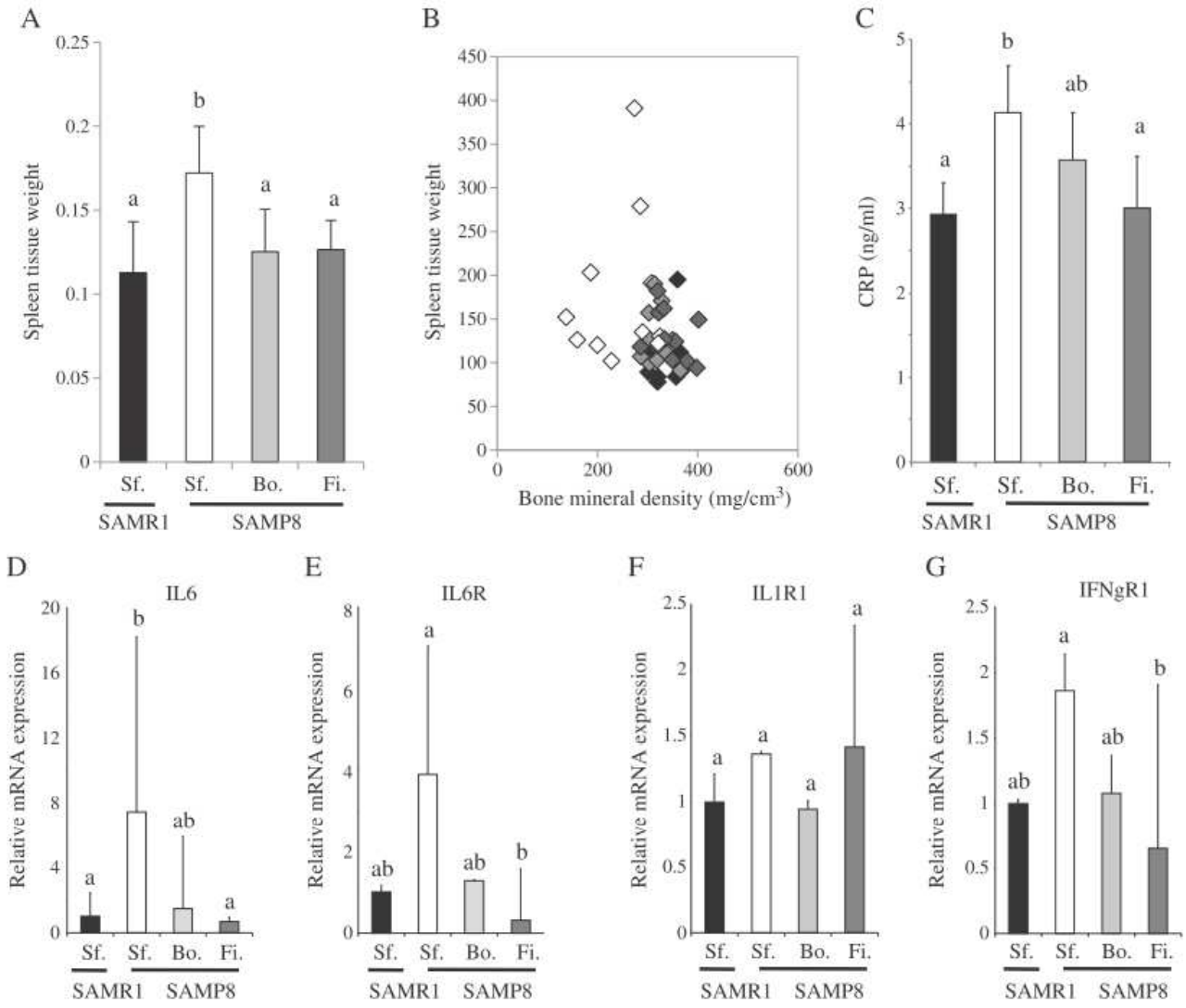


Fig. 4. Inflammation parameters: A: spleen weight measurements. B: Pearson correlation between spleen weight and bone mineral density. C: C-reactive protein analysis in blood samples. D–G: transcriptomic analyses on bone tissues. Expression of inflammatory markers were determined by Taqman Low density Arrays (IL-6, IL-6R, IL-1R1, IFNgR1). Only significant variations and relevant markers are presented. (Sf: Sunflower diet; Bo: borage diet; Fi: fish diet). Significant different groups are represented by different letters as analyzed by ANOVA ($p<0.05$).

expression of inflammation markers. For instance, expression of cytokines and corresponding receptors responsible for mediating inflammatory responses such as IL-6, IL-6R and IFNgR1 were increased in SAMP8 under sunflower diet and brought back to SAMR1 values when SAMP8 were fed a borage or fish enriched diet (Fig. 4D, E, G). In contrast, nutritional treatment did not affect other inflammatory mediators such as IL-1R1 (Fig. 4F) or TNFα (data not shown). Despite dissimilarities in statistic significances, taken together, the results strongly suggest that both diets impact inflammation establishment that may account in return for bone loss prevention.

Fish and borage diets affect bone remodeling markers

Keeping on searching for the origin of diet-induced bone rescue, efforts were focused to question whether bone remodeling or bone differentiation markers may be impacted by one diet or another. Then, to evaluate the rate of bone matrix degradation, blood samples were harvested for CTX1 quantification (Fig. 5A). Despite the lack of significance due to a great amplitude in the data obtained from the SAMP8 sunflower group, the results show that both borage and fish diets tend to prevent from the CTX-1 level increase. This trend supports a lesser osteoclastic activity and consequently a lower resorption rate and bone loss.

Thus, in support to blood investigations, transcriptomic analyses were performed on bone tissue samples (whole femorae). Using, TLDA database and ANOVA analysis, both fish and borage groups' expressions for studied markers were compared to SAMP8 and SAMR1 sunflower diet groups. Due to the amount of targets, only remarkable bone markers expressions with the most significant differences were represented. As showed in Fig. 5B–I expression of both pro-resorption and pro-formation markers was impacted. Expression of key enzymes contributing to bone resorption was altered. MMP2 and MMP9, involved in collagen degradation showed decreased expression as compared to the SAMP8 sunflower group (MMP9: −55% for fish diet, MMP2: −50% under both borage and fish diets) (Fig. 5B,C). Besides collagen degradation, osteoclast differentiation was also down-regulated by both borage and fish as demonstrated by the drop in the calcitonin receptor (CTR) expression (−80% and−60% respectively) (Fig. 5D). This

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

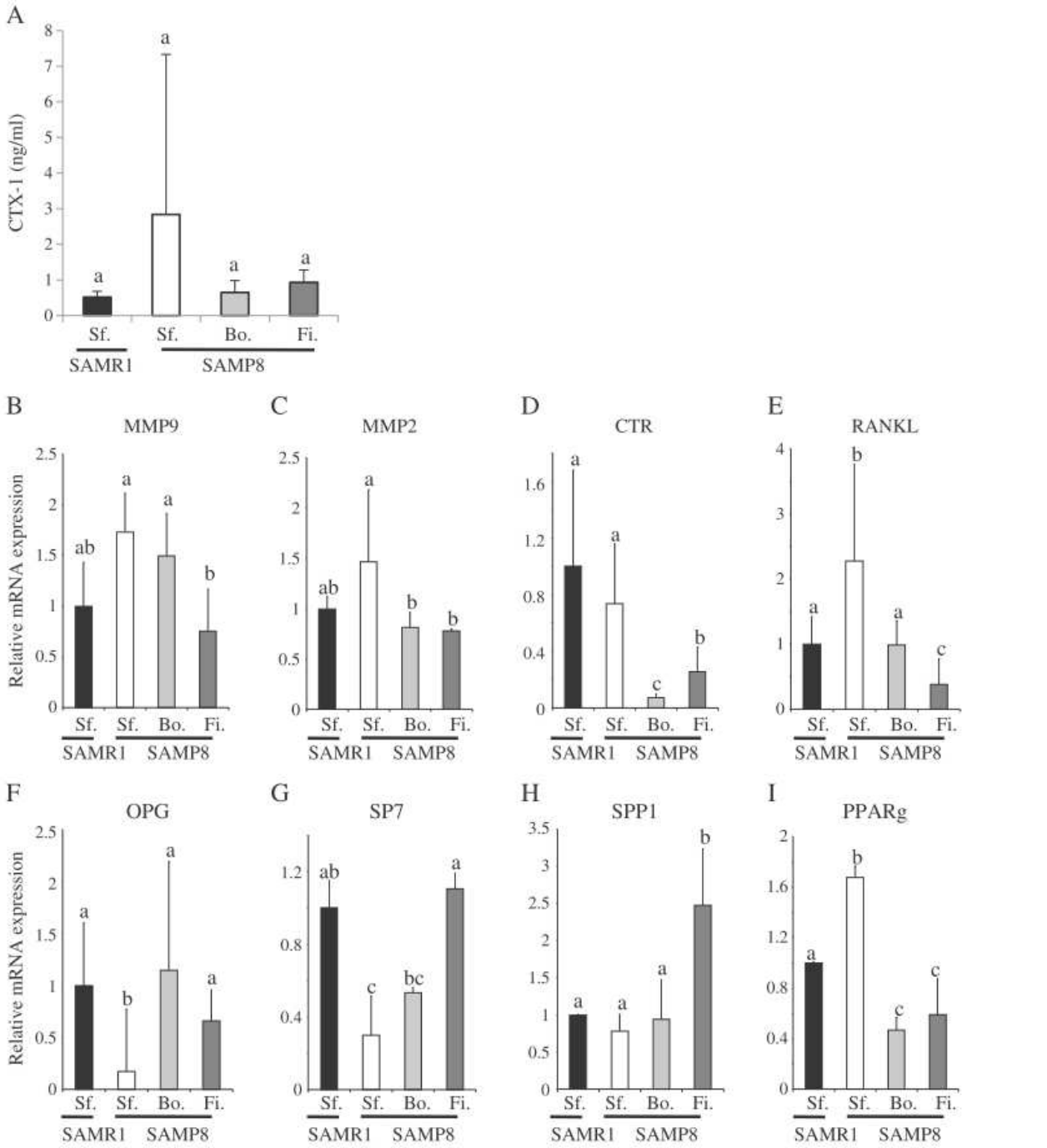


Fig. 5. Bone remodeling parameters. A: resorption marker CTX-1 determination. B–I: transcriptomic analysis on bone tissues. Expression of the bone cell markers were determined by Taqman Low density Arrays (MMP9, MMP2, CTR, RANKL, OPG, SP7, SPP1, PPARg). Only significant variations and relevant markers are presented. (Sf: Sunflower diet; Bo: borage diet; Fi: fish diet). Significant different groups are represented by different letters as analyzed by ANOVA ($p<0.05$).

decrease in mature osteoclast markers may be associated with a modulation of the osteoblast-induced osteoclast differentiation. As a matter of fact, borage and fish induced higher OPG and lower RANKL transcription levels leading to a higher OPG/RANKL ratio (Fig. 5E, F).

In contrast to osteoclast markers, expression of osteoblast differentiation markers such as SP7 (osterix) and SPP1 (osteopontin) was stimulated as compared to SAMP8 sunflower group by fish diet (+310% and +370% respectively) (Fig. 5G, H). In addition, both diets reduced PPAR expression (approximately −70%), a transcription factor involved in osteoblast/adipocyte commitment thus favoring osteoblast differentiation (Fig. 5I).

Discussion

Osteoporosis represents a major health concern for aging populations that might be prevented by nutritional strategies [13]. In our study, we investigated the effect of different fat-supplemented diets on a mice progeria model. Taken together, our data demonstrate that both borage and fish oil enriched diets were able to counteract aging-

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

associated bone loss, mostly by preventing inflammation establishment and its consequences on bone cell behavior.

In this work, we first established that our experimental model reproduced osteoporosis without affecting bone length. Indeed, SAMP6 mice are classically described for their osteoporotic features rather than SAMP8. However in a context of multi disciplinary approaches, the SAMP8 model was chosen for exhibiting both muscle and bone weakness [11,12]. In addition, SAMP8 mice exhibit neurological disorders resembling to Alzheimer disease that is associated with aging complications. In this light, SAMP8 correctly mimics aging features and patho-physiologic environment regarding senile osteoporotic establishment in humans.

In this model, sunflower oil diet significantly exacerbated bone loss only in SAMP8. This observation is consistent with the literature on ω6 effects on bone as demonstrated by Martinez-Ramirez showing that risk of fracture in elderly is positively associated with ω6 fatty acid intake [14]. As a matter of fact, sunflower oils exhibit a high Linoleic acid/α-linolenic acid ratio. This high ratio is acknowledged for its pro-inflammatory effects. Given the fact that aging-associated bone loss is also widely related to increased inflammation, the study aimed at deciphering whether fish or borage enriched sunflower-based oil could counteract pro-inflammatory mechanisms and prevent senile osteoporosis in conditions that mimic western food habits.

The high Linoleic acid/α-linolenic acid ratio was proven to lead to increased production of PGE-2 by cyclooxygenases [15]. In return, PGE2 contributes to bone loss by favoring inflammatory context establishment [10] and osteoclastogenesis [5,6]. In our study, sunflower-associated bone loss and inflammation were counteracted both by borage and fish oil supplementation.

Fat diets may induce weight gain that favors bone formation by mechanical loading [16]. In our model, only fish diet induced weight gain. Since, this gain was significantly associated with BMD, this parameter should be taken into account to explain BMD rescue. Weight gain may result from fat or lean mass gain. In our hand, weight gain induced by fish diet was only correlated with fat mass gain. In contrast, investigation on muscle tissues showed weight decrease for all SAMP8 groups. As osteoporosis, sarcopenia is associated with aging. Characterized by muscle weakness and loss, sarcopenia accelerates osteoporotic features [17]. In this context, this strain effect suggests that BMD rescue may not be associated with a better muscular health. However, fat increase is associated with estrogen production by adipocyte [18] which might explain part of fish oils effects on BMD rescue.

Besides mechanical loading, inflammation plays a major role in the modulation of bone remodeling. The literature widely acknowledges that inflammatory context greatly stimulates bone resorption while inhibiting formation [19,20]. This process includes an increased of RANKL production by osteoblast and activated immune cells that promotes osteoclast formation, activation and survival. As an indicator of the global inflammatory status, spleen weight analysis and determination of CRP concentration in serum were performed and negatively correlated with BMD. As a matter of fact, both spleen weight and CRP concentration were increased in SAMP8 versus SAMR1. These data are consistent with enhanced inflammatory status observed with aging [21]. However, in our model, this raise was not only related to aging but associated with aging in the presence of sunflower diet (see supplementary data). Therefore, inhibition, by borage and fish diets, suggests that both borage and fish may rescue BMD phenotype via a potent anti-inflammatory effect during aging process. These data are consistent with the literature. As mentioned above, sunflower oils present a low ω3/ω6 ratio favoring PGE2 synthesis, inflammatory context establishment and subsequent bone resorption [5,6,10]. In contrast, in fish oils, ω3/ω6 ratio is higher and preferentially leads to PGE3 synthesis [8]. PGE2 and PGE3 exhibit the same effect on bone resorption, however, conversion to prostaglandins from ω3 PUFAs is less effective than from ω6, resulting in lower PGE3 levels [7,8]. Borage effects on BMD might originate from higher concentration of GLA. As a matter of fact, conversion of GLA by cyclooxygenases leads to PGE1 production which presents anti-inflammatory effects that may benefit to bone and rescue BMD [9]. In support to this hypothesis, spleen weight and CRP levels were correlated with a decrease in IL-6 and IL-6R expressions in bone tissues.

To further investigate cellular mechanisms responsible for BMD rescue, bone marker expression for cell differentiation, activity and communication was harvested by Taqman Low Density Arrays. As expected, borage and fish impacted bone cell transcription patterns. Both decreased RANKL expression. This down-regulation is consistent with the reduced inflammatory context [19,20]. However, transcriptomic analyses were done on the whole femur which does not allow distinguishing the origin of RANKL (osteoblast, stromal cells or immune cells). Together with OPG increase expression these results suggest that borage and fish oil supplementation leads to decreased osteoblast-induced osteoclastogenesis [22,23]. Effects of fish oils on BMD in our model are consistent with Rahman et al.'s study [24]. They demonstrated that DHA, decreases osteoclast formation and activity by reducing NFkB expression and DNA binding thus preventing from RANKL signaling and associated osteoclastogenesis. DHA is a ω3 highly represented in fish oils and its presence may contribute to explain the observed result on BMD protection. However, our results contrast with the work from Poulsen's group [25] revealing that GLA may stimulate RANKL expression in the murine MC3T3-E1 [5] osteoblast-like cell line. This discrepancy may originate from the model. As mentioned above, transcriptomic analyses were performed on total femorae, which includes not only osteoblasts but also fibroblasts, dendritic cells and lymphocytes that produce RANKL in bone marrow environment [26]. Our hypothesis, that fish and borage target osteoclastogenesis was further supported by the observed decrease in CTR expression a marker of mature and activated osteoclasts. Together with the decrease of bone resorbing enzyme expression such as MMP-2 and MMP-9, these results provide a potent explanation for the BMD rescue by inhibiting OC differentiation and activity.

Besides, both osteoblasts and adipocytes are derived from bone-marrow mesenchymal stem cells (MSCs) [27]. It has been postulated that age associated bone loss partly comes from a decreased number of bone forming osteoblasts, and an increased number of adipocytes in the bone marrow, resulting from a preferential adipocytic differentiation of the MSCs [28]. The adipocyte-specific transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ) acts as a critical positive regulator of marrow adipocyte formation and as a negative regulator of osteoblast development [29]. In vivo, increased PPAR-γ activity leads to bone loss, similar to the bone loss observed with aging [30,31], whereas decreased PPAR-γ activity results in increased bone mass [32]. In our model, PPAR-γ mRNA expression is reduced in the femurs of fish oil and borage oil fed mice which may contribute to the beneficial effect of these diets on bone.

We demonstrated for the first time that borage and fish diets restored inflammation and bone parameters using an original model of senile osteoporosis that mimics clinical features of aging in humans. This study enlightens the duality of fat diets. In one hand, sunflower oil exacerbated bone loss, in another, borage and fish oils protected bone health.

Our data strongly parallel with clinical observation and provide further explanation on cellular mechanisms of action, suggesting that fish and borage oil supplementation may act through inhibition of osteoclastic resorption by abrogating increased inflammatory context and osteoblast-induced osteoclast formation. Then, this encouraging data suggest that nutritional approaches are relevant regarding osteoporosis and offer promising alternatives in the design of new strategies for preventing aged-related locomotor dysfunctions.

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

Nevertheless, further in vitro studies are now required to fully determine components responsible for biological effects and to decipher underneath molecular mechanisms.

Supplementary materials related to this article can be found online at doi:10.1016/j.bone.2011.05.030.

Author disclosures

Fabien Wauquier, Valentin Barquissau, Laurent Léotoing, Marie-Jeanne Davicco, Patrice Lebecque, Sylvie Mercier, Claire Philippe, Elisabeth Miot-Noirault, Jean-Michel Chardigny, Béatrice Morio, Yohann Wittrant and Véronique Coxam have no conflicts of interest.

Acknowledgments

This work was supported in part by the Departement Alimentation Humaine —ANSSD —INRA. The authors would like to thank Dr. Jean Michel Chardigny (UMR INRA-ENESAD Flaveur, Vision et Comportement du consommateur, 21065 Dijon Cedex, France.) for providing the SAM mice with the authorization of Dr. Hosakawa, Aichi, Japan. The authors are as well grateful to (1) C. Jouve and JP. Rigaudière for their help in collecting the samples and data; (2) to the people from the "Animal lab: Installation Experimental de Nutrition" who provided every day cares to the mice; and (3) Latifa Rbah for her assistance in conducting analysis of bone mineral density.

References

[1] Cooper C. Osteoporosis: disease severity and consequent fracture management. Osteoporos Int Jun 2010(Suppl 2):S425–9.
[2] Ozgocmen S, Kaya H, Fadillioglu E, Aydogan R, Yilmaz Z. Role of antioxidant systems, lipid peroxidation, and nitric oxide in postmenopausal osteoporosis. Mol Cell Biochem Jan 2007;295:45–52.
[3] Mann V, Huber C, Kogianni G, Collins F, Noble B. The antioxidant effect of estrogen and selective estrogen receptor modulators in the inhibition of osteocyte apoptosis in vitro. Bone Mar 2007;40:674–84.
[4] Valverde P. Pharmacotherapies to manage bone loss-associated diseases: a quest for the perfect benefit-to-risk ratio. Curr Med Chem 2008;15:284–304.
[5] Ono K, Kaneko H, Choudhary S, Pilbeam CC, Lorenzo JA, Akatsu T, et al. Biphasic effect of prostaglandin E2 on osteoclast formation in spleen cell cultures: role of the EP2 receptor. J Bone Miner Res Jan 2005;20:23–9.
[6] Tsutsumi R, Xie C, Wei X, Zhang M, Zhang X, Flick LM, et al. PGE2 signaling through the EP4 receptor on fibroblasts upregulates RANKL and stimulates osteolysis. J Bone Miner Res Oct 2009;24:1753–62.
[7] Laneuville O, Breuer DK, Xu N, Huang ZH, Gage DA, Watson JT, et al. Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)-octadecatrienoic acids from alpha-linolenic acid. J Biol Chem Aug 18 1995;270:19330–6.
[8] Raisz LG, Alander CB, Simmons HA. Effects of prostaglandin E3 and eicosapentaenoic acid on rat bone in organ culture. Prostaglandins May 1989;37:615–25.
[9] Corwin RL. Effects of dietary fats on bone health in advanced age. Prostaglandins Leukot Essent Fatty Acids Jun 2003;68:379–86.
[10] Calder PC. n−3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr Jun 2006;83:1505S–19S.
[11] Derave W, Eijnde BO, Ramaekers M, Hespel P. No effects of lifelong creatine supplementation on sarcopenia in senescence-accelerated mice (SAMP8). Am J Physiol Aug 2005;289:E272–7.
[12] Lo WC, Chiou JF, Gelovani JG, Cheong ML, Lee CM, Liu HY, et al. Transplantation of embryonic fibroblasts treated with platelet-rich plasma induces osteogenesis in SAMP8 mice monitored by molecular imaging. J Nucl Med May 2009;50:765–73.
[13] Kruger MC, Coetzee M, Haag M, Weiler H. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. Prog Lipid Res Oct 2010;49:438–49.
[14] Martinez-Ramirez MJ, Palma S, Martinez-Gonzalez MA, Delgado-Martinez AD, de la Fuente C, Delgado-Rodriguez M. Dietary fat intake and the risk of osteoporotic fractures in the elderly. Eur J Clin Nutr Sep 2007;61:1114–20.
[15] Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res Jan-Mar 2001;40:125–48.
[16] Felson DT, Zhang Y, Hannan MT, Anderson JJ. Effects of weight and body mass index on bone mineral density in men and women: the Framingham study. J Bone Miner Res May 1993;8:567–73.
[17] Saller A, Maggi S, Romanato G, Tonin P, Crepaldi G. Diabetes and osteoporosis. Aging Clin Exp Res Aug 2008;20:280–9.
[18] Reid IR. Relationships among body mass, its components, and bone. Bone Nov 2002;31:547–55.
[19] Hardy R, Cooper MS. Bone loss in inflammatory disorders. J Endocrinol Jun 2009;201:309–20.
[20] Mundy GR. Osteoporosis and inflammation. Nutr Rev Dec 2007;65:S147–51.
[21] Bruunsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. Curr Opin Hematol May 2001;8:131–6.
[22] Theoleyre S, Wittrant Y, Tat SK, Fortun Y, Redini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev Dec 2004;15:457–75.
[23] Wauquier F, Leotoing L, Coxam V, Guicheux J, Wittrant Y. Oxidative stress in bone remodelling and disease. Trends Mol Med Oct 2009;15:468–77.
[24] Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. J Cell Physiol Jan 2008;214:201–9.
[25] Poulsen RC, Wolber FM, Moughan PJ, Kruger MC. Long chain polyunsaturated fatty acids alter membrane-bound RANK-L expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells. Prostaglandins Other Lipid Mediat Feb 2008;85:42–8.
[26] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature May 15 2003;423:337–42.
[27] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature Jul 4 2002;418:41–9.
[28] Moerman EJ, Teng K, Lipschitz DA, Lecka-Czernik B. Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. Aging Cell Dec 2004;3:379–89.
[29] Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JM, Manolagas SC, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. Endocrinology Jun 2002;143:2376–84.
[30] Ali AA, Weinstein RS, Stewart SA, Parfitt AM, Manolagas SC, Jilka RL. Rosiglitazone causes bone loss in mice by suppressing osteoblast differentiation and bone formation. Endocrinology Mar 2005;146:1226–35.
[31] Rzonca SO, Suva LJ, Gaddy D, Montague DC, Lecka-Czernik B. Bone is a target for the antidiabetic compound rosiglitazone. Endocrinology Jan 2004;145:401–6.
[32] Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung UI, Kubota N, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Investig Mar 2004;113:846–55.

Please cite this article as: Wauquier F, et al, Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis, Bone (2011), doi:10.1016/j.bone.2011.05.030

Article numéro 3:

The free fatty acid receptor GPR40 protects from bone loss through inhibition of osteoclast differentiation

Fabien Wauquier, Claire Philippe, Laurent Léotoing, Sylvie Mercier, Marie-Jeanne Davicco, Patrice Lebecque, Jérome Guicheux, Paul Pilet, Vincent Poitout, Véronique Coxam, Yohann Wittrant

XXXX, 2011

L'article numéro 2 a permis de confirmer l'effet bénéfique de certains acides gras à longues chaînes dans la préservation du capital osseux *in vivo.* Ce rôle protecteur semble être lié, au moins pour partie, aux propriétés anti-inflammatoires des AGPI testés. Cependant, il est peu probable que l'effet des acides gras sur l'os se résume à une action anti-inflammatoire et les mécanismes régissant les interactions entre acides gras et tissu osseux sont encore loin d'être tous élucidés.

Parmi ces mécanismes potentiels, les effets directs des acides gras au niveau cellulaire par l'activation de récepteur spécifique occupent une place croissante dans la littérature. Récemment, les acides gras à longues chaînes ont été identifiés comme ligands naturels d'un récepteur membranaire couplé à une protéine G, le GPR40 (Briscoe, Tadayyon et al. 2003). Le rôle de ce récepteur est, à ce jour, essentiellement connu dans les cellules β des ilots de Langherans où il participe à la potentialisation par les acides gras de la sécrétion d'insuline en réponse au glucose (Itoh, Kawamata et al. 2003) (Latour, Alquier et al. 2007) (Brownlie, Mayers et al. 2008) (Vettor, Granzotto et al. 2008) (Doshi, Brahma et al. 2009) (Schmidt, Liebscher et al. 2011). Il pourrait également intervenir au niveau des cellules de l'intestin où il contribuerait à la sécrétion des incrétines (Edfalk, Steneberg et al. 2008) en réponse au acides gras ainsi qu'au niveau du système nerveux central où il serait le médiateur de l'induction, par l'acide docosahéxaénoique (DHA), de la différenciation de certaines populations neuronales (Ma, Tao et al. 2007) (Kaplamadzhiev, Hisha et al. 2010) (Ma, Zhang et al. 2010).

De manière intéressante, nous avons pu confirmer l'expression du GPR40 au niveau de cellules de la lignée ostéoclastique (Cornish, MacGibbon et al. 2008) et nous avons donc émis l'hypothèse qu'il pourrait contribuer à une modulation directe du remodelage osseux par les acides gras.

Ce travail a permis de mettre en évidence l'existence d'un phénotype ostéoporotique chez la souris invalidée pour le GPR40. L'effet protecteur de ce récepteur semble lié principalement à un effet inhibiteur de son activation sur la différenciation des ostéoclastes (Fig 23). En effet, l'utilisation de l'agoniste spécifique du GPR40 empêche in vitro la différenciation ostéoclastiques par RANKL de deux modèles cellulaires de manière GPR40-dépendante. De surcroit, cet agoniste est également capable in vivo de s'opposer à une perte osseuse induite chez la souris. Ces résultats révèlent pour la première fois l'implication du récepteur GPR40 dans la physiologie osseuse et apportent la connaissance d'une nouvelle possibilité de modulation directe des cellules osseuses par les acides gras.

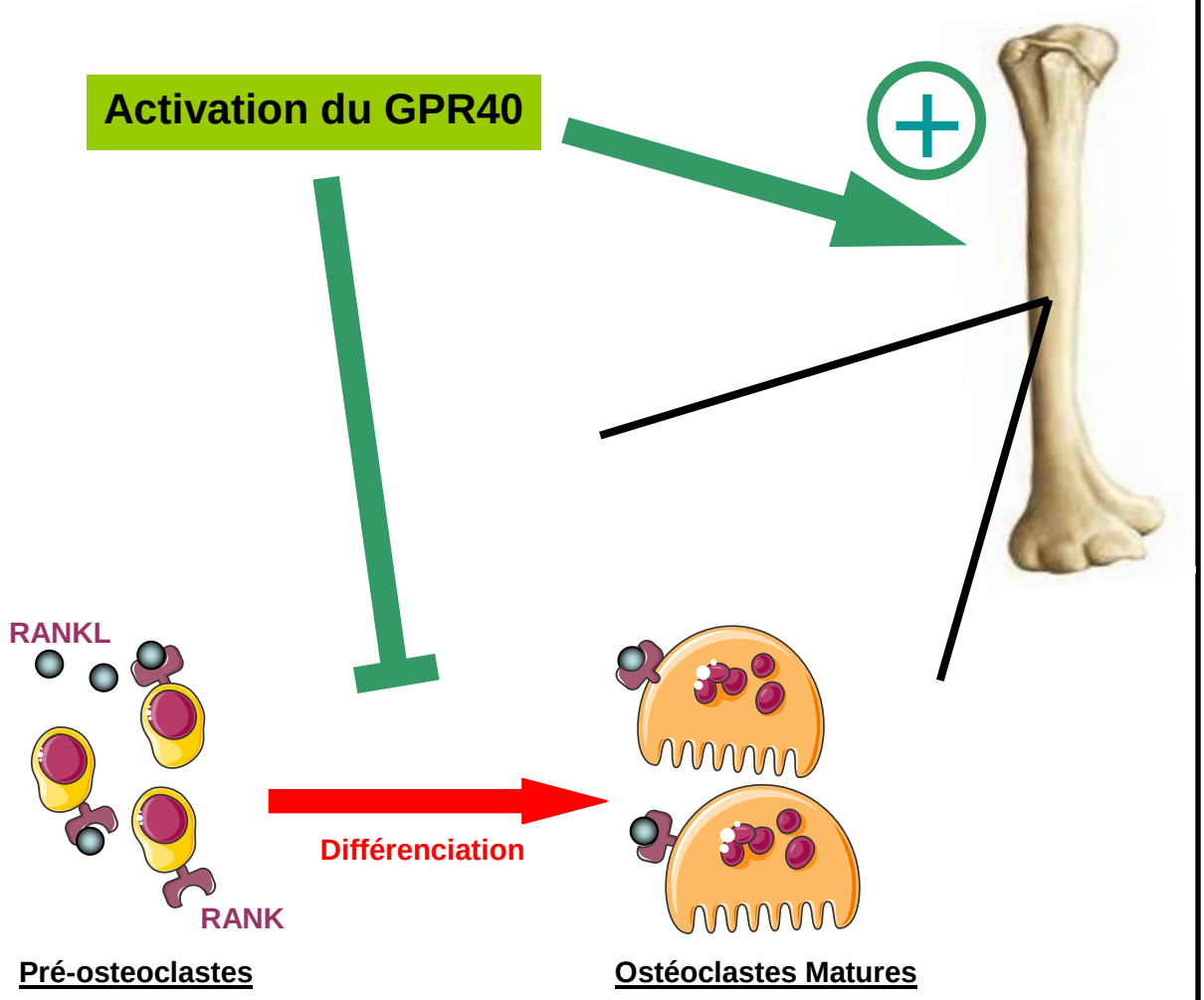


Figure 23 : Le rôle ostéoprotecteur de l'activation de GPR40 impliquerait une inhibition de la différenciation des ostéoclastes induite par RANKL

The free fatty acid receptor GPR40 protects from bone loss through inhibition of osteoclast differentiation

Fabien Wauquier[1,2,3], Claire Philippe[1,2,3], Laurent Léotoing[1,2,3], Sylvie Mercier[1,2,3], Marie-Jeanne Davicco[1,2,3], Patrice Lebecque[1,2,3], Jérôme Guicheux[4,5], Paul Pilet[4,5], Vincent Poitout[6], Véronique Coxam[1,2,3] and Yohann Wittrant[1,2,3*]

1. INRA, UMR 1019, UNH, CRNH Auvergne, F-63009 CLERMONT-FERRAND, FRANCE
2. Clermont Université, Université d'Auvergne, Unité de Nutrition Humaine, BP 10448, F-63000 CLERMONT-FERRAND, FRANCE
3. Equipe Alimentation, Squelette et Métabolismes
4. Inserm, UMR971, F- 44042 NANTES, FRANCE
5. Université de Nantes, UFR Odontologie, 1 Place Alexis Ricordeau, F- 44042 NANTES, FRANCE
6. Montréal Diabetes Research Center, MONTREAL, CANADA

* To whom correspondances should be addressed : e-mail : yohann.wittrant@clermont.inra.fr ; mail: UMR 1019 - Centre de Recherche INRA de Clermont-Ferrand/Theix ; Phone : +33 (0)4 73 62 47 84 ; Fax : +33 (0)9 57 05 99 12

Keywords:
GPR40, osteoclast, nutrition, fatty acids, osteoporosis

Introduction

Literature has widely linked lipid intake and inflammation status, a key protagonist involved in bone resorption (Ono, Kaneko et al. 2005) (Tsutsumi, Xie et al. 2009). As a matter of fact, inflammation favors bone degradation by stimulating osteoclast activity while inhibiting osteoblast bone formation leading to unbalanced bone remodeling and subsequent bone loss. Regarding inflammation, lipids exhibit a duality, with both pro- and anti-inflammatory effects depending on their structure and metabolism (Laneuville, Breuer et al. 1995) (Raisz, Alander et al. 1989). In this light, a growing body of evidence has revealed that ω-6 increase bone loss while ω-3 are believed to protect bone health (Corwin 2003) (Calder 2006) (Ono, Kaneko et al. 2005) (Tsutsumi, Xie et al. 2009). Nevertheless, this debate remains controversial and rather reductive. In fact, metabolites deriving from free fatty acids might not be the only ones responsible for mediating effects of fatty acids on bone remodeling. For the last decade, different receptors including nuclear and membrane-bound receptors have been extensively described for their involment in transducing a specific signal when interacting with free fatty acids. The mainly documented receptors belong to the PPAR, TLR and GPR receptor families.

Diascro et coll. were the first to demonstrate that a mixture of palmitic, oleic, and linoleic acids, activate the PPARs and induce adipocyte-like differentiation of both ROS17/2.8 and SaOS-2/B10 pre-osteoblast cell lines suggesting that fatty acids can initiate the switch from osteoblasts to adipocyte-like cells (Diascro, Vogel et al. 1998). Molecular mechanisms responsible for this unbalance were later described. At a cellular level, a fatty acids rich environment was proven to stimulate PPARγ activity while drastically reducing Runx2 expression and function, a transcription factor responsible for inducing osteoblastogenesis, in MC3T3-E1 and primary osteoblast precursors (Maurin, Chavassieux et al. 2005) (Jeon, Kim et al. 2003). Taken together, these data strongly support that our food habit in terms of fatty acids intake may have a dramatic impact on bone metabolism through PPARs activation. Besides, TLRs were recently acknowledged for binding saturated fatty acid and activate different intracellular signaling pathways (Hwang 2001). These interactions have been mainly described in the establishment of metabolic syndrome (Shi, Kokoeva et al. 2006). Indeed, mice model invalidated for TLR2 and 4 were shown to be protected against induced inflammation, increased adiposity and insulin resistance upon enriched saturated fatty acids diet (Coenen, Gruen et al. 2009) (Himes and Smith 2010).

In 2003, two teams demonstrated that GPR40, 41 and 43 were able to interact with free fatty acids and to transduce a specific signal across the cell membrane. Using Ca^{2+} mobilization probes and cellular models transfected with either GPR40, 41 or 43, Brown et al. showed that short chain fatty acids activated GPR41 and GPR43 while Briscoe demonstrated that GPR40 activation was restricted to medium and long chain fatty acids (Briscoe, Tadayyon et al. 2003) (Brown, Goldsworthy et al. 2003) (Schmidt, Liebscher et al. 2011). The roles of these fatty acids receptors were mainly documented in pancreatic tissues for their involvement in β-cell functions (Covington, Briscoe et al. 2006). Indeed, free fatty acids were found to regulate *in vitro* insulin secretion from pancreatic beta cells through GPR40 (Itoh, Kawamata et al. 2003). Using GPR40 deficient mice, Latour et al. lately showed that GPR40 stimulation contributes to fatty acid potentiation of glucose induced insulin secretion (Latour, Alquier et al. 2007). More recently, oxidative stress was proven as a key protagonist in the control of insulin secretion by palmitate through GPR40 stimulation (Graciano, Santos et al. 2011).

As expected regarding their role in pancreatic islets functions, GPRs expression was also evidenced in other tissues related to food metabolism for their role in nutra-sensing and fat storage. In another hand, G-coupled fatty acids receptors are expressed by immune cells such as leukocytes and monocytes (Nilsson, Kotarsky et al. 2003) (Oh, Talukdar et al. 2010). As bone tissues share common precursor cells with both the hematopoietic and the mesenchymal lineage, GPRs expression were investigated in bone cells (Cornish, MacGibbon et al. 2008). In our hand we confirmed for the first time the expression of GPR40 in bone cells both *in vivo* and *in vitro*. In addition, we hypothesized that GPR40 counteracts bone loss associated with detrimental properties of lipids and demonstrated that GPR40 is a key modulator of bone remodeling. Thus, our data bring new insight in the roles of GPRs in bone microenvironment and confirm the relevance of GPR40 as potent target in designing new therapeutic and/or nutritional strategies in the prevention of excessive bone loss.

Materials and Methods

Ethics.

All animal procedures were approved by the institution's animal welfare committee and were conducted in accordance with the National Research Council's guidelines for the care and use of laboratory animals. Animals were housed in the animal laboratory of the INRA Research (Center, Nguyen et al.) for Human Nutrition (http://www1.clermont.inra.fr/unh/telechargementinternet/ienplaquette.pdf). Throughout the study, animals were housed in a controlled environment (12:12 h light-dark cycle, 20-22°C, 50-60% relative humidity / 5 mice per plastic cage with free access to water / except for ovariectomy: mice were housed one per cage). Animals were delivered to our facility 2 weeks before study for acclimatization to our animal environment. At the end of the protocol, blood was withdrawn on anesthetized animals. Then, animals were sacrificed by cervical dislocation and tissues were harvested, frozen and stocked prior to investigation.

Animals.

GPR40-/- mice. GPR40-/- (knockout [KO]) mice were obtained from Dr. Vincent Poitout (Montréal Diabetes Research Center, Université de Montréal, Canada) in collaboration with AMGEN Inc. As described by Latour et al. (17395749), GPR40−/− mice on a mixed C57BL/6/129 background were generated by homologous recombination in embryonic stem cells at Lexicon Genetics (The Woodlands, TX). Exon 2 of the GPR40 gene was replaced with a LacZ gene. Pups were screened using PCR of genomic DNA. Wild-type (WT) littermates were used as controls.

Ovariectomized mice. After an acclimatization period, 9 weeks old C57BL/6jRj females were subjected to ovariectomy. After surgery, mice were housed one per cage for the total duration of the experiment and randomly divided into 3 distinct groups (n=7 per group; Sham operated/control vehicule; OVX/control vehicule; OVX/GW9508). Gavage started after a 24 hour recovery period. Mice received, three times per week, 100µl of either DMSO as control vehicule or GW9508 at the concentration of 8mg/kg of body weight/day for 5 weeks.

Primary bone marrow cultures for osteoclast formation

Bone marrow cells were isolated from 3- to 5-weeks-old wild-type or GPR40-/- C57/BL mice. Briefly, femurs were excised, and the marrow cavity was flushed with α-MEM

supplemented with 10% FCS. Cells were washed and plated in 1 ml of the same medium at a density of $2.5*10^6$ cells/cm^2. Cultures were grown either in complete medium alone (control) or in differentiation medium [rm-RANKL (50 ng/ml)(R&DSystems)]. Every two days, half the medium was removed and replaced with fresh medium with twice the final concentration of the treatment agent. After six days cultures were harvested for tartrate-resistant acid phosphatase (TRACP) activity or total RNA.

Cell lines

Raw264.7. The murine osteoclast precursor cell line RAW264.7 was obtained obtained from the American Type Culture Collection (ATCC® Number: TIB-71™). For osteoclast differentiation experiments, cells were seeded at a density of $3*10^4$ cells/cm² in α-MEM medium supplemented with10% FCS in the presence of 50ng/ml of recombinant murine RANKL (R&D Systems) for 6 days in combination or not with either GW9508 or DMSO as control vehicule; medium being changed every two days. For signaling experiments, cells were grown in α-MEM medium supplemented with10% FCS until about 80% confluency then serum starved for a total period of 48 hours for each condition.

MC3T3-E1 (4). As for RAW cells, MC3T3-E1 (4) pre-osteoblast cell line was obtained from the American Type Culture Collection (ATCC® Number: CRL-2593™). For viability assays, cells were seeded at a density of 10^4 cells/cm² in α-MEM medium supplemented with 2% FCS and cultured in the presence of either GW9508 or DMSO control vehicule for 24 hours prior harvesting.

Bone micro-architecture analysis

Micro-architecture (secondary spongiosa) was analyzed of left femurs of 14 weeks old mice. After removing soft tissues, femurs were placed into PBS buffer with 10% formaldehyde at 4°C for one week. All samples were imaged using X-ray radiation micro-CT (SkyScan 1072). To perform a measurement, the specimen was mounted on a turntable that could be shifted automatically in the axial direction (angular step: 0.675° / reconstruction angular range: 186.30°). Aluminum filter (0.5mm thick) was placed between X-rays source and sample. Pictures of 1024*1024 pixels were obtained using 37kV and 215µA. According to camera settings final pixels measured 5.664µm leading to a voxel of $1.817*10^{-7}mm^3$. Calculation of histomorphometric parameters was performed using CTAn ® and Nrecon® softwares, version 1.11. and 1.6.1.7 respectively.

Bone mineral density architecture

Bone morphological analysis was done using an eXplore CT 120 scanner (GE Healthcare, Canada). Frozen femora, cleaned of soft tissues, were scanned. Acquisition consisted of 360 views acquired in 1° increments collected in one full gantry rotation, with a 20 ms exposure/view and X-ray tube settings being 100 kV and 50 mA. CT images were reconstructed using a modified conebeam algorithm with an isotropic voxel of 0.045 x 0.045 x 0.045 mm^3. CT scans were analyzed using MicroView® version 2.3 software (GE Healthcare, Canada). A hydroxyapatite calibration phantom (SB3, Gamex RMI, WI) was used to convert gray-scale levels to HA density values. Trabecular bone of the distal femur was selected for bone mineral density and bone volume fraction (BVF = Bone Volume / Total volume) analyses by fitting a cylindrical region (r = 0.7mm) of interest in the centre of the femur, starting 0.1 mm proximally from the growth plate and extending a further 0.32mm in the proximal direction. Bone mineral density was estimated as the mean converted gray-scale level within the region of interest.

Body mass and composition

Sham and OVX mice filled with GW9508 or control vehicule were weighted every week throughout the experimental period. In addition, mice were subjected to body composition before on day 0 and 27 using QMR EchoMRI-900™ system. Whole body fat mass, lean tissue mass, free water, and total body water were measured in live animals without anesthesia or sedation. Results were expressed in grams and compared to total body mass.

Cell proliferation assays.

RAW264.7 and MC3T3-E1 (subclone 4) cells were seeded in a 96-well-plate at a density of 3500 cells per well, and cultured for 24h supplemented with 2% serum in the presence of vehicule (DMSO) or 10-100µM of GW9508 (Cayman Chemical - CAS 885101-89-3). The cell proliferation was determined by an XTT based method, using the Cell Proliferation Kit II (Sigma-Aldrich) according to the supplier's recommendations. The OD was determined at 450 nm.

TRAP assays

Enzymatic activity: TRAP activity was assayed according to standard methods using *p*-nitrophenyl phosphate as a substrate (22). Medium was aspirated from cultures, and cell

lysates were prepared using NP40 lysis buffer [50 mM Tris, pH 7.4; 250 mM NaCl; 5 mM EDTA; 50 mM NaF; 1 mM; Na3VO4; 1% Nonidet P40 (NP40); 0.02% NaN3; PMSF 1mM; protease inhibitor cocktail (Sigma Cat. # P-2714)]. Samples were incubated in 100 µl buffer solution [125 mM sodium acetate buffer (pH 5.2), 100 mM *p*-nitrophenyl phosphate (Sigma-Aldrich), and 1 mM L(+) sodium tartrate]. The production of *p*-nitrophenol was determined in 96-well plates by measuring the absorbance at 405 nm at 37 C. In the mean time, protein amounts were quantified to express data as the mean OD per minute per milligram protein.

Cell staining: In parallel experiments, cellular TRAP activity was analyzed using the leukocyte acid phosphatase kit (Sigma-Aldrich). Briefly, the cells were washed twice with PBS, fixed for 5 min with citrate/paraformaldehyde/acetone solution, and stained according to the manufacturer's instructions.

Cell transfection and NF-κB-dependent luciferase activity

RAW264.7 cells were transfected at approximately 80% confluency with 80 ng/cm2 of NF-κB-RE/pGL3 Basic-luciferase vector using Fugene HD transfection reagent (Roche) according to published methods (12620896). 24 hours post-transfection, cells were incubated with RANKL (50ng/ml) in the presence of vehicule (DMSO) or 50µM of GW9508 (Cayman Chemical - CAS 885101-89-3). At 6 and 24 hours after treatment, cell lysates were prepared and luciferase activity was quantified using the Luciferase assay kit (Promega). For each condition, data are expressed as relative light units/mg protein.

GPR40 silencing

GPR40 was specifically knockdown into RAW264.7 cells using the SureSilencing™ RNA Interference technology (SABiosciences, Qiagen). Briefly, at 80% confluency, RAW264.7 cells grown in 6-wells plates were transfected with either one of the four supplied mouse GPR40 specific neomycin/plasmid-based shRNA or a negative control. Fugene HD transfection reagent (Roche) was used as transfection reagent according to published methods (12620896). 48 hours post-transfection, cells were incubated with neomycin antibiotics (Sigma-Aldrich) to selectively grow cells carrying sh-plasmids and establish a long-term specific GPR40 knockdown RAW264.7 cell line. After one month of antibiotic treatment, growing cells were harvested for GPR40 expression by real-time RT-PCR. Colonies exhibiting the most knockdown efficiency were selected for further investigations.

Western blot analysis

Bone and visceral adipose tissues from wild-type and GPR40-/- mice as well as cultured cells were lysed and subjected to western blot analysis. Following harvesting dissected tissues and cultured cells were immediately chilled on ice and lysed for 30min using NP40 lysis buffer [50 mM Tris, pH 7.4; 250 mM NaCl; 5 mM EDTA; 50 mM NaF; 1 mM; Na3VO4; 1% Nonidet P40 (NP40); 0.02% NaN3; PMSF 1mM; protease inhibitor cocktail (Sigma Cat. # P-2714)]. Then, lysates were centrifuged at 13000g for 15min and pellets were discarded. Proteins were quantified using BCA kit (Sigma-Aldrich) according to manufacturer's protocol and 20µg of total protein were loaded and resolved on an 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Invitrogen). GPR40, IKK, phospho-IKK, IkBα, phosphor-IkBα (SER32), ERK1/2, phosphor-ERK1/2 and β-actin proteins were detected using specific antibodies from Santa Cruz Biotechnology (Santa Cruz, CA; GPR40: sc-28416 / β-actin: sc-47778), and Cell-signaling (2682; 2697; 4814; 2859; 9102; 9101), respectively. After exposure with the indicated primary antibody, the immunoblots were washed and incubated with goat antirabbit/mouse-coupled horseradish peroxidase (Santa Cruz) followed by chemiluminescence using ECL reagent (Amersham).

Real-time RT-PCR

Modifications of osteoclast markers in treated cell cultures were examined by RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with deoxyribonuclease I (1 U/µg) to remove any contaminating genomic DNA. RNA concentration was determined using Nanodrop technology. cDNA was obtained from 1µg total RNA using SuperScript® VILO™ cDNA synthesis kit according to the manufacturer's protocol. Then, RT reaction mixture is diluted 1/10 in autoclaved dionized water and two microliters are subjected to PCR using specific primers (200nM final each) and EXPRESS SYBR® GreenER™ qPCR Supermix Universal consistently with supplier's instructions. Primers were designed as following: cak : f : cgaaaagagcctagcgaaca / r : tgggtagcagcagaaacttg; ctr : f : tgcgaggggatctatcttca / r : gttggcactatcgggaacc; trap: f : ccagcgacaagaggttcc / r : agagacgttgccaaggtgat; mmp9: f : cgacatagacggcatccag / r : ctgtcggctgtggttcagt; nfatc1: f : gggtcagtgtgaccgaagat / r : ggaagtcagaagtgggtgga. Primers for 18S were used to ascertain that an equivalent amount of cDNA was synthesized. PCR amplification was performed using Eppendorf Mastercycler® ep Realplex. Reaction conditions were as follows: 2 min at 50°C (one cycle), 2 min at 95°C (one cycle), and 15 sec at 95°C and 1 min at 60°C (40 cycles). The relative expression was calculated using the

comparative ΔΔCt method after normalization against the expression of 18S rRNA. Data are expressed as relative mRNA expression where control signal was arbitrarily set at 1.

Taqman Low Density Arrays (TLDA)

Left femurs were isolated from mice and cleaned from any soft tissues as fast as possible on day 28 after the experimental period of treatment. Then, samples were immediately frozen into liquid nitrogen and crushed into powder prior to RNA extraction using Trizol method (Invitrogen). 1 μg of total RNA was converted to cDNA using the high capacity cDNA reverse transcription (RT) kit (Applied Biosystems). Ten microliters of total extract were incubated with 10 μl of 2× RT master mix. RT was performed as following (step 1: temperature 25 °C, time 10 min; step 2: temperature 37 °C, time 120 min; step 3: temperature 85 °C, time 5 min). The resulting cDNA was used immediately for TaqMan® low-density arrays (TLDAs) (Applied Biosystems 7900HT real-time PCR system), a real-time polymerase chain reaction (PCR) in a low-density array format, to assess simultaneously the expression of multiple genes.

Each TLDA card of the low-density array has 8 separate loading ports, each with 48 separate wells, for a total of 384 wells per card. Each 2-μl well contains specific, user-defined primers and probes, capable of detecting a single gene. 18S ribosomal RNA and actin served as the housekeeping genes. Each cDNA sample (100 μl) was added to an equal volume of 2× TaqMan® universal PCR master mix (Applied Biosystems). After gentle mixing and centrifugation, the mixture was transferred to a loading port on a TLDA card. The array was centrifuged twice for 1 min, each at 1200 rpm, to distribute the samples from the loading port to each well. The card was then sealed and PCR amplification performed using an Applied Biosystems Prism 7900HT sequence detection system (equiped with a TaqMan® low density array upgrade). Thermal cycler conditions were as follows: 2 min at 50 ° C, 10 min at 94.5 °C, 30 s at 97 ° C, and 1 min at 59.7 ° C for 40 cycles. Expression values were calculated using the comparative threshold cycle (C_T) method as directed by the manufacturer (User Bulletin No. 2, Applied Biosystems). Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the relative expression of transcripts for each gene. C_T values for each gene and for 18S and actin both as housekeeping genes, were calculated. The ΔC_T value [$\Delta C_T = C_T$ (Sam R1 sunflower fed mice) − C_T(18S and actin)] was used as the calibrator. Assigning to the calibrator value of 1.00, the relative expression of each gene was calculated in arbitrary units (AU) with the formula: $\Delta\Delta C_T = \Delta C_T$(sample) − ΔC_T(calibrator).

Statistics.

Data obtained were analyzed either by ANOVA Tukey's or Newman's Kell test (ExcelStat Pro software - Microsoft Office 2007).

Results

Lack of GPR40 is associated with bone loss

GPR40 function was originally described in pancreatic β-cells. Its expression was lately extended to other organs dealing with fatty acids metabolism such as gut; however the role of this receptor regarding lipids and bone interaction remains to be studied. Using a mouse model invalidated for global expression of GPR40 we investigated the potent role of this G-coupled protein receptor in bone tissues behavior. According to µCT analsis, mice lacking GRP40 expression exhibited osteoporotic features with reduced bone mineral density, bone volume and altered microarchitecture as revealed by a decreased trabecular thickness (Fig.1A). Thus when compared to control wild-type littermates mice, one may speculate a protective role of GPR40 on bone remodeling processes.

Then, to further investigate this hypothesis, we questioned the expression of GPR40 in bone tissue to determine whether GPR40 may directly influence bone cells activities. As a matter of fact, we showed for the first time that this receptor was expressed at the protein level in bone tissues. In the same time, we confirmed its absence in bone in GPR40-/- mice.

GPR40 agonist GW9508 inhibits RANKL-induced osteoclast differentiation

Using primary BMM cultures and the osteoclast precursor cell line RAW264.7 we confirmed the expression of GPR40 in osteoclasts and tested the effects of a GPR40 agonist the GW9508 on RANKL-induced osteoclastogenesis models. As shown by TRACP staining, addition of RANKL induced giant multinucleated TRACP+ cells formation (Fig2.A upper line). In contrast, osteoclast number was dramatically reduced in wild-type bone marrow GW9508 treated cultures as compared to control (Fig2.B left column). Regarding octeoclast markers, RANKL-induced TRAP, CTR, MMP9 and CatK expressions were down-regulated in the presence of the GPR40 agonist, thus supporting TRACP staining observations (Fig.2.C).

To further analyze this hypothesis, experiments were repeated in another model using the RAW264.7 cells as a pre-osteoclast cell line model. Data obtained paralleled with bone marrow cultures. In fact, although inhibition did not reach TRACP staining observations in bone marrow cultures, TRAP activity as measured by enzymatic assay was significantly altered by GW9508 (Fig.3.B). Moreover, RANKL-induced osteoclast marker expression was

powerfully inhibited by GW9508, thus validating both the hypothesis and the models (Fig.3.C).

GPR40 is required to mediate inhibition of osteoclastogenesis by GW9508

Despite the specificity of the GW9508 for the GPR40 receptor, invalidation models were analysed to fully investigate the involvement of the receptor activation in modulating osteoclastogenesis. Thus, as shown in figure 2, the absence of GPR40 in bone marrow cultures obtained from GPR40-/- mice led to the complete abolition of GW9508 effects. In this context, osteoclastogenesis parameters were kept induced in the presence of RANKL as demonstrated by both TRACP staining (Fig.2.A-B) and osteoclast marker expression (Fig.2.C).

In RAW264.7 cells, GPR40 was partially knock-downed by shRNA interference technologies. Inhibition of GPR40 was routinely estimated around 60% as shown in figure 3A. Then, cells were tested for GW9805 influence in the presence of RANKL. Interestingly, results paralleled with the bone marrow cultures with an overall inhibition of GW9508 effects on osteoclastogenesis associated with the receptor knock-down (Fig. 3B-C). However, regarding osteoclast markers expression, abolition of GW9508 effects was less effective with the highest doses of 50µM (Fig.3.C).

Altered cell differentiation by GW9508 is not related to any impact on cell viability

To insure that GW9508 inhibition of osteoclastogenesis was not related to cytotoxicity, cultures were tested for cell viability in the presence of increasing doses of GW9508. XTT analyses revealed that GW9508 did not exhibit significant toxicity within the range of concentration used for all experiments. Cytotoxicity only became remarkable from 100µM in RAW264.7 cells thus supporting a specific effect of the GPR40 agonist on cell differentiation at 10 and 50 µM (Fig.4.A).

GW9508 down-regulates osteoclastogenesis by blocking RANKL-induced signaling pathways

To further decipher molecular targets linking GPR40 activation to osteoclast differentiation inhibition, we haversted cell cultures for modulation of RANKL-induced signaling pathways. The main downstream pathway for RANK activation is the NF-κB system. Interestingly, GW9508 abrogated RANKL-induced phosphorylation of IkB and IKK (Fig.4.C) suggesting that GW9508 prevents from NF-κB activation, nuclear translocation and

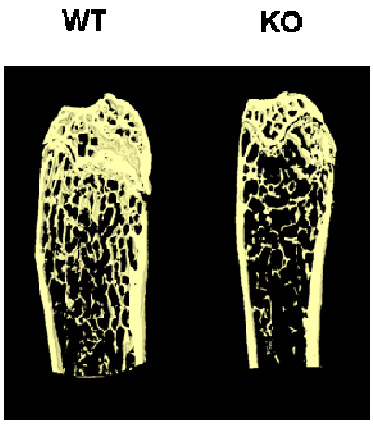
subsequent transcriptional activities. As a matter of fact, using RAW264.7 cell cultures transfected with a luciferase construct under the control of NF-κB response-elements, incubation with GW9508 in the presence of RANKL led to a drastic decrease luciferase activity, thus further supporting a global inhibition of the NF-κB pathway. GW9508 also altered ERK1/2 phosphorylation (Fig.4.D). Furthermore, GW9508 abrogated the RANKL-induced NFATc1 expression. Such inhibition did not occurred when GPR40 was knock-downed, thus strongly supporting a GPR40 dependent mechanism (Fig.4.E).

GW9508 counteracts bone loss *in vivo* in an ovariectomized mouse model

To further conclude with the potent bone protective effect of GPR40 agonist, mice were ovariectomized to induce bone loss and were given orally GW9508 or DMSO control vehicule to test whether *in vitro* results may translate to pre-clinical observations. As expected, ovariectomy significantly altered bone microarchitecture and mineral density (Fig.5.A-B). However, GW9508 was able to prevent from OVX-associated bone loss thus confirming the encouraging data obtained *in vitro*. Taken together, it is very tempting to speculate that observations may even translate to clinic. In addition, data from µCT analysis were reinforced by transcriptomic analyses exhibiting a decrease in osteoclast markers expression. In contrast, along with the rescued bone phenotype, OPG expression was restored in the presence of the GPR40 agonist (Fig.5.C). In another hand, analyses of body composition revealed that GW9508 counteracted the fat mass gain associated with ovariectomy without affecting lean body mass thus contributing to further neutralize clinical features associated with ageing and related bone loss.

A.

	GPR 40 WT	GPR 40 KO
BMD (mg/cm³)	359.96 ± 44.73	299.78 ± 54.29 *
BV/TV (%)	22.497 ± 4.576	17.886 ± 4.518 *
Tb.Th (mm)	0.158 ± 0.016	0.130 ± 0.039 *
Tb.N (1/mm)	1.425 ± 0.287	1.462 ± 0,526
Tb.Sp (mm)	0.179 ± 0.073	0.193 ± 0.036
Bone surface density (1/mm)	10.843 ± 1.664	9.233 ± 1.207*



B.

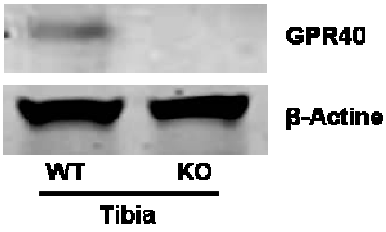


Figure 1. GPR40-/- mice exhibit an osteoporotic phenotype. A: MicroCT analysis of left femurs from wild-type (WT) and GRP40-/- (KO) mice (n=11); B: Western blot analysis of GPR40 expression in bone (tibia).

.

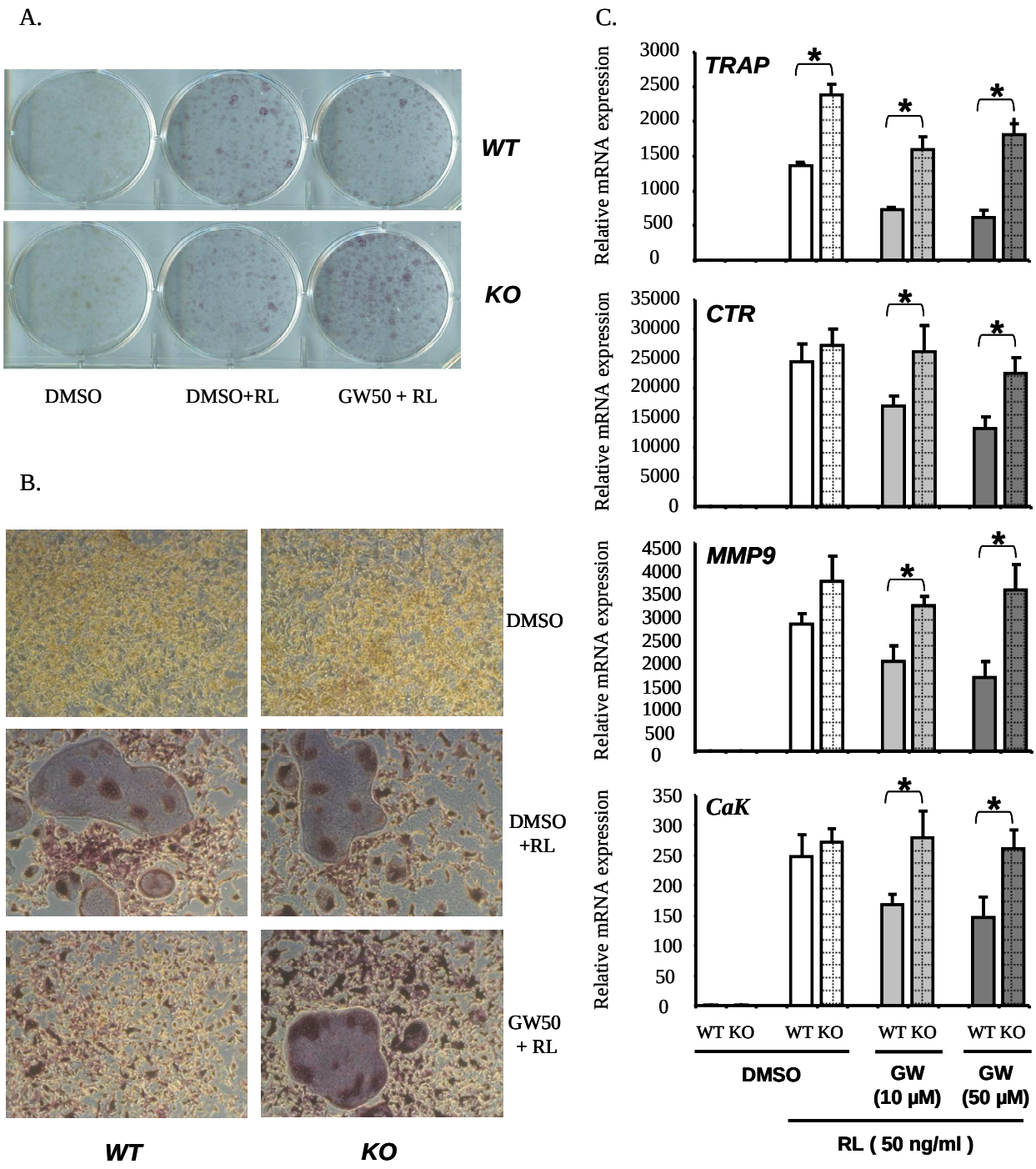


Figure 2. Osteoclast primary cell cultures obtained from bone marrow tissues. A and B: TRAP staining wild-type (WT) versus GPR40-/- mice (KO). Cells were either undifferentiated or treated with murine recombinant RANKL at the concentration of 50ng/ml to induce osteoclast differentiation. In these conditions, cells were either incubated with control vehicule (DMSO) or GPR40 agonist (GW9508; 50µM). C: Osteoclast marker expression analyzed by real-time RT-PCR; cells were treated as described above (* $p < 0.05$).

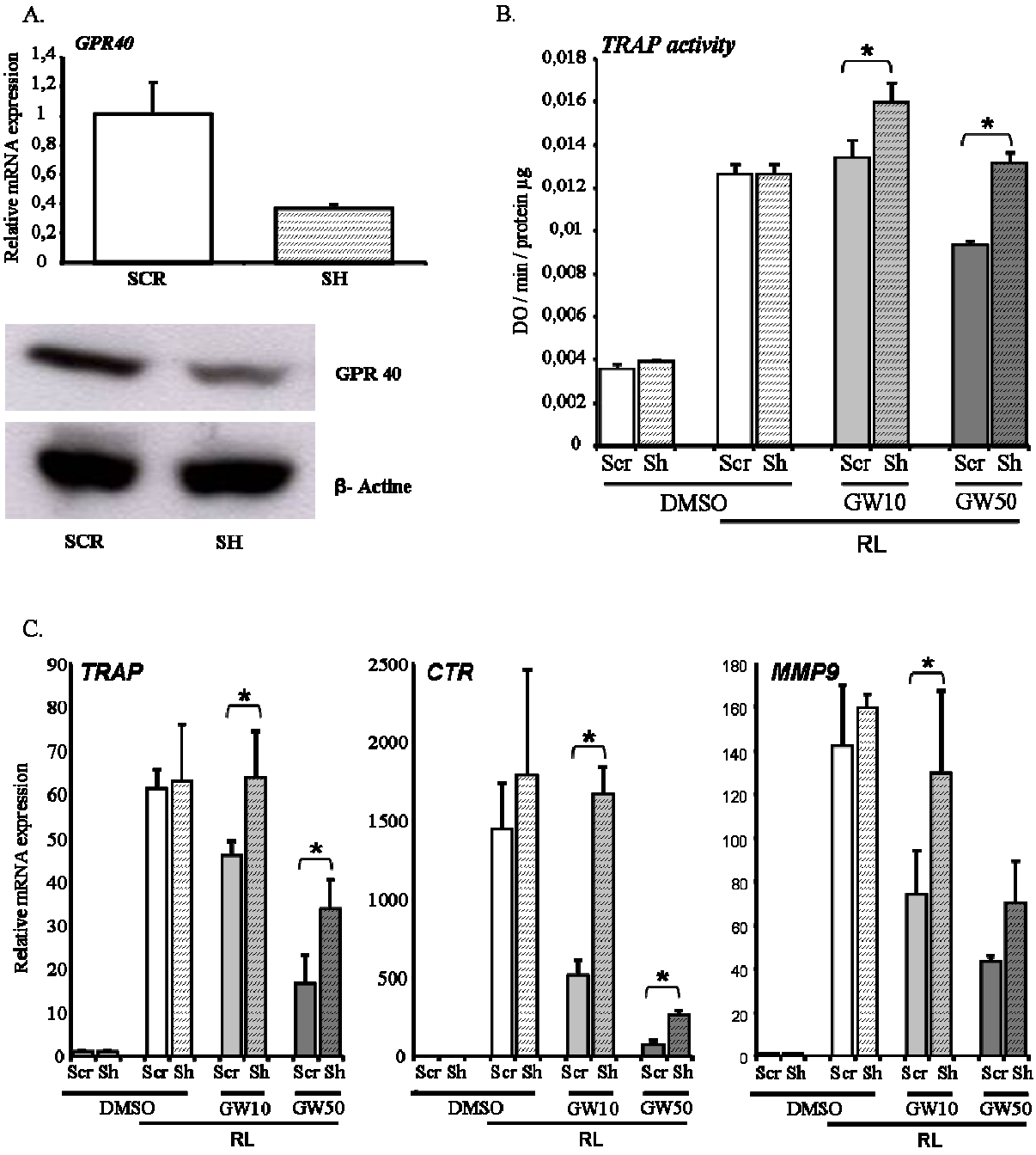


Figure 3. RAW264.7 cell cultures. A: Western blot analysis of cells invalidated for GPR40 expression (SH) or transfected with a non-targeting plasmid (SCR; control condition). B: TRAP enzymatic activity in SCR versus SH RAW264.7 cells. Cells were either undifferentiated or treated with murine recombinant RANKL at the concentration of 50ng/ml to induce osteoclast differentiation. In these conditions, cells were either incubated with control vehicule (DMSO) or GPR40 agonist (GW9508; 50µM). C: Osteoclast marker expression analyzed by real-time RT-PCR; cells were treated as described above (* $p < 0.05$).

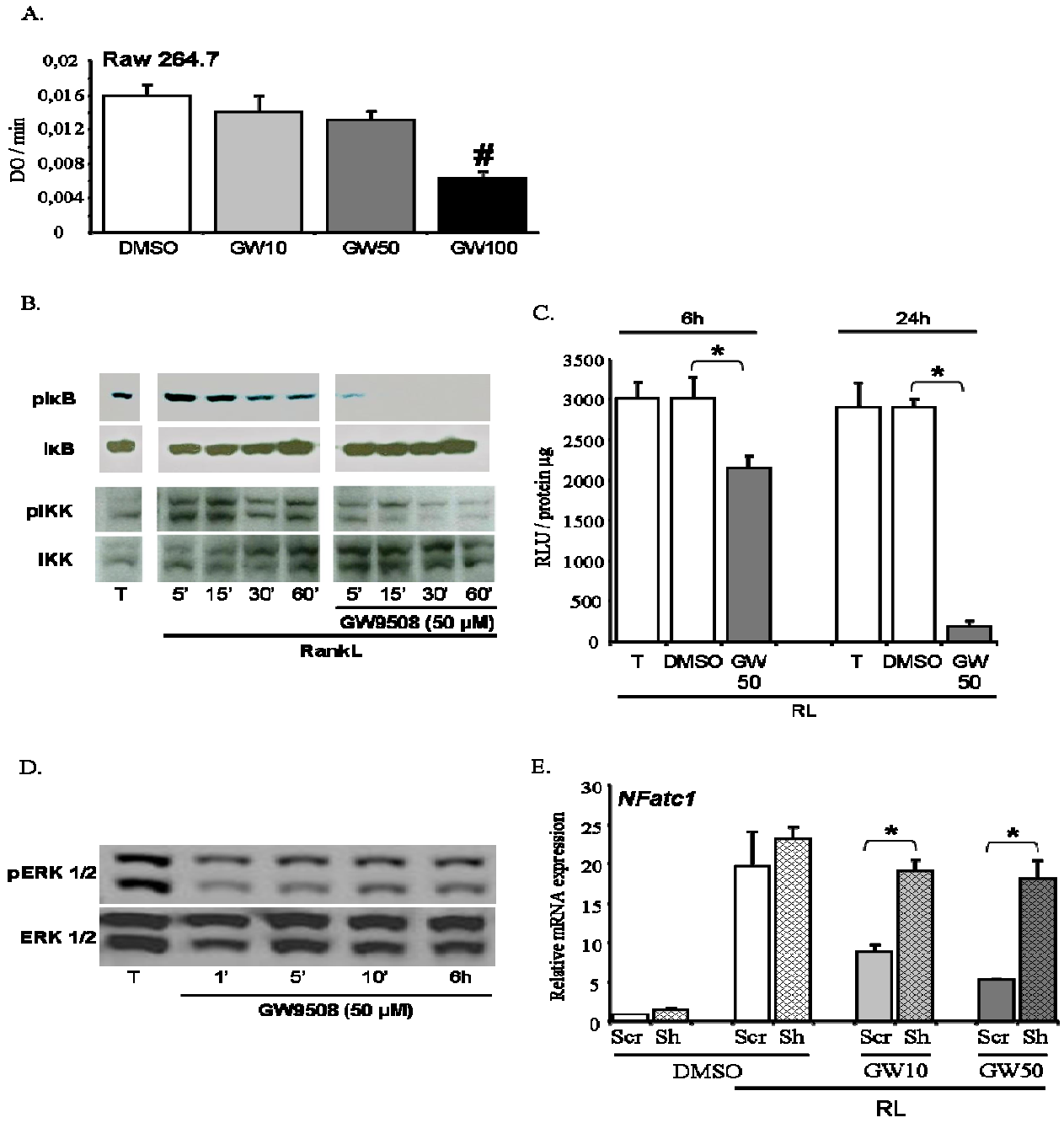


Figure 4. GPR40 agonist effects on RAW264.7 signaling pathways. A: XTT viability assay (# $p < 0.05$ vs DMSO control). C: NF-κB-dependent luciferase assay. Cells were treated with murine recombinant RANKL at the concentration of 50ng/ml to induce osteoclast differentiation. In these conditions, cells were incubated with GPR40 agonist (GW9508; 50µM) for increasing period of time (* $p< 0.05$). B and D: Western blot analysis of IkB, IKK and ERK1/2 phosphorylation; cells were treated as described above. E: NFatc1 expression analyzed by real-time RT-PCR in Scr versus Sh RAW264.7 cells upon GW9508 incubation (* $p < 0.05$).

A.

	Sham DMSO	OVX DMSO	OVX GW
BV/TV (%)	10.992 ± 2.041 [a]	7.381 ± 1.174 [b]	10.011 ± 1.473 [a]
Tb.Th (mm)	0,231 ± 0,029	0,205 ± 0,014	0,221 ± 0,027
Tb.N (1/mm)	0,482 ± 0,109 [a]	0,361 ± 0,059 [b]	0,457 ± 0,09 [ab]
Tb.Sp (mm)	1,919 ± 0,407 [a]	2,625 ± 0,436 [b]	2,026 ± 0,373 [a]
Bone surface density (1/mm)	7.501 ± 0.184 [a]	5.468 ± 1.001 [b]	6.724 ± 0.18 [ab]

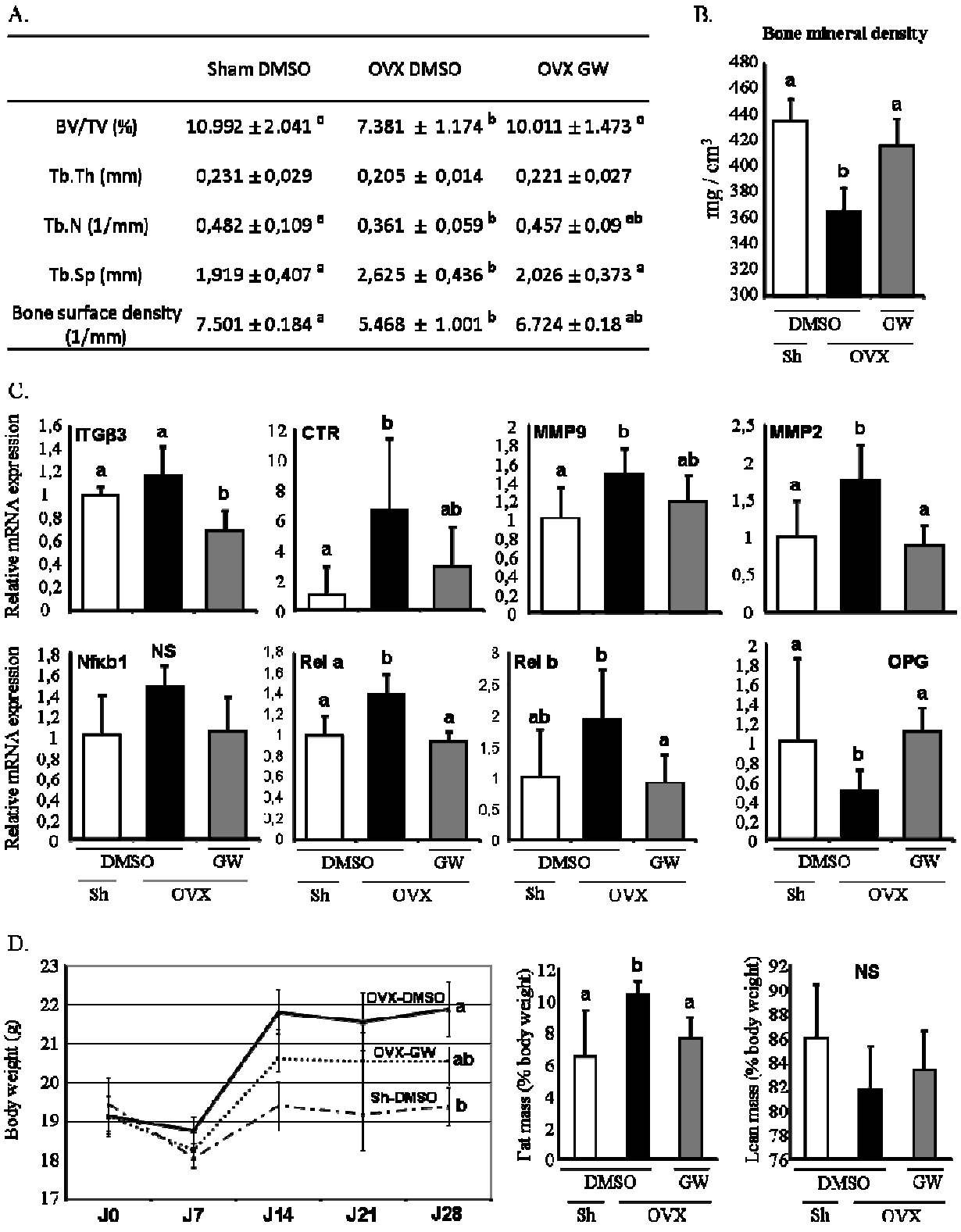


Figure 5. GPR40 agonist as a protective agent against OVX-induced bone loss. A and B: MicroCT analysis. C: TLDA analysis on tibiae. D: Mouse body weight and echoMRI body composition analysis. Significant different groups are represented by different letters as analyzed by ANOVA ($p < 0.05$)

Discussion / Conclusion

Besides their role in modulating inflammation parameters through metabolite production, free fatty acids have recently been acknowledged for directly binding membrane bound receptors. In this light, GPR40 have been identified as a key partner. However, the roles of such receptor at the body level are far from being fully determined. In this work, we showed for the first time that bone cells not only express the receptor GRP40 at a protein level but we further demonstrated its functionality and its crucial role in osteoclast behavior and subsequent bone remodeling.

Interestingly, our results further complete a previous study from Cornish et al. showing an inhibitory effect of saturated fatty acids (C:14 to C:18) on osteoclastogenesis (Cornish, MacGibbon et al. 2008). In their work authors showed expression of GPR40 and most notably GPR120 at the mRNA levels in RAW264.7 cells and hypothesized a potent role of these receptors in the saturated fatty acids mediated effects. Along with this study, Oh et al. recently demonstrated that GPR120 was responsible for mediating DHA anti-inflammatory impact when using the RAW264.7 cells in a macrophage differentiation system (Oh, Talukdar et al. 2010). Taken together, these data strongly correlate with ours, confirming the relevant interest of GPRs in mediating fatty acids effects on bone cells. However, Oh et al. conclude on a GPR40 independent effect due to the lack of GPR40 expression in their model. In our hand, we found that GPR40 was not only express at the mRNA level, but we confirm its expression also at the protein level. This discrepancy may result from both the heterogeneity of the cell line and the experimental design. Indeed, in Oh's study, RAW264.7 cells were used in a macrophage differentiation protocol rather than an osteoclast differentiation procedure. Moreover, consistently with the GPR40-/- mice bone phenotype, ex vivo and *in vitro* invalidation experiments, further demonstrated that GPR40 was required for mediating GW9508 effects on cells. Therefore, our results clearly enlighten the crucial role of GPR40 in regulating osteoclast maturation and strongly support a highly potent contribution of this receptor in bone and lipid nutrition relationships. However, primary cultures using GPR40-/- bone marrow cells showed greater evidence of GPR40 involvement in mediating GW9508 than data obtained from RAW 264.7 cells experiments. These results may originate from the incomplete invalidation of the receptor in the cell line (60%), thus leading to a potent activation of the remaining expressed GPR40.

In another hand, GPR40-/- mice were invalidated at a whole body level. Then, *in vivo* bone phenotype may also result from GPR40 invalidation impact on other organs that are known to influence bone metabolism. As a matter of fact, GPR40 function was mainly described from potentializing insulin secretion in the presence of long chain fatty acids in pancreatic β-cells (Latour, Alquier et al. 2007). Interestingly, insulin is a bone anabolic agent known to stimulate osteoblast function. Thus, one may speculate that pancreas lack of GPR40 expression may decrease insulin secretion and therefore contribute to the observed osteoporotic features. In the same way, GPR40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion (Edfalk, Steneberg et al. 2008). In return, incretins stimulate both insulin secretion and bone formation (Xie, Zhong et al. 2007) (Yamada, Yamada et al. 2008) (Ding, Shi et al. 2008). Taken together, these data reinforce the potent involvement of other organs interactions with GPR40-/- bone phenotype. Nevertheless, as demonstrated by cell cultures experiments (primary and cell lines), GPR40 was expressed and required in bone cells to mediate inhibition of osteoclast differentiation in the presence of a GPR40 agonist. These data perfectly correlate with the osteoporotic phenotype of invalidated mice and without excluding potent indirect influences, they strongly support the fact that GPR40 directly impact bone cells differentiation and functions.

GPR40 is not the only receptor described for directly binding long chain fatty acids and share this ability along with Toll Like Receptors and Peroxysome Proliferation Activator Receptors (Berger, Bailey et al. 1996) (Still, Grabowski et al. 2008) (Hwang 2001) (Shi, Kokoeva et al. 2006) (Coenen, Gruen et al. 2009) (Himes and Smith). PPARγ has been extensively studied for its crucial role in mensemchymal cells commitment and several groups have speculated that PPARs stimulation by fatty acids may control the switch between adipocyte, myoblast and osteoblast differentiation (Diascro, Vogel et al. 1998) (Maurin, Chavassieux et al. 2005) (Jeon, Kim et al. 2003). PPARs influence on osteoclastogenesis was investigated as well. However, PPARs effects on osteoclast remain controversial may depend on PPAR type. In fact, osteoclastogenesis was found to be inhibited upon PPARγ activation (Chan, Gartland et al. 2007; Hounoki, Sugiyama et al. 2008), whereas antisense strategies for PPARδ/β revealed a pro-resorbing impact of these two isoforms (Mano, Kimura et al. 2000). Considering PPARγ, Wan et al. uncovered a pro-osteoclastogenic effect of PPARγ by using a Tie2Cre/flox mouse model in which PPARγ is deleted in osteoclasts but not in osteoblasts (Wan, Chong et al. 2007). Interestingly, PPARγ agonists such as thiazolidinediones and rosiglitazone also stimulate GRP40 signaling pathways, therefore inhibition of osteoclastogenesis by PPARγ activators may in fact result from an uninvestigated GPR40

activation (Smith, Stoddart et al. 2009) (Gras, Chanez et al. 2009). Regarding osteoblasts, the question remains. As a matter of fact, in contrast to GPR40-/- mice, heterozygous PPARγ deficient mice were recently shown to exhibit enhanced bone formation with increased osteoblastogenesis (Viccica, Francucci et al. 2010). Taken together, these data suggest a strong antagonism between PPARγ and GPR40 in terms of impact on bone, thus challenging even more the determination of mechanisms linking bone and lipids relationships.

Toll-like receptors (TLRs) are known to play a crucial role in the immune response. Interestingly, in bone tissues, Johson et al. reported that mice deficient for TLR4 exhibit increased bone size with increased bone mineral content and density (Johnson, Riggs et al. 2004). In parallel, TLR4-/- mice were protected against induced inflammation, increased adiposity and insulin resistance upon enriched saturated fatty acids diet (Coenen, Gruen et al. 2009) (Himes and Smith 2010). These data strictly contrast with ours, and suggest that TLR4 and GPR40 activation by fatty acids may stimulate counteracting signaling pathways. Indeed, saturated fatty acids binding TLR4 at the cell surface of osteoclasts was demonstrated to increase activation of NF-κB (Oh, Sul et al. 2009) an essential transcription factor for osteoclast differentiation. In our hands, GW9508 induced a GPR40-dependent inhibition of the NF-κB transcriptional activity that stringently correlates with the GPR120-dependent anti-inflammatory effect of DHA as demonstrated by Oh et al. (Oh, Talukdar et al. 2010). Taken together, our data strongly suggest a potent inflammatory role of GPR40 activation. Then, it is very tempting to speculate that this potent anti-inflammatory role of GPRs may either contribute to anti-inflammatory effects of ω3 or counteract with pro-inflammatory potential of long chain saturated and ω6 metabolites (Wauquier, Barquissau et al. 2011).

Along with inflammatory parameters, body fat mass represents an additional risk factor for both sarcopenia and osteoporosis. Then, the reduction of fat mass gain upon GW9508 treatment may contribute to further explain how GPR40 may impact bone remodeling. However, unlike invalidated models, data do not allow us to definitely exclude a GPR120 contribution. Therefore, a similar experiment is now required using GPR40-/- ovariectomized mice.

In addition, it would be of major interest to determine whether modulation of fatty acids quality may impact on GPR40 activation. So far the literature data do not allow distinguishing between different LCPUFAs in terms of differential GPR40 binding and activation. Such investigations are now required to determine any preferential activation of GPR40 by different fatty acids type. Taken together, combined data from our study and the literature reveal the complexity the relationships between bone metabolism and lipid intake

thus requiring an integrated point of view. Moreover, investigations on osteoblastic cells are now needed to fully determine GPR40 impact on bone cells and lipids interactions.

References

Berger, J., P. Bailey, et al. (1996). "Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in db/db mice." Endocrinology **137**(10): 4189-95.

Briscoe, C. P., M. Tadayyon, et al. (2003). "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids." J Biol Chem **278**(13): 11303-11.

Brown, A. J., S. M. Goldsworthy, et al. (2003). "The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids." J Biol Chem **278**(13): 11312-9.

Calder, P. C. (2006). "n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases." Am J Clin Nutr **83**(6 Suppl): 1505S-1519S.

Center, J. R., T. V. Nguyen, et al. (1999). "Mortality after all major types of osteoporotic fracture in men and women: an observational study." Lancet **353**(9156): 878-82.

Chan, B. Y., A. Gartland, et al. (2007). "PPAR agonists modulate human osteoclast formation and activity in vitro." Bone **40**(1): 149-59.

Coenen, K. R., M. L. Gruen, et al. (2009). "Impact of macrophage toll-like receptor 4 deficiency on macrophage infiltration into adipose tissue and the artery wall in mice." Diabetologia **52**(2): 318-28.

Cornish, J., A. MacGibbon, et al. (2008). "Modulation of osteoclastogenesis by fatty acids." Endocrinology **149**(11): 5688-95.

Corwin, R. L. (2003). "Effects of dietary fats on bone health in advanced age." Prostaglandins Leukot Essent Fatty Acids **68**(6): 379-86.

Covington, D. K., C. A. Briscoe, et al. (2006). "The G-protein-coupled receptor 40 family (GPR40-GPR43) and its role in nutrient sensing." Biochem Soc Trans **34**(Pt 5): 770-3.

Diascro, D. D., Jr., R. L. Vogel, et al. (1998). "High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells." J Bone Miner Res **13**(1): 96-106.

Ding, K. H., X. M. Shi, et al. (2008). "Impact of glucose-dependent insulinotropic peptide on age-induced bone loss." J Bone Miner Res **23**(4): 536-43.

Edfalk, S., P. Steneberg, et al. (2008). "Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion." Diabetes **57**(9): 2280-7.

Graciano, M. F., L. R. Santos, et al. (2011). "NAD(P)H oxidase participates in the palmitate-induced superoxide production and insulin secretion by rat pancreatic islets." J Cell Physiol **226**(4): 1110-7.

Gras, D., P. Chanez, et al. (2009). "Thiazolidinediones induce proliferation of human bronchial epithelial cells through the GPR40 receptor." Am J Physiol Lung Cell Mol Physiol **296**(6): L970-8.

Himes, R. W. and C. W. Smith (2010). "Tlr2 is critical for diet-induced metabolic syndrome in a murine model." Faseb J **24**(3): 731-9.

Hounoki, H., E. Sugiyama, et al. (2008). "Activation of peroxisome proliferator-activated receptor gamma inhibits TNF-alpha-mediated osteoclast differentiation in human peripheral monocytes in part via suppression of monocyte chemoattractant protein-1 expression." Bone **42**(4): 765-74.

Hwang, D. (2001). "Modulation of the expression of cyclooxygenase-2 by fatty acids mediated through toll-like receptor 4-derived signaling pathways." Faseb J **15**(14): 2556-64.

Itoh, Y., Y. Kawamata, et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-6.

Jeon, M. J., J. A. Kim, et al. (2003). "Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts." J Biol Chem **278**(26): 23270-7.

Johnson, G. B., B. L. Riggs, et al. (2004). "A genetic basis for the "Adonis" phenotype of low adiposity and strong bones." Faseb J **18**(11): 1282-4.

Laneuville, O., D. K. Breuer, et al. (1995). "Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)- octadecatrienoic acids from alpha-linolenic acid." J Biol Chem **270**(33): 19330-6.

Latour, M. G., T. Alquier, et al. (2007). "GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo." Diabetes **56**(4): 1087-94.

Mano, H., C. Kimura, et al. (2000). "Cloning and function of rabbit peroxisome proliferator-activated receptor delta/beta in mature osteoclasts." J Biol Chem **275**(11): 8126-32.

Maurin, A. C., P. M. Chavassieux, et al. (2005). "Expression of PPARgamma and beta/delta in human primary osteoblastic cells: influence of polyunsaturated fatty acids." Calcif Tissue Int **76**(5): 385-92.

Nilsson, N. E., K. Kotarsky, et al. (2003). "Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids." Biochem Biophys Res Commun **303**(4): 1047-52.

Oh, D. Y., S. Talukdar, et al. (2010). "GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects." Cell **142**(5): 687-98.

Oh, S. R., O. J. Sul, et al. (2009). "Saturated fatty acids enhance osteoclast survival." J Lipid Res.

Ono, K., H. Kaneko, et al. (2005). "Biphasic effect of prostaglandin E2 on osteoclast formation in spleen cell cultures: role of the EP2 receptor." J Bone Miner Res **20**(1): 23-9.

Raisz, L. G., C. B. Alander, et al. (1989). "Effects of prostaglandin E3 and eicosapentaenoic acid on rat bone in organ culture." Prostaglandins **37**(5): 615-25.

Schmidt, J., K. Liebscher, et al. (2011). "Conjugated linoleic acids mediate insulin release through islet G protein coupled receptor FFA1/GPR40." J Biol Chem.

Shi, H., M. V. Kokoeva, et al. (2006). "TLR4 links innate immunity and fatty acid-induced insulin resistance." J Clin Invest **116**(11): 3015-25.

Smith, N. J., L. A. Stoddart, et al. (2009). "The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1." J Biol Chem **284**(26): 17527-39.

Still, K., P. Grabowski, et al. (2008). "The peroxisome proliferator activator receptor alpha/delta agonists linoleic acid and bezafibrate upregulate osteoblast differentiation and induce periosteal bone formation in vivo." Calcif Tissue Int **83**(4): 285-92.

Tsutsumi, R., C. Xie, et al. (2009). "PGE2 signaling through the EP4 receptor on fibroblasts upregulates RANKL and stimulates osteolysis." J Bone Miner Res **24**(10): 1753-62.

Viccica, G., C. M. Francucci, et al. (2010). "The role of PPARgamma for the osteoblastic differentiation." J Endocrinol Invest **33**(7 Suppl): 9-12.

Wan, Y., L. W. Chong, et al. (2007). "PPAR-gamma regulates osteoclastogenesis in mice." Nat Med **13**(12): 1496-503.

Wauquier, F., V. Barquissau, et al. (2011). "Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis." Bone.

Xie, D., Q. Zhong, et al. (2007). "Glucose-dependent insulinotropic peptide-overexpressing transgenic mice have increased bone mass." Bone **40**(5): 1352-60.

Yamada, C., Y. Yamada, et al. (2008). "The murine glucagon-like peptide-1 receptor is essential for control of bone resorption." Endocrinology **149**(2): 574-9.

Troisième partie :

Conclusions, Discussion

Sur la base de la littérature, il ne fait aucun doute que les acides gras contenus dans la fraction lipidique de l'alimentation peuvent moduler le devenir du tissu osseux ***(Article 1 : Fatty acids and bone)***. Les possibilités d'adaptation du squelette reposent sur sa capacité à intégrer des signaux locaux et systémiques pour « s'ajuster » aux besoins via le processus du remodelage osseux. En parallèle, les acides gras sont utilisés de manière ubiquitaire dans l'organisme et peuvent donc potentiellement influencer la plupart des organes et systèmes biologiques. Au final, à l'échelle de l'organisme, les acides gras peuvent aussi bien moduler le remodelage du squelette de manière directe en agissant sur les cellules osseuses que de manière indirecte en agissant sur d'autres organes eux-mêmes impliqués dans la régulation de la physiologie osseuse **(Fig.24)**.

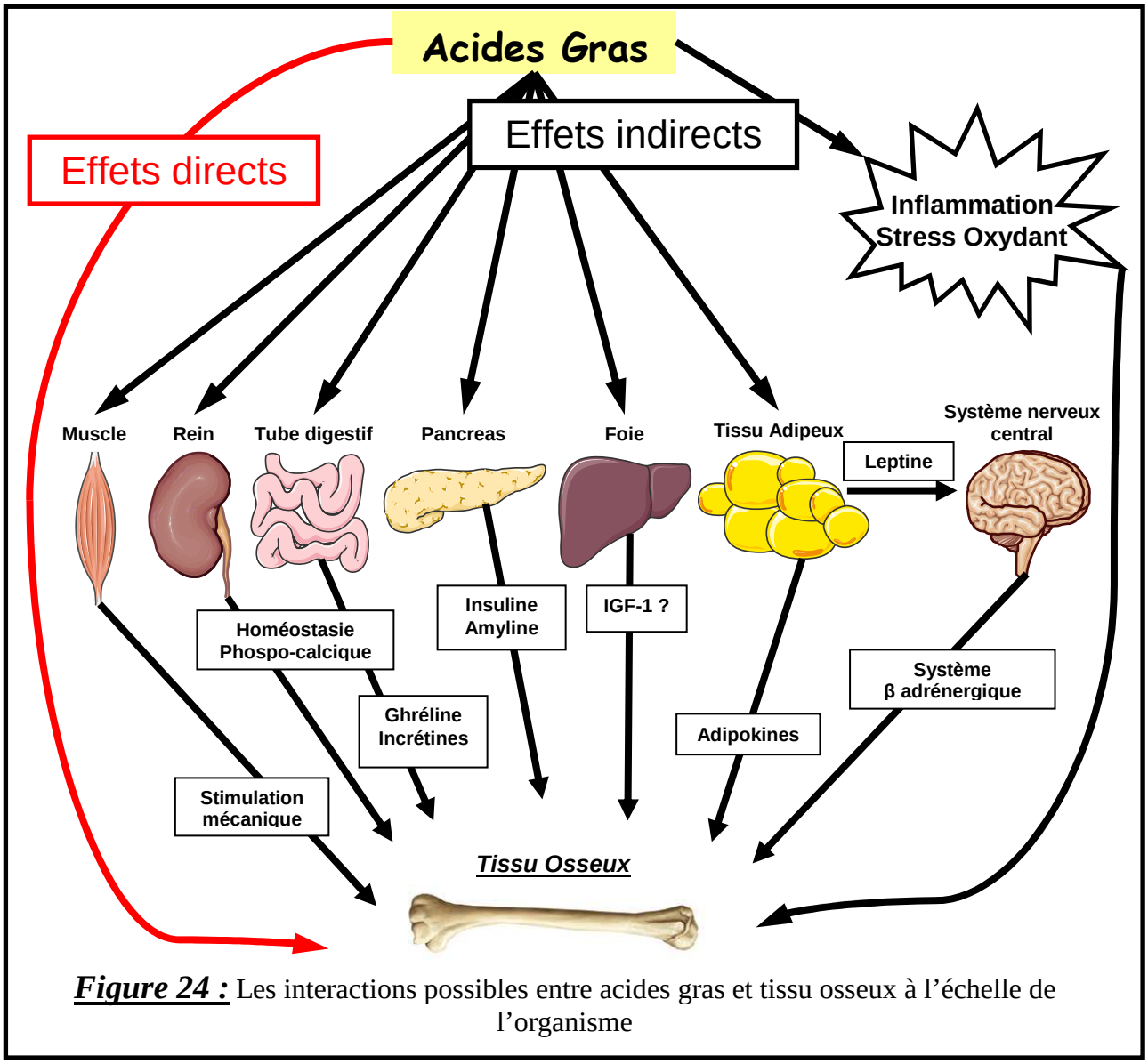


Figure 24 : Les interactions possibles entre acides gras et tissu osseux à l'échelle de l'organisme

Cette première observation révèle à la fois l'importance et la complexité des relations existant entre les acides gras et le métabolisme osseux. Ainsi, la modulation, en quantité ou en qualité, de la composition en acide gras du régime va avoir un effet au niveau de l'os qui sera la résultante de la modulation d'au moins une partie de ces différentes voies de régulation potentielles.

Une analyse détaillée des données établies concernant les effets santés des AGPI de la sous famille des ω3, incluant notamment l'acide éicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA), révèle un intérêt tout particulier de ces molécules dans la prévention de la perte osseuse (Sun, Krishnan et al. 2003) (Banu, Bhattacharya et al. 2010). Ainsi, les ω3 en quantités adaptées dans le régime sont susceptibles d'entraîner :

- une prévention, au cours de certaines formes d'obésité, des stéatoses rénales (Chin, Fu et al. 2010) (Cappelli, Di Liberato et al. 1997) et hépatiques (Kim, Lee et al. 2008) (Tanaka, Sano et al. 2008) qui peuvent évoluer vers des altérations graves de ces organes associées à des perturbations du métabolisme osseux.,

- une prévention du dysfonctionnement et de l'apoptose des cellules β du pancréas (Wei, Li et al. 2010) (Bellenger, Bellenger et al. 2011) maintenant ainsi le signal ostéoanabolique de l'insuline,

- une action anti-inflammatoire (Seki, Tani et al. 2009) (Galli and Calder 2009) permettant de limiter la production des facteurs associés qui sont connus par ailleurs pour favoriser la résorption osseuse,

- une amélioration de l'absorption intestinale de calcium (Gilman and Cashman 2007) et une augmentation de la sécrétion basale de ghréline au niveau de l'estomac (Burghardt, Kemmerer et al. 2010).

De fait, l'effet osseux des AGPI de la sous famille des ω3 impliquent probablement plusieurs organes et systèmes biologiques ce qui illustre parfaitement la complexité des relations existant entre la nutrition lipidique et le métabolisme osseux. Ainsi, si l'impact bénéfique de ces acides gras sur le squelette est bien établi notamment au travers d'études cliniques et pré-cliniques, il est difficile de déterminer quel (s) est (sont) le (s) mécanisme (s) essentiel (s) impliqué (s) dans le processus.

Au cours d'une étude d'intervention pré-clinique dans un modèle de progeria chez la souris, nous avons pu associer la protection osseuse induite par les ω3 à une réduction des paramètres inflammatoires osseux et systémiques. Par ailleurs, l'acide γ-linolénique connu pour « mimer » les effets anti-inflammatoires des ω3 notamment par une perturbation similaire de la voie de synthèse des prostaglandines (Kapoor and Huang 2006) s'est avéré, au

cours de cette étude, avoir les mêmes effets bénéfiques que les ω3 sur les paramètres inflammatoires et osseux. Ces résultats suggèrent donc une importance toute particulière de la modulation du status inflammatoire dans la médiation des effets des acides gras sur l'os, au moins dans ce modèle de perte osseuse associée à un vieillissement avancé de l'organisme ***(Article 2 : Borage and fish oils lifelong supplementation decreases inflammation and improves bone health in a murine model of senile osteoporosis).***

Par ailleurs, les effets directs des acides gras au niveau des cellules osseuses font l'objet d'études de plus en plus nombreuses, ces mécanismes vennant ajouter un niveau de complexité supplémentaire au système. Ainsi, en plus de moduler les régulations systémiques du remodelage osseux, les acides gras peuvent agir directement sur les cellules impliquées dans le processus.

Dans ce cadre, nous nous sommes intéressés au rôle d'un récepteur membranaire aux acides gras à longues chaînes dont nous avons pu démontrer l'expression au niveau des cellules osseuses : le GPR40. Le phénotype ostéoporotique des souris invalidées pour ce récepteur a permis de révéler le rôle protecteur de ce dernier pour l'os. Ce phénotype semble lié à un rôle inhibiteur de l'activation du GPR40 au niveau des pré-ostéoclastes, sur l'ostéoclastogénèse induite par RANKL ce qui a été observé *in vitro* et *ex vivo*. Enfin, l'activation *in vivo* du GPR40 par son agoniste administré par gavage permet de tempérer la perte osseuse induite par ovariectomie chez la souris ***(Article 3 : The free fatty acid receptor GPR40 protects from bone loss through inhibition of osteoclast differentiation)***.

Ce travail suggère donc un nouveau mode d'interaction direct entre les acides gras à longues chaînes et le métabolisme osseux via l'activation du récepteur GPR40. Si la corrélation entre le phénotype des souris invalidées et les effets de l'agoniste, *in vitro* comme *in viv*o, est excellente, il reste un certain nombre de points à éclaircir pour définitivement valider ce mécanisme.

D'abord, comme évoqué dans la discussion de l'article 3, on ne peut pas complètement exclure à ce stade du travail, un effet indirect de l'invalidation du GPR40 via ses conséquences sur un autre organe. En effet, les souris invalidées pour GPR40 le sont de manière totale et le GPR40 a déjà été décrit comme étant le médiateur de l'effet positif des acides gras sur la sécrétion, par d'autres organes, de facteurs anaboliques pour l'os. En effet, le GPR40 participe à la potentialisation par les acides gras de la sécrétion d'insuline en réponse au glucose au niveau des cellules β du pancréas (Latour, Alquier et al. 2007) ainsi qu'à la sécrétion des incrétines par les cellules intestinales (Edfalk, Steneberg et al. 2008). Dans ces conditions, même si nos résultats obtenus *in vitro* suggèrent fortement une action

directe sur les cellules osseuses, seule la réalisation d'un modèle de souris présentant une abolition de l'expression de GPR40 spécifiquement au niveau des cellules osseuses permettra de définitivement valider l'importance de l'expression de ce récepteur au niveau de l'os.

Du point de vue de la signalisation cellulaire, notre travail a permis de mettre en évidence le fait que l'inhibition de la différenciation ostéoclastique par le GPR40 est associée à l'altération de la voie essentielle impliquée dans le processus, la voie NF-κB. Ainsi, les phosphorylations des facteurs Inhibitor of NF-κB (Iκ-B) et Iκ-B kinase (IKK), nécessaires à la transduction du signal sont inhibées par l'activation du GPR40 tout comme l'activité transcriptionelle NF-κB qui en découle. Cependant, les mécanismes permettant de relier, d'un point de vue moléculaire, l'activation du GPR40 à cette inhibition de la voie NF-κB restent à démontrer.

Les voies de signalisation induites par l'activation du GPR40 ont à l'heure actuelle principalement été décrite au niveau de la cellule β. A ce niveau, la protéine G couplée au récepteur est majoritairement de type Gαq, et l'activation du GPR40 induit l'activation de la phospholipase C (PLC) qui est suivie par une augmentation du calcium intra cytoplasmique dont l'origine serait principalement extracellulaire et viendrait de l'activation de canaux calciques de type L (LTCC) au niveau de la membrane plasmique (Fujiwara, Maekawa et al. 2005) (Shapiro, Shachar et al. 2005). De manière intéressante, il a été montré que l'activation des LTCCs par un agoniste, au niveau de pré-ostéoclastes primaires de souris, induisait une inhibition de la différenciation ostéoclastique induite par RANKL notamment en altérant la voie NF-κB (Noh, Park et al. 2011). Par ailleurs, l'activation des LTCCs dans ce modèle est également associée à une réduction de l'expression du facteur de transcription NFATc1 ce que nous avons également observé en réponse à l'agoniste du GPR40. En outre, le récepteur à la calcitonine, un inhibiteur connu à la fois de la différenciation et de l'activité des ostéoclastes, peut également être couplé à une protéine G de type Gq (Del Fattore, Teti et al. 2008). En revanche, l'activation de la PLC et les oscillations du calcium intra cytoplasmique qui l'accompagne, sont classiquement connues pour faire partie du processus de différenciation ostéoclastique induit par RANKL et sont notamment important pour l'expression de NFATc1 (Hwang and Putney 2011). Au final, si ces données permettent d'envisager l'implication de la voie de signalisation classique induite par le GPR40 dans la médiation de l'effet inhibiteur du récepteur sur la différenciation des ostéoclastes, cela reste entièrement à démontrer.

Au-delà de cette voie habituellement décrite pour le GPR40, une étude récente conduite par Oh et al a montré que l'activation d'un autre GPR proche du GPR40 (le GPR120, également récepteur membranaire des acides gras à longues chaînes) était capable d'induire une altération de la voie NF-κB via un processus nettement moins standard. Dans ce modèle, l'activation du GP120 par son ligand (en l'occurrence le DHA) entraîne son intéraction avec la β-Arrestin 2 et son internalisation. Le complexe β-Arrestin 2 / GPR120 peut ensuite interagir avec la protéine TAK1 Binding Protein 1 (TAB1) ce qui va empêcher l'intéraction normale de cette dernière avec la protéine TGF-β Activated Kinase 1 (TAK1) qui ne pourra alors pas jouer son rôle de manière efficace dans l'activation du facteur IKK et donc du reste de la voie NF-κB (Oh, Talukdar et al. 2010). Comme beaucoup de GPRs (Barak, Ferguson et al. 1997), il est tout à fait envisageable que le GPR40 puisse intéragir avec la β-Arrestin 2, ce mécanisme représente donc une autre voie moléculaire qui permettrait d'expliquer l'effet inhibiteur du GPR40 sur la différenciation des ostéoclastes.

Au niveau de l'os, le GPR40 est exprimé par les ostéoclastes mais aussi par les cellules responsables de la formation osseuse, les ostéoblastes. Nous avons pu montrer que l'activation du GPR40, délétère à forte dose pour la viabilité de précurseurs ostéoclastiques, est sans effet sur celle de la lignée ostéoblastique MC3T3-E1. Cette « toxicité » spécificique est tout à fait cohérente avec l'effet ostéoprotecteur du GPR40 **(Fig 25)**. L'effet de l'agoniste du GPR40 sur la différenciation de cette lignée ostéoblastique a également été étudié. Là encore, ces expériences ont permis de suggérer un effet positif du GW9508 sur la différenciation ostéoblastique associé à une augmentation de l'expression et de l'activité de la phosphatase alcaline ainsi que de l'expression de l'ostéocalcine. **(Fig.26)**

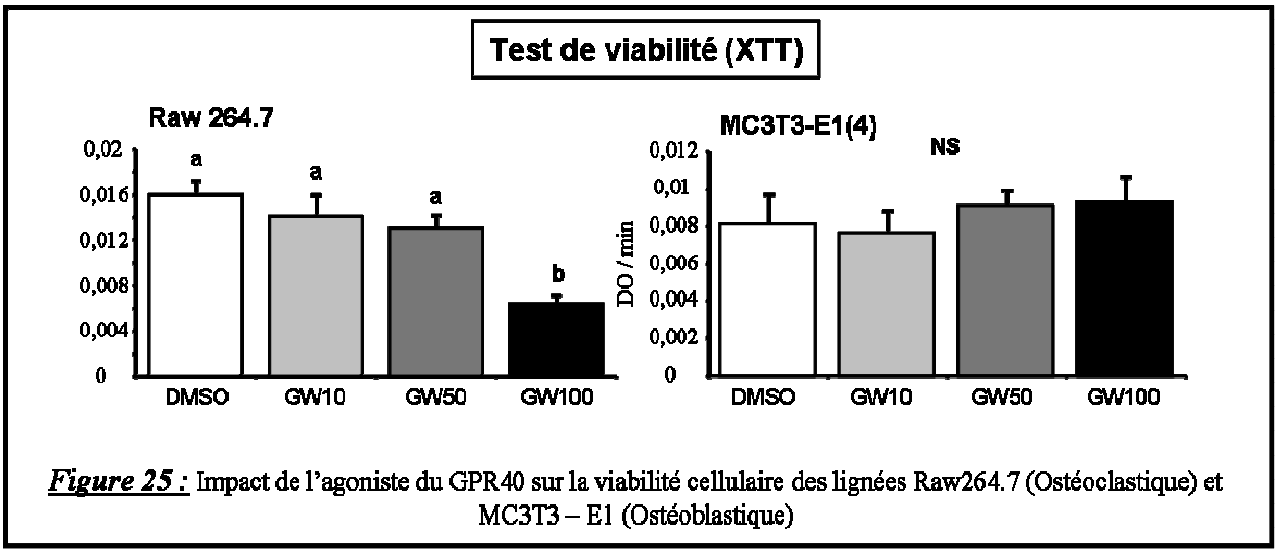


Figure 25 : Impact de l'agoniste du GPR40 sur la viabilité cellulaire des lignées Raw264.7 (Ostéoclastique) et MC3T3 – E1 (Ostéoblastique)

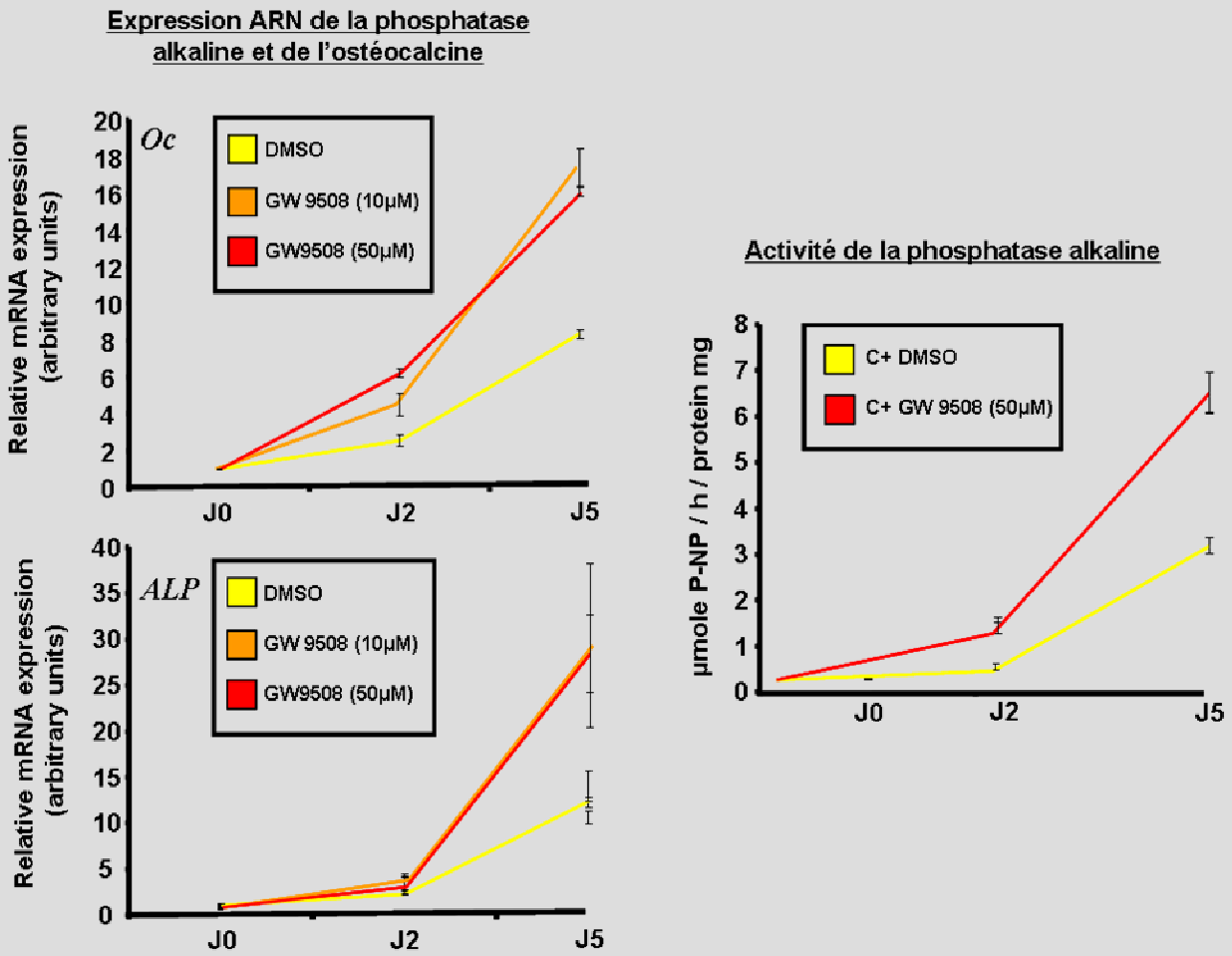


Figure 26 : Effet de l'agoniste du GPR40 sur la différentiation ostéoblastique de cellules MC3T3-E1 cultivées dans un milieu α-MEM +10% de sérum de veau fétal + Vitamine C + β-glycérophosphate.

En revanche, le traitement de ces cellules par le GW9508 semble altérer leur capacité à former des nodules de minéralisation **(Fig.27)**

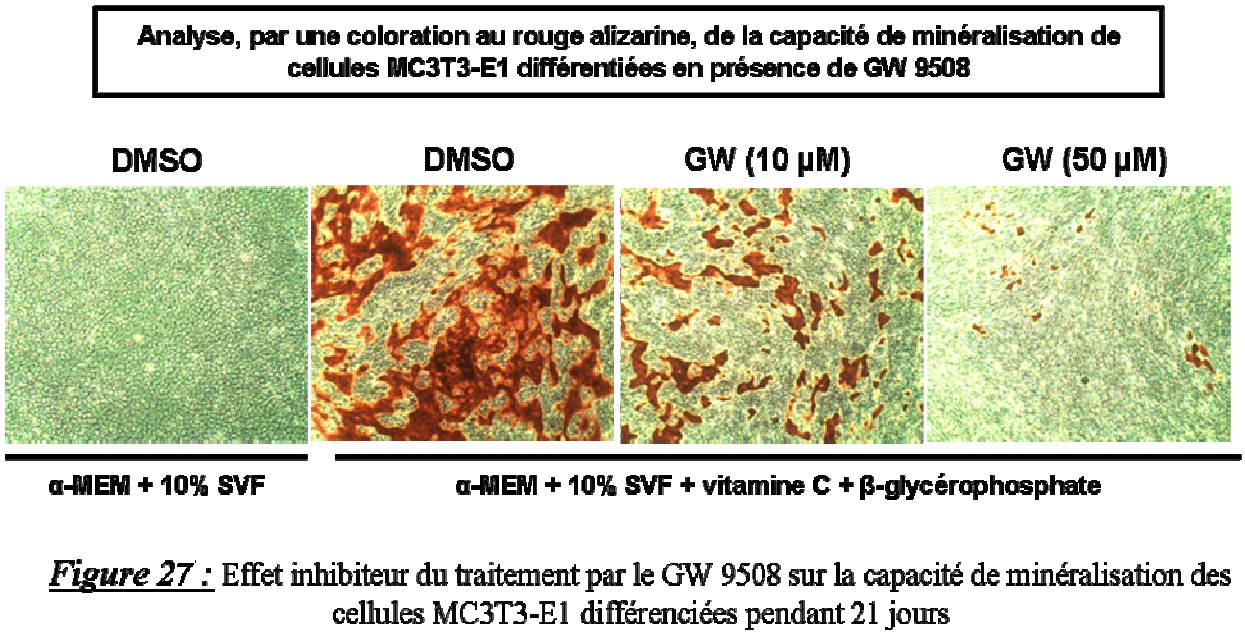


Figure 27 : Effet inhibiteur du traitement par le GW 9508 sur la capacité de minéralisation des cellules MC3T3-E1 différenciées pendant 21 jours

Contrairement à son action inhibitrice sur la différenciation ostéoclastique, ces effets de l'agoniste du GPR40 sur la différenciation et l'activité des ostéoblastes *in vitro*, n'ont pas été confirmé *in vivo* et le gavage par le GW9508 s'est avéré sans effet sur les marqueurs de la différenciation ostéoblastique chez la souris. Par ailleurs, la validation de l'implication du récepteur GPR40 par l'étude de la réponse au GW9508 dans des cellules invalidées pour ce récepteur n'a pas non plus été réalisée à ce jour. En conséquence, ces résultats préliminaires qui suggèrent clairement que le rôle osseux du GPR40 ne se limite pas à son influence sur la différenciation des ostéoclastes vont nécessiter un certain nombre de confirmation avant de pouvoir être intégrés au modèle.

Le récepteur GPR40 est donc un récepteur membranaire aux acides gras à longues chaînes dont l'activation semble clairement bénéfique pour le maintien de la masse osseuse via une réduction de l'ostéoclastogénèse. Si on essaie de replacer ce rôle du GPR40 dans un contexte physiologique, il pourrait être impliqué dans un certain nombre de situations biologiques où une protection du squelette est associée à la disponibilité d'une grande quantité d'acides gras et vice versa. Sur ce principe, le GPR40 pourrait contribuer à la relation positive parfois observée entre quantité de tissu adipeux et densité minérale osseuse. Par

ailleurs, l'augmentation de la résorption osseuse associé à une restriction calorique (Ihle and Loucks 2004) pourrait également trouvée son origine dans une signalisation limitée de GPR40. Enfin, de la même façon, la diminution des niveaux de résorption osseuse consécutive au repas (Clowes, Hannon et al. 2002) (Bjarnason, Henriksen et al. 2002) serait également susceptible de faire intervenir ce récepteur.

Cependant, l'impact d'un acide gras sur une cellule ne se limite pas à l'activation d'un récepteur membranaire. D'abord, il existe plusieurs récepteurs membranaires dont l'activité est modulée par les acides gras (GPR40, GPR120, Toll-like Receptors (TLRs)). De plus, de par leur nature hydrophobe, les acides gras peuvent franchir la membrane plasmique et peuvent donc aussi bien agir à l'extérieur qu'à l'intérieur de la cellule. En intracellulaire, il peut activer des récepteurs nucléaires comme les Peroxysome Proliferator-Activated Receptors (PPARs), servir de substrat énergétique, servir de substrat à la formation des prostaglandines, participer à la génération de céramides, ou contribuer à modifications post-traductionnelles de certaines protéines. La réponse cellulaire à un acide gras donné, va dépendre de sa structure et sera donc la résultante des différents mécanismes qu'il aura induits **(Fig.28).**

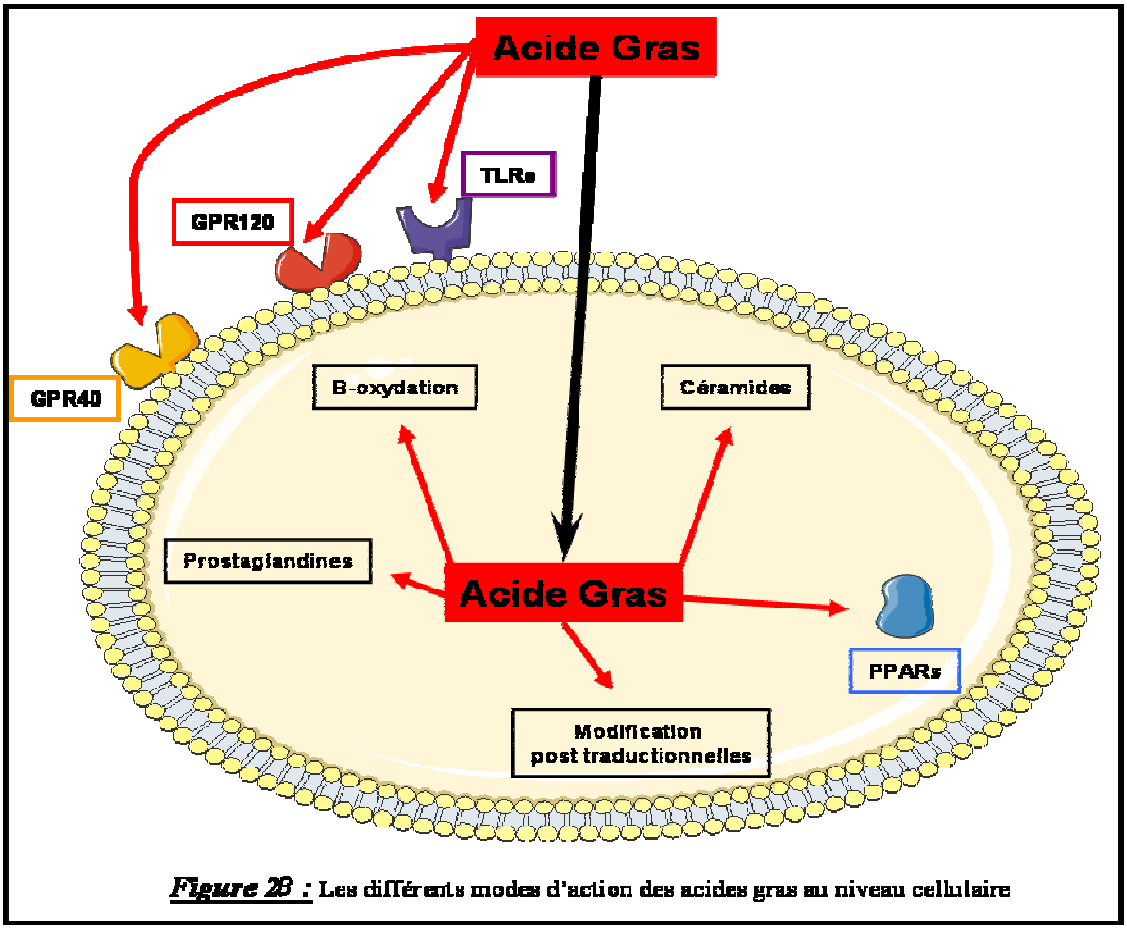


Figure 28 : **Les différents modes d'action des acides gras au niveau cellulaire**

En conséquence, il apparaît difficile de relier les situations biologiques évoquées à la simple activation du récepteur GPR40, d'autant plus que l'augmentation, ou la diminution selon le cas, des acides gras présents dans la circulation n'a pas non plus été démontrée comme étant le mécanisme causal de la modification du remodelage osseux dans ces situations.

La pluralité des mécanismes potentiellement déclenchés par les acides gras au niveau cellulaire est à associer à celle des régulations systémiques altérant le remodelage osseux et modulées par ces mêmes acides gras. Au final, la complexité de ce système met en avant la nécessité d'approches intégrées tenant compte d'un maximum de paramètres afin de permettre une meilleure compréhension des interrelations existantes entre la fraction lipidique contenue dans l'alimentation et le métabolisme du tissu osseux.

La découverte de ce nouveau mode d'action des acides gras sur le remodelage de l'os est doublement intéressante en termes de valorisation présentant à la fois un potentiel nutritionnel et thérapeutique.

Comme évoqué précédemment, les stratégies de préventions nutritionnelles de l'ostéoporose sont en cours d'évolution. Pour permettre leur bon développement, la connaissance précise des mécanismes à l'origine des effets santé de certains nutriments est essentielle. Dans le cas particulier du GPR40, l'intérêt nutritionnel direct de sa découverte peut paraître a priori limité en raison de son activation par tous les acides gras à longues chaînes sans discrimination (Briscoe, Tadayyon et al. 2003). En effet, il apparaît difficile de recommander une augmentation de la fraction lipidique du régime alimentaire pour favoriser la santé osseuse dans la mesure où les régimes hyperlipidiques sont classiquement associés à un effet délétère pour le squelette (Parhami, Tintut et al. 2001) (Xiao, Cui et al. 2010) (ainsi que pour d'autres organes essentiels). D'ailleurs, cette ambiguïté illustre de nouveau parfaitement la complexité des relations entre acides gras et os qui vont bien au-delà de l'activation d'un récepteur sur les ostéoclastes.

De manière intéressante, le GPR120, qui avait initialement été décrit comme activé par tous les acides gras à longues chaînes à l'instar du GPR40, a été récemment présenté comme liant plus spécifiquement les AGPI de la sous famille des ω3 (Oh, Talukdar et al. 2010). Par ailleurs, au sein de certaines études, le GPR40 s'est également avéré, dans certaines conditions, capable de présenter une spécificité pour certains acides gras à longues chaînes en particulier (Yonezawa, Katoh et al. 2004) (Hardy, St-Onge et al. 2005). Ces études suggèrent

fortement que l'activation équivalente du GPR40 par tous les acides gras à longues chaînes est une idée fausse mais ne permettent malheureusement pas, par leur nombre limité et leur manque de consensus, de déterminer une hiérarchie. Dans ce contexte, identifier, s'ils existent, les acides gras activant préférentiellement le GPR40 apparaît comme une perspective fondamental de ce travail.

Indépendamment de la nutrition, la découverte de ce rôle osseux du GPR40 identifie ce récepteur comme une cible très intéressante pour le développement de thérapeutiques contre l'ostéoporose. En effet, comme en témoigne la prévention de la perte osseuse observée chez les souris ovariectomisées traitées avec l'agoniste du GPR40, l'activation pharmacologique de ce récepteur possède un potentiel très prometteur. Par ailleurs, l'activation du GPR40 présente en parallèle un intérêt thérapeutique au niveau du pancréas où elle potentialise la sécrétion d'insuline en réponse au glucose et elle ne présente pas, en l'état actuel des connaissances d'effets délétères notables. En outre, il est d'ailleurs possible que le GPR40 soit déjà utilisé depuis longtemps dans certaines thérapies de manière involontaire. En effet, les thiazolidinédiones et la rosiglitazone, qui sont utilisés pour leurs vertus préventives du développement du diabète de type 2 via l'activation de PPARγ, pourraient être, en parallèle, des activateurs du GPR40 dans certaines conditions (Smith, Stoddart et al. 2009) (Gras, Chanez et al. 2009). Ainsi, la potentialisation de la sécrétion d'insuline en réponse au glucose par les thiazolidinédiones, observée *in vitro* comme *in vivo* (Cavaghan, Ehrmann et al. 1997) (Ohtani, Shimizu et al. 1996), pourrait impliquer l'activation du GPR40.

De même, d'un point de vue osseux, ce manque de spécificité pourrait expliquer les résultats contradictoires obtenus dans la littérature concernant l'effet de PPARγ et/ou de certains de ses agonistes, sur la différenciation ostéoclastique. En effet, si son rôle activateur de la différenciation des ostéoclastes a pu être mis en évidence via des études utilisant la génétique de la souris (Wan, Chong et al. 2007), ses agonistes ont bien souvent été décrits comme étant des régulateurs négatifs de ce processus de différenciation (Okazaki, Toriumi et al. 1999) (Yang and Lai 2010) (Chan, Gartland et al. 2007). Dans ce contexte, l'activation du GPR40, et ses conséquences délétères sur la différenciation des ostéoclastes pourrait être le mécanisme à l'origine de ces ambigüités.

Quatrième partie :

Annexes

Annexe numéro 1:

Oxydative stress in bone remodeling and disease

Fabien Wauquier, Laurent Léotoing, Véronique Coxam, Jérôme Guicheux, Yohann Wittrant

Trends in Molecular Medicine, 2009

Review

Cell PRESS

Oxidative stress in bone remodelling and disease

Fabien Wauquier[1,2,3,4], Laurent Leotoing[1,2,3,4], Véronique Coxam[1,2,3,4], Jérôme Guicheux[5] and Yohann Wittrant[1,2,3,4]

[1] Inra, UMR 1019 Nutrition Humaine, F-63122 Saint Genès Champanelle, France
[2] Clermont Université, UFR Médecine, UMR 1019 Nutrition Humaine, F-63000, Clermont-Ferrand, France
[3] CRNH Auvergne, F-63000 Clermont-Ferrand, France
[4] Equipe Alimentation, Squelette et Métabolismes, F-63122 Saint Genès Champanelle, France
[5] Inserm, U791, Université de Nantes, LIOAD, Centre for osteoarticular and dental tissue engineering, group ''Physiopathology of skeletal tissues and cartilage engineering'' – Nantes, France

Oxidative stress is characterised by an increased level of reactive oxygen species (ROS) that disrupts the intracellular reduction–oxidation (redox) balance. Although initially shown to be involved in aging, physiological roles for ROS in regulating cell functions and mediating intracellular signals have emerged. In bone tissues, recent studies have demonstrated that ROS generation is a key modulator of bone cell function and that oxidative status influences the pathophysiology of mineralised tissues. Here, we review the crucial role of oxidative stress in bone pathophysiology, and discuss the possibility that ROS production might be a relevant therapeutic target under certain conditions. Further studies will be needed to investigate whether manipulation of the redox balance in bone cells represents a useful approach in the design of future therapies for bone diseases.

Oxidative stress – Dr Jekyll or Mr Hyde?

During the past decade, studies have linked oxidative stress (Box 1, Figure 1) with the pathophysiology of almost all organs. In fact, depending on their concentration, reactive oxygen species (ROS) can either have beneficial or deleterious effects on tissues.

Oxidative stress has been acknowledged as a major contributor to the immune response [1]. Activation of immune mechanisms is characterised by the establishment of an inflammatory response and recruitment and activation of immune cells leading to pathogen killing. These processes widely involve the production of ROS [2,3]. This local oxidative stress plays a pivotal role in lymphocyte function. Following activation, lymphocytes produce increased levels of ROS that participate in the initiation and amplification of antigenic signals [4]. In addition, recent studies report the crucial role of ROS in dendritic cell maturation and related T-cell proliferation [5,6], supporting the importance of oxidative stress in the prevention of infectious diseases.

ROS have been broadly described to play roles in aging and associated complications [7]. Indeed, with increasing age many kinds of degenerative diseases can develop because of oxidative stress. These pathologies include cancers, cardiovascular diseases, ischemia–reperfusion injury, arthritis, diabetes and neurological disorders [8]. Deleterious effects of oxidative stress result from damage to cell structures and dysfunction because of lipid and protein oxidations. In addition, ROS alter mitochondrial and nuclear DNA integrity by increasing the risk of mutations. When DNA repair mechanisms are overwhelmed, cells undergo apoptosis or necrosis, which can lead to tissue damage [9].

Accumulating evidence suggests that bone biology is particularly affected by redox balance regulation and that targeting ROS production in bone cells might be an important approach in the prevention of bone damage. Bone is a dynamic organ with a well-regulated turnover to allow bone tissue functions such as growth, locomotion, organ protection or calcium/phosphate homeostasis. Bone remodelling is regulated by consecutive formation and resorption sequences supported by osteoblast and osteoclast activities, respectively (Figure 2). Because osteoclasts and immune cells such as macrophages and dendritic cells share some common features, ROS production in bone cells has also been investigated in relation to these activities. Recent studies have examined the association between ROS production and bone pathophysiology, contributing to define the cellular and molecular basis of the biological

Glossary

Oxidative stress: the production of higher than normal levels of ROS that might originate from the respiratory chain or other enzymatic complexes such as Nox (Figure 1).
Osteoporosis: pathology characterised by decreased bone mass and altered bone micro-architecture, leading to excessive skeletal fragility.
Bone remodelling: a dynamic, lifelong process of reshaping bone. Bone remodelling results in adequate alternate sequences of bone resorption and bone formation, allowing bone growth and tissue repair after injury.
Bone mineral density (BMD): refers to the quantity of mineral in bone tissues, expressed in grams per square centimetres.
Osteoblast: a cell originating from mesenchymal stem cells. Osteoblasts are responsible for the synthesis of bone matrix and are characterised by the expression of differentiation markers such as RANKL, OPG, ALP, collagen and Runx2.
Osteoclast: a multinucleated cell of haematopoietic origin that derives from monocyte and macrophage precursors in the presence of RANKL. Osteoclasts are characterised by TRACP activity, RANK and calcitonin receptor expression and the ability to resorb bone.
Antioxidants: molecules that counteract oxidative stress. They might depend on the activities of endogenous enzymes (catalases, peroxidases, SODs) or exogenous molecules with antioxidant properties (mostly represented by micronutrients from the diet, such as polyphenols and vitamins).

Corresponding author: Wittrant, Y. (yohann.wittrant@clermont.inra.fr).

1471-4914/$ – see front matter doi:10.1016/j.molmed.2009.08.004 Available online xxxxxx

Box 1. Oxidative stress and ROS

Oxidative stress results from the deregulation of the redox balance in cells and tissues and is associated with an overall increase in levels of ROS. ROS are mainly represented by the superoxide radical anion (O_2^{-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) in cells. ROS are produced in cells at various sites including the plasma membrane, mitochondria, endoplasmic reticulum and cytoplasm. Several enzymatic complexes are responsible for ROS production, including NADPH oxidase, cytochrome P450, xanthine oxidase, monoamine oxidase, cyclo-oxygenase and lipoxygenase. The largest amounts of ROS are produced in mitochondria by the transfer of unpaired electrons from the respiratory chain to O_2. This results in O_2^{-} formation, which is then converted to H_2O_2 by manganese-dependent superoxide dismutase (MnSOD) in the mitochondrial matrix and by Cu- and Zn-dependent SOD in the cytosol. H_2O_2 is detoxified by either catalase or glutathione peroxidase activity [77].

activities of ROS. Here, we review the role of oxidative stress in bone remodelling and disease.

Oxidative stress in the establishment of bone disease

ROS production is particularly involved in mineral tissue homeostasis and contributes mostly to bone remodelling by promoting bone resorption [10–15]. Recent observations have also suggested that oxidative stress might be involved in bone pathogenesis including osteoporosis, bone tumour development, diabetes-induced bone complications and joint inflammatory diseases (Table 1).

Osteoporosis

Numerous studies have provided evidence for the positive correlation between oxidative and osteoporotic status (Box 2). Decreased bone mineral density (BMD), a general feature of osteoporosis, was shown to be associated with higher oxidative stress index values and total plasma oxidant status in osteoporotic patients [16]. Sendur et al. recently confirmed this observation by showing a negative correlation between plasma lipid oxidation and BMD values in osteoporotic postmenopausal women compared with a healthy group [17]. Clinical manifestation of osteoporosis mainly occurs in the aging population, with a higher prevalence when associated with oestrogen deficiency. *In vivo* experiments support the role of ROS in age-related osteoporosis. In aging mice, both male and female C57BL/6 mice showed decreased bone formation rates, whereas ROS levels were upregulated [18]. In this study, Almeida et al. pointed out that the observations might rely on antioxidant defence failure and that the same changes in oxidative stress were reproduced by gonadectomy [18]. It is interesting to note that, besides aging, ovariectomy induces oxidative stress in rat femurs together with a decreased activity of antioxidant systems [18,19]. These findings provide new insight into the role of oestrogen deficiency during postmenopausal osteoporosis.

Oxidative stress status depends on the balance between oxidant and antioxidant enzymatic activities. Catalase and glutathione peroxidase are major antioxidant enzymes that detoxify hydrogen peroxide. In postmenopausal women with osteoporosis, catalase and glutathione peroxidase activity were found to be lowered [20,21]. Accordingly, the treatment of ovariectomised mice with pegylated catalase inhibits bone loss [22], suggesting a crucial role for hydrogen peroxide in postmenopausal osteoporosis. In parallel, the studies of Jagger et al. and Almeida et al. confirmed that oestrogen deficiency generates a lowering of thiol antioxidant defences in rodent bone, leading to accelerated bone loss with aging [18,23]. This failure of antioxidant defences against

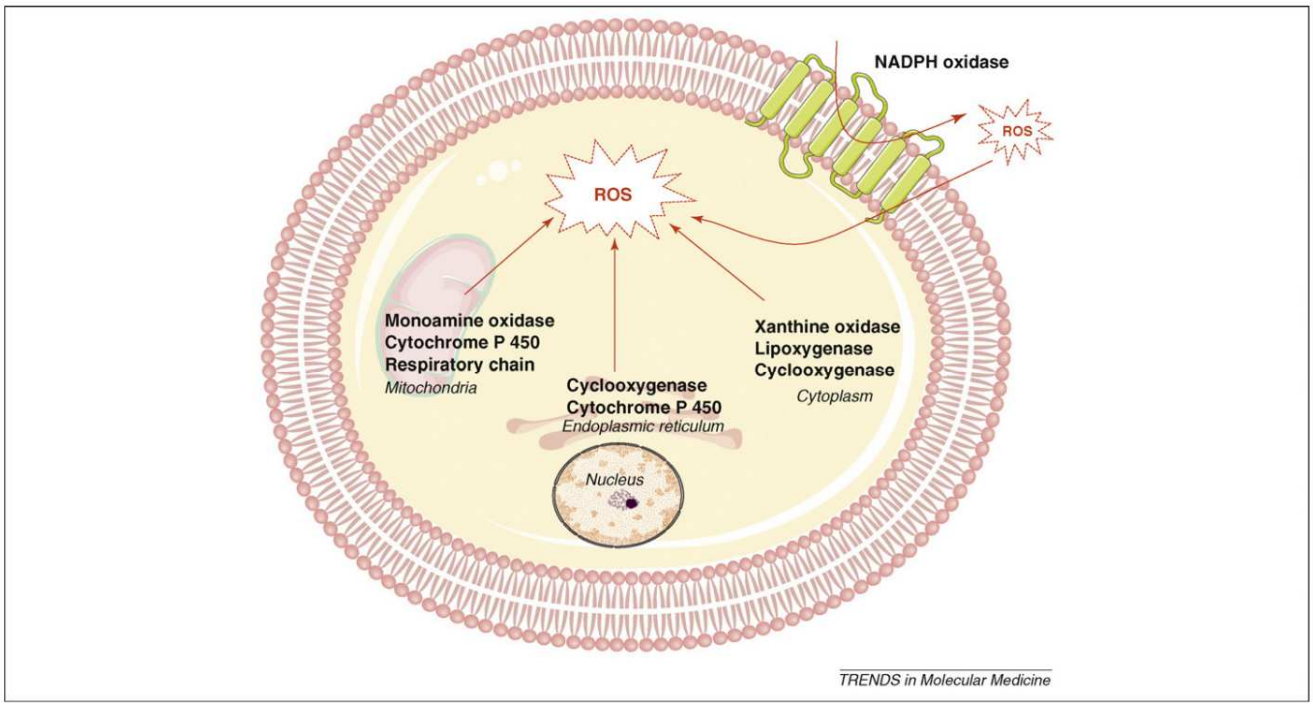


Figure 1. Cellular localisation of ROS production. ROS are produced in cells at various sites including the plasma membrane, mitochondria and cytoplasm. The largest amounts of ROS are produced in mitochondria because of electron leaks in the respiratory chain. At the surface of the cell, NADPH oxidases transport electrons across the plasma membrane to generate superoxide ($O_2^{\cdot-}$). Superoxide is unstable and so is rapidly converted to H_2O_2, which can diffuse through the cell membrane.

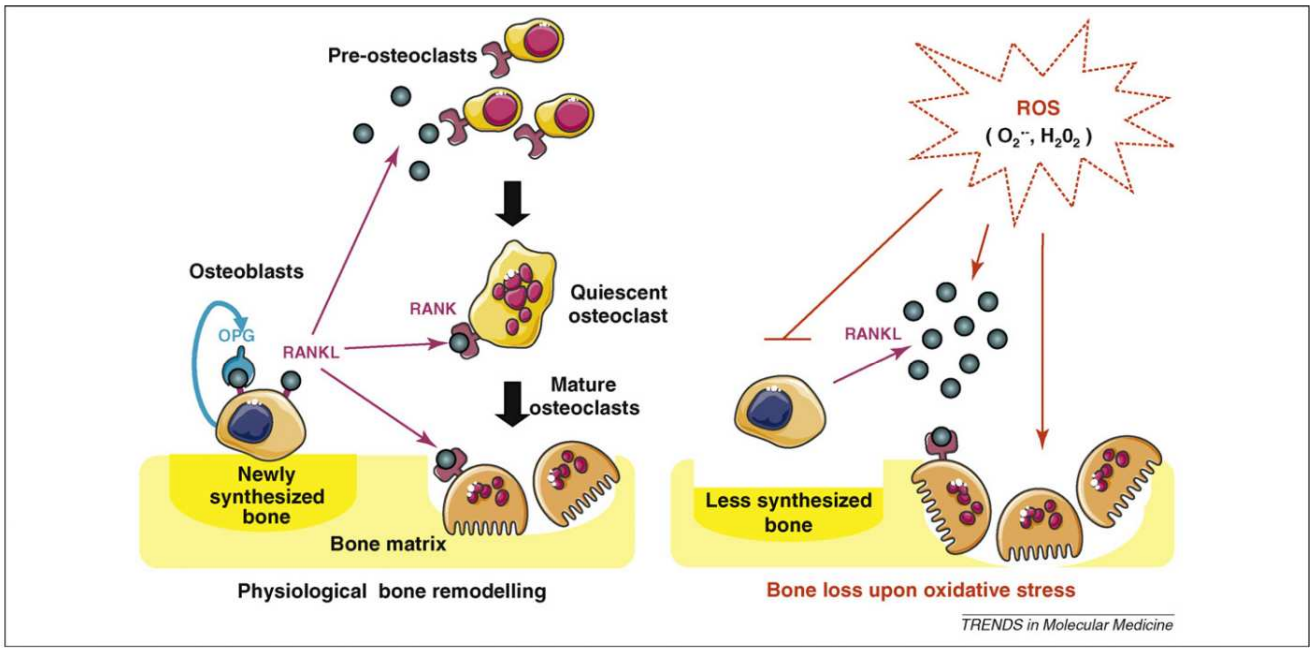


Figure 2. Bone remodelling and oxidative stress. In the presence of the receptor activator of NF-κB ligand (RANKL), expressed by osteoblasts and BMSCs, haematopoietic precursors differentiate into multinucleated osteoclasts. RANKL binding to the receptor RANK at the surface of pre-osteoclasts stimulates cell fusion, activates resorption capabilities and enhances cell survival. OPG, a decoy receptor for RANKL, prevents osteoclast differentiation. By degrading bone, osteoclasts create lacunae that are filled with newly synthesised matrices by osteoblasts. As a result of oxidative stress this bone coupling is unbalanced, and bone formation by osteoblasts is reduced, whereas osteoclast differentiation and activities and subsequent bone resorption are enhanced directly or indirectly through an increased RANKL production.

increased oxidative stress was shown to induce bone loss through a tumour necrosis factor alpha (TNFα)-dependent signalling pathway [23]. In addition, plasma level and activity of superoxide dismutase (SOD), an enzyme that converts the superoxide anion to hydrogen peroxide, were negatively associated with lumbar BMD in humans [20,24,25]. Taken together, these data support the possibility that hydrogen peroxide has a deleterious effect on bone health and highlight the importance of oxidative stress in the development of osteoporosis. Therefore, it is tempting to suggest that antioxidants should be investigated as a potential approach for the treatment of age-related bone loss.

Bone tumour development

Oxidative stress has been shown to be associated with bone tumour development. Prostate cancer cells metastasise to bone tissues and can cause secondary bone tumours. Interestingly, patients with advanced but not localised prostate cancer are subject to high levels of oxidative stress [26]. However, glutathione (GSH) depletion by buthionine sulphoximine sensitises Ewing's sarcoma family tumour cells to fenretinide-induced cell death [27]. In addition, mitochondrial superoxide generation enhanced by thymoquinone, a phytochemical compound, induces p53-independent apoptosis in human osteosarcoma cell lines [28]. These data reveal a potential conflict: on one hand increased ROS levels might be involved in carcinogenesis, but on the other hand many current chemotherapies used to treat bone tumours rely on the ability of ROS to promote cancer cell apoptosis. Therefore, although targeting ROS production might be a relevant approach to investigate for treating bone tumour establishment, the design of such strategies would also need to investigate the potential effects on chemotherapy, and should be considered with extreme caution.

Table 1. Relationships between oxidative stress and the development of bone disease

Bone pathology	Observations	ROS involvement
Osteoporosis	• ↓ Bone formation rate	• ↑ ROS production [18] • ↓ Thiol antioxidant defences [18,23]
	• ↓ Bone mineral density	• ↑ Total plasma oxidant status [16,80] • ↑ Plasma lipid oxidation [17,20,21] • ↓ Catalase and glutathione peroxidase activity [20,21] • ↑ Plasma level and activity of super oxide dismutase [20,24,25]
	• ↓ Serum osteocalcin level	• Catalase gene polymorphism [81]
Bone tumours	• Advanced prostate cancer	• ↑ Oxidant status [26]
	• Ewing's sarcoma	• GSH depletion sensitises tumour cells to fenretinide-induced cell death [27]
Diabetes	• Streptozotocin-induced osteopenia	• ↑ Oxidant status [30]
Joint inflammatory diseases	• Rheumatoid arthritis	• ↑ ROS in synovial cells [32,33] • ↑ ROS in senescent chondrocytes [34]
	• Ankylosing spondylitis	- ↑ Total oxidant status [36–38]

Box 2. Osteoporosis

Osteoporosis is characterised by bone loss and excessive skeletal fragility that leads to an increased risk of fractures. Clinical manifestations include a decrease in BMD, bone cortical thickness and bone trabecular number, inducing altered bone architecture and impaired mechanical bone properties. In osteoporosis, bone loss results from an imbalance between osteoclastic bone resorption and osteoblastic bone formation activities and is positively correlated with inflammatory and oxidative context. Osteoporosis mostly affects elderly populations, with a high prevalence in postmenopausal women [20,21,25,78]. This is a major concern for public health systems as it results in disabilities and significantly affects quality of life and life expectancy.

Diabetes-associated bone complications

Diabetes is associated with increased levels of oxidative stress and an increased risk of fracture [29]. However, a causal relationship between diabetes and bone loss has not been shown. *In vivo* experiments demonstrate that streptozotocin-induced diabetic mice exhibit osteopenia together with increased levels of oxidative stress [30]. In addition, the treatment of osteoblastic cells (MC3T3) with metformin, an insulin-sensitising drug, prevents the damage caused by advanced glycation endproducts (AGE)-induced oxidative stress by blocking the upregulation of AGE receptors [31]. Thus, further studies of the role of oxidative stress and the potential relationship between altered bone health and diabetic status are needed.

Joint inflammatory diseases

Rheumatoid arthritis (RA) is the most studied model of joint inflammatory diseases. It is characterised by a loss of cartilage and enhanced bone resorption that mainly occurs in knee and hip articulations. Gene expression profiles suggest that oxidative stress mechanisms are enhanced in synovial cells during RA [32]. These findings were confirmed by cellular ROS measurements and suggest that synovial cells generate oxidative stress, supporting RA establishment [32]. In synovial fluid from patients with osteoarthritis, increased ROS levels were shown to stimulate vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and -2) expression, leading to cartilage degradation through the induction of matrix metalloproteinase activities [33]. These data are consistent with the hypothesis that chondrocyte senescence is driven by oxidative stress. Indeed, articular chondrocytes exhibit an age-related decline in proliferative and synthetic capabilities while maintaining the ability to produce proinflammatory mediators and matrix-degrading enzymes [34].

Besides RA, prosthetic loosening results from bone resorption induced by wear and debris particles generated from artificial joint articulations. Using synovial fibroblasts cultures, Wei et al. demonstrated that titanium particles are responsible for stimulating cyclo-oxygenase 2 (COX-2) expression through an oxidative stress-induced pathway [35]. In parallel, patients suffering ankylosing spondylitis, a bone inflammatory disease localising in the lower part of the spine, showed an increase in total oxidant status, including advanced oxidation of protein products and lipid peroxidation [36–38]. Accordingly, these studies support a role for oxidative stress in mediating local inflammation and enhancing bone resorption, leading to prosthetic failure, pain and ultimately increased morbidity. It would be interesting to investigate whether ROS production and oxidant status might be useful as predictive biomarkers in bone pathology. Such studies might also provide insight into the basic mechanisms underlying oxidative stress in bone biology and disease.

Oxidative stress in bone remodelling

As described above, a large body of emerging evidence indicates a negative correlation between ROS production and bone health (Figure 2). Bone tissues are constantly remodelled to allow growth and mineral homeostasis. Bone remodelling results from the close communication and balance between osteoblast and osteoclast activities (bone coupling), which leads to either bone formation or bone resorption, respectively. The roles of ROS in promoting bone diseases have mainly been investigated by assessing the effects of the superoxide anion and hydrogen peroxide on bone cell function and bone remodelling.

Bone resorption

In 1990, Garrett et al. first described the relationships between oxygen-derived free radicals, particularly the superoxide anion, and the formation and activation of osteoclasts [15]. These findings were confirmed by Lee et al. in 2005. They demonstrated that the receptor activator of nuclear factor-kappaB ligand (RANKL)-induced osteoclastogenesis requires ROS production, using bone marrow (BM) precursor cells as an osteoclast differentiation model [13]. Similarly, glutathione peroxidase 1 (Gpx1) is the main antioxidant enzyme expressed in osteoclasts and is responsible for the degradation of hydrogen peroxide. Its overexpression in the osteoclastic cell line RAW264.7 prevents RANKL-induced osteoclastogenesis [22], suggesting a crucial role for hydrogen peroxide in osteoclast formation. These authors demonstrated that 17β-estradiol stimulates Gpx1 expression in BM-derived osteoclasts and that oestrogen deficiency was a key step in the ROS-mediated stimulation of TNFα expression, leading to enhanced osteoclastogenesis and bone resorption [39].

Furthermore, patients with hyperhomocysteinemia showed a higher risk of developing osteoporosis and bone fractures. Interestingly, in mouse BM cells, the intracellular generation of ROS was found to be mediated by homocysteine treatment. In addition, compared with controls, homocysteine-treated cultures exhibited enhanced levels of osteoclast differentiation and activities, supported by a higher number of tartrate-resistant acid phosphatase (TRACP)-positive multinucleated cells with increased numbers of nuclei per cell, stimulation of actin ring formation and upregulation of integrin beta3 mRNA levels [12].

In diabetes, hyperglycaemia is associated with enhanced ROS generation in endothelial cells and altered bone metabolism. In contrast to endothelial cells, osteoclast precursors incubated with high concentrations of D-glucose inhibited ROS production through a metabolic pathway and were prevented from RANKL-induced osteoclast formation [14]. Taken together, these studies indicate that ROS impact

Box 3. Molecular basis of coupling in bone remodelling

RANKL, a member of the tumour necrosis factor (TNF) cytokine family, has been shown to both activate mature osteoclasts and mediate osteoclastogenesis. Within the bone system, RANKL is expressed by osteoblast lineage cells and exerts its biological effect by binding to the RANK receptor at the surface of osteoclasts. Osteoprotegerin (OPG), is produced by osteoblastic/stromal cells and acts as a decoy receptor for RANKL, preventing it from binding to and activating RANK on the osteoclast surface. Thus, the biological effects of OPG on bone cells include the inhibition of terminal stages of osteoclast differentiation, suppression of the activation of mature osteoclasts and induction of apoptosis [40,41,79].

bone resorption by directly promoting osteoclast formation and activity.

Bone formation/resorption coupling

The molecular triad of osteoprotegerin (OPG), the receptor activator of nuclear factor-kappaB (RANK) and the receptor activator of nuclear factor-kappaB ligand (RANKL) plays a crucial role in bone remodelling and functions as a pivotal molecular link for osteoblast and osteoclast coupling [40,41] (Box 3, Figure 2). In the human osteoblast-like MG63 cell line, primary mouse bone marrow stromal cells (BMSCs) and calvarial osteoblasts, increased intracellular ROS levels (H_2O_2 or the xanthine/xanthine oxidase-generated superoxide anion) were shown to stimulate RANKL mRNA and protein expression. These data strongly support the involvement of ROS such as H_2O_2 or the superoxide anion in bone loss-related diseases by stimulating osteoclast differentiation and bone resorption [10].

In ovariectomised mice, increased production of TNFα, a cytokine that promotes osteoclast differentiation, was observed in BM in response to both oxidative stress and T-cell activation. This effect might contribute to the mechanisms underlying bone loss during menopause [42]. More recently, using osteoclast precursors from BM and osteoblast/pre-osteoclast co-culture, Chen et al. found that ethanol-induced RANKL expression relies on increased intracellular levels of ROS through enhanced NADPH oxidase activity [43]. These data provide new insights into the potential sources of ROS that promote bone cell communication and reveal that even indirectly, through RANKL expression by osteoblasts, the superoxide anion and hydrogen peroxide support osteoclast differentiation and subsequent bone resorption.

Bone formation

Bone loss can result either from enhanced osteoclast bone resorption or decreased osteoblast bone formation. Despite its influence on the stimulation of RANKL expression in osteoblasts, recent investigations of the role of H_2O_2 in osteoblast activities have demonstrated an overall inhibition of cell differentiation. In 2004, Liu et al. showed that hydrogen peroxide induced oxidative stress and suppressed the osteoblastic differentiation process in primary mouse BMSCs. This inhibition of cell differentiation was characterised by a reduction in alkaline phosphatase (ALP) activity, a key enzyme involved in mineral apposition. The presence of metallothionein, a ROS inhibitor, restored cell differentiation, suggesting a crucial role for oxidative stress in the inhibition of osteoblastic differentiation [44]. Subsequently, Bai et al. confirmed that similar levels of H_2O_2 induced oxidative stress and suppressed osteoblastic differentiation of primary rabbit BMSCs [45]. In this study, H_2O_2-treated osteoblasts exhibited a reduction in levels of differentiation markers including ALP, type I collagen, colony-forming unit-osteoprogenitor (CFU-O) formation, and nuclear phosphorylation of the transcription factor Runx2. More recently, the relationship between the antioxidant system and bone formation was examined in MC3T3-E1 cells. When incubated with H_2O_2, mineralisation levels were diminished and gene expression of the osteogenic markers Runx2, ALP and bone sialoprotein was decreased [46].

Apart from affecting the differentiation process, ROS also influence osteoblast lifespan. Glutaredoxin 5 (Grx5) is a highly expressed protein in bone that might play a role in ROS protection. When overexpressed, Grx5 prevents ROS production and protects osteoblastic cells from ROS-induced apoptosis through a mechanism depending on manganese superoxide dismutase activity (MnSOD) [47]. Conversely, ROS are believed to be involved in chondrocyte hypertrophy, a crucial step for the longitudinal growth and development of long bones. This hypothesis is supported by *in vivo* and *in vitro* experiments, demonstrating that treatment with N-acetyl cysteine enhances endogenous antioxidant levels and protects cells from oxidative stress, thereby promoting chondrocyte proliferation while inhibiting hypertrophy [48]. Therefore, in contrast to what is observed in osteoblast cells, ROS seem to stimulate chondrocyte terminal differentiation and promote endochondral ossification.

Taken together, these data demonstrate that ROS not only directly promotes osteoclastogenesis but also supports bone loss by inducing apoptosis and the decreased differentiation and activities of osteoblasts while stimulating RANKL-induced osteoclast formation. Thus, by affecting both cell types as well as bone cell communication, oxidative stress seems to be of major importance in bone cell function. These findings also suggest that targeting the development of oxidative stress and the design of potential antioxidant strategies for the treatment of bone diseases should be investigated.

Molecular redox mechanisms in bone cells

Understanding the mechanisms by which ROS or oxidative stress might modulate intracellular signalling pathways in bone cells is essential for identifying potential molecular targets for manipulation of cellular redox status.

Sources

Redox homeostasis is crucial for many cell functions. In particular, oxidative stress is involved in altered gene expression caused by the deregulation of redox-sensitive transcription factor activities. Oxidative stress results from the excessive production of ROS that can originate from less well-regulated oxidative energy metabolisms in the mitochondrial electron-transport chain (due to electron leak in the respiratory chain) or can be generated at the surface of the cells by enzymatic complexes such as

NADPH oxidases (Nox) [49]. Previously, NADPH oxidase activities were only considered to produce superoxide anions (O^{2-}) in phagocytes. Recently, however, several homologues were found in different tissues, suggesting an important role for Nox in inducing oxidative stress in many cell types including bone cells [50]. Indeed, Nox-1, -2 and -4 were found to be expressed both in osteoclast precursors and stromal osteoblasts [13,43].

General mechanisms

ROS play an important role as regulatory mediators in signalling processes. For a given signalling protein, an oxidative attack induces a loss of function, a gain of function or a switch to a different function. Superoxide anions produced in the extracellular space are unstable and are converted into more stable hydrogen peroxide (H_2O_2) [4,51]. H_2O_2 is neutral and can diffuse through cell membranes to oxidise cysteine-rich regions in cytoplasmic proteins [4]. ROS are also known to be involved in modulating tyrosine kinase phosphorylation pathways either directly by stimulating tyrosine kinase receptors or indirectly by inhibiting tyrosine phosphatases [4,52,53]. This mechanism has been well characterised for the insulin receptor [52].

In bone, H_2O_2 has been shown to oxidise those proteins involved in cell differentiation and modulate their activity, either by inhibition or stimulation. Among the redox-sensitive signalling pathways involved in bone cell differentiation, mitogen-activated protein kinases (MAPKs), Wnt/β-catenin and nuclear factor-kappaB (NF-κB) are the most studied (Figure 3).

Mitogen-activated protein kinases

MAPKs have important roles in cell proliferation, differentiation and apoptosis. Therefore, extracellular signal-regulated kinase (ERK1/2), p38 and c-Jun N terminal kinase (JNK) activities have been studied under conditions of oxidative stress. In 2005, Lee et al. showed that osteoclast differentiation stimulated by RANKL was mediated by ROS production and the subsequent activation of JNK, p38 and ERK1/2. These data were supported by studies of inhibition of ROS production using Nox-1 RNAi (RNA interference) in a primary osteoclast precursor cell model of BM macrophages [13], and suggested that the superoxide anion generated by Nox-1 is involved in osteoclast formation. In primary osteoblasts and BMSCs, treatment with H_2O_2 stimulates ERK1/2 phosphorylation as well as phospholipase C-γ1 and ERK-dependent NF-κB activation, resulting in impaired osteoblastic differentiation [45]. Recently, Chen et al. reported the involvement of Nox enzymes in ethanol induction of ERK phosphorylation and nuclear translocation. They demonstrated that inhibition of Nox enzyme activity abolished ERK activation and prevented the stimulation of RANKL expression in mouse stromal osteoblasts [43]. These data are consistent with the study from Bai et al. showing that induction of RANKL expression by ROS was mediated through ERK activation [10].

Wnt/β-catenin

Wnts are secreted lipid-modified proteins known to bind their cognate receptor and co-receptor, Frizzled and LRP5/LRP6, respectively. These molecules are widely involved in bone formation and osteoblast functions. Wnt signalling promotes the accumulation of β-catenin in the cytoplasm by preventing its proteosomal degradation. Once translocated into the nucleus, β-catenin is involved in the stimulation of osteoblastic markers, thereby promoting osteoblast differentiation [54]. In conditions of oxidative stress, as occurs with aging or oestrogen deficiency, ROS production is increased and proteins such as p53 and p66shc are phosphorylated [18,55]. Most notably, activation of the

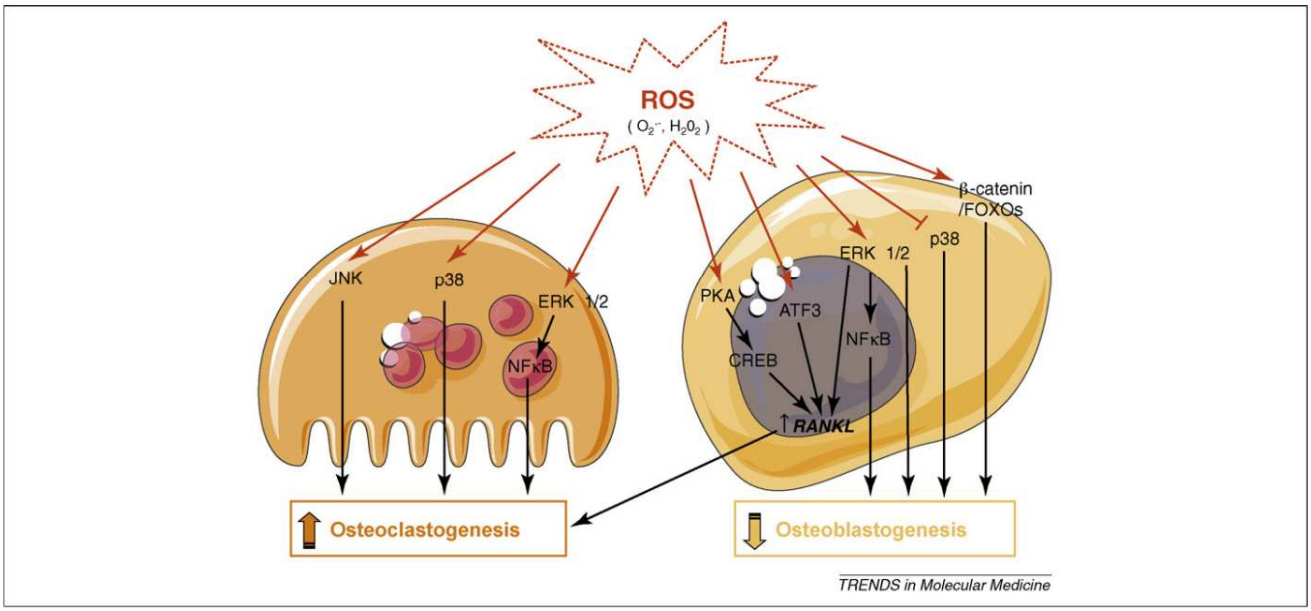


Figure 3. ROS modulation of signalling pathways in bone cells. ROS promote bone loss by inhibiting osteoblast differentiation and enhancing osteoclastogenesis. ROS-induced bone resorption occurs directly or indirectly (increased RANKL expression) through the modulation of kinases and transcription factor activities in both osteoclasts and osteoblasts.

adapter protein p66shc by oxidative stress promotes the association between Forkhead box-O (FOXO) transcription factors and β-catenin. Thus, by diverting the pool of β-catenin, ROS inhibit Wnt-induced osteoblastic gene expression and can play a role in causing increased osteoblast and osteocyte apoptosis [54].

Nuclear factor-kappaB

RANKL binds to its cognate receptor RANK to induce NF-κB activation, leading to osteoclast differentiation, activation and maturation. Therefore, interactions between ROS and NF-κB have been investigated. Alpha-lipoic acid (LA), a strong antioxidant, was found to suppress RANKL-induced osteoclastogenesis from BM-derived precursor cells. This phenomenon was mediated through the inhibition of ROS production and NF-κB activation in osteoclast precursor cells. Specifically, LA reduced DNA binding of NF-κB but did not inhibit inhibitory kappaB kinase (IKK) activation [56]. In addition, pretreatment of osteoclasts with the antioxidants N-acetyl-l-cysteine and GSH reduced RANKL-induced NF-κB activation. However, in this case, the reduced NF-κB activity was associated with decreased IKK activity and IkB-α phosphorylation [11]. These data provide evidence that ROS play an important role in osteoclast differentiation through NF-κB regulation. In osteoblasts, the addition of exogenous metallothionein impaired H_2O_2-stimulated NF-κB signalling and prevented the hydrogen peroxide-induced inhibition of osteoblastic differentiation of primary mouse BMSCs [44]. More recently, high D-glucose concentration was shown to prevent RANKL-induced osteoclast formation by inhibiting NF-κB activity through an antioxidative mechanism, suggesting that high glucose levels might alter bone turnover by decreasing osteoclast differentiation and function in diabetes [14].

Other signalling pathways: ATF, CREB and HSF

Besides MAPKs, Wnt and NF-κB, other signalling pathways have also been shown to be regulated by ROS. As described for ERK, Chen et al. showed that activating transcription factor 3 (ATF-3) contributed to increased gene expression of RANKL in mouse osteoblasts following the stimulation of NADPH oxidase activity and ROS production by ethanol [43]. Further analysis by Bai et al. revealed that ROS promote phosphorylation of cAMP response element-binding protein (CREB)/ATF-2 and its binding to the CRE domain in the murine RANKL promoter region. Moreover, using protein kinase A (PKA) inhibitor KT5720 and CREB1 RNAi transfection, the PKA–CREB signalling pathway was shown to be necessary for the ROS stimulation of RANKL expression in mouse osteoblasts. However, in human MG63 cells, ROS promoted heat shock factor 2 (HSF2) binding to the heat shock element in the human RANKL promoter region, but PKA activation was not required for this, as revealed by KT5720 treatment and HSF2 RNAi experiments [10]. These data provide some explanation for the imbalance between osteoblast and osteoclast activities during aging and increased oxidative stress leading to altered bone health.

Among the ROS, the superoxide anion and hydrogen peroxide are predominantly implicated in interfering with osteoclast and osteoblast signalling, respectively. In conditions of oxidative stress, many key pathways involved in bone cell proliferation, differentiation and apoptosis are affected. Each pathway represents a potential therapeutic avenue, and suggests further studies for the design of antioxidant strategies to prevent excessive bone loss.

Prevention of redox-associated bone disease

Because of the crucial roles of ROS and oxidative stress in bone turnover, interest in the use of antioxidants in potential treatments for osteoporosis and bone inflammatory diseases has increased. Different experimental protocols have been studied using either pharmacological or nutritional approaches.

Current treatments for osteoporosis

Hormone replacement therapy (HRT) has shown clear benefits for bone health in postmenopausal osteoporotic women. *In vitro* experiments have indicated the possible underlying mechanisms. Pretreatment of osteocyte cell lines with 17β-estradiol resulted in the inhibition of H_2O_2-induced apoptosis and correlated with a decrease in intracellular ROS production [57]. In postmenopausal women, lower catalase activity was observed; treatment with raloxifene, a selective oestrogen-receptor modulator (SERM), was shown to correct catalase activity and reduce oxidative status [21], as demonstrated by decreased protein carbonyl levels in the serum [58]. However, a higher prevalence of thromboembolic events, uterine and breast cancers has also been reported for HRT [59].

Nutritional approaches

Epidemiological studies have provided evidence of a link between nutrient, antioxidant intake and bone health, and have led to investigations of the antioxidant properties of nutrients. When fed with grapefruit pulp, orchidectomised male rats showed higher antioxidant status and improved bone density [60]. Polyphenols, a major antioxidant component in grapefruit, are the most abundant antioxidants in the diet and are widespread constituents of fruits, vegetables, cereals, dry legumes, chocolate, tea, coffee and wine. Experimental studies in animals or cultured cell lines have supported roles for polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes or osteoporosis [61]. Green tea polyphenols were reported to prevent ovariectomy-induced bone loss in female rats by inhibiting a decrease in glutathione peroxidase activity [62]. Myricetin, a naturally occurring flavonoid, was reported to prevent oxidative damage in osteoblastic MC3T3-E1 cells [63], and grape seed proanthocyanidin extract decreased ROS production in lipopolysaccharide-stimulated murine osteoclast-like cells [64]. In addition, quercetin, a plant-derived flavonoid, was found to be protective against gestational oxidative stress-related bone damage, supporting a potential benefit of antioxidant nutrients in bone pathophysiology [65].

Besides polyphenols, pigments such as lycopene, present in red tomatoes, have also exhibited health benefits

from their antioxidant properties. Recently, a cross-sectional study of 33 postmenopausal women showed that high lycopene consumption was associated with a low serum concentration of N-telopeptide of collagen type 1 (NTx) and lower serum protein oxidation, suggesting a decrease in ROS-induced bone resorption [66].

In addition, lipids are not only targets for ROS but also have a protective effect in bone tissue. Mice fed with fish oil are protected from ovariectomy-induced bone loss because of the inhibition of osteoclastogenesis. In fish oil-fed mice, plasma omega-3 fatty acid concentration was significantly increased compared with omega-6 fatty acids. These findings are consistent with *in vitro* studies; selected n-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid) were demonstrated to cause a significant decrease in TRACP activity and TRACP+ multinuclear cell formation from BM cells compared with selected n-6 fatty acids (linoleic acid and arachidonic acid) [67]. Interestingly, arachidonic acid, an omega-6 fatty acid found in high proportions in western diets, was shown to be an effective activator of ROS production. By contrast, eicosapentaenoic acid and docosahexaenoic acid reduced superoxide production catalysed by the NADPH oxidase in neutrophils [68]. The antioxidant effects were more pronounced with increasing carbon chain length and polyunsaturation [68]. However, whether prevention of bone loss by omega-3 fatty acids involves the inhibition of ROS production remains to be determined.

In other studies, collagen-induced arthritis in mice was shown to be associated with an increased intracellular ROS production in lymphocytes from inguinal lymph nodes, together with inflammatory cytokines. Treatment with LA decreased ROS production and inflammatory properties, and suggested a role for LA in the antioxidant prevention of inflammatory joint diseases [69]. In addition, pretreatment with LA inhibited both TNFα- and H_2O_2-induced intracellular ROS production and oxidative stress-associated apoptosis of human BM stem cells [70].

Recently, a study of the relationships between vitamin E serum concentration (in the form α-tocopherol), oxidant status and BMD was conducted in elderly men. Vitamin E is a fat-soluble vitamin that can scavenge peroxyl radicals. The authors demonstrated a positive correlation between α-tocopherol serum concentration, low urinary 8-iso-PGF2α (an *in vivo* biomarker of oxidative stress) and higher BMD [71].

Taken together, several studies have provided support for nutritional approaches to antioxidant strategies for the prevention of bone loss. However, despite some epidemiological evidence, clinical studies of antioxidant supplementation in established diseases have so far been inconclusive overall [72].

Concluding remarks

In this review, we have considered several key questions: what is the importance of oxidative stress in bone tissue function? What are the underlying mechanisms of oxidative stress in bone tissue? And could oxidative stress be a relevant target in the design of new therapeutic approaches for bone diseases?

Clinical studies have determined a positive correlation between plasma oxidant status and bone disease development, most notably in pathologies inducing bone loss, such as osteoporosis. *In vivo* and *in vitro* experiments have shown that oxidative stress affects both osteoclastic and osteoblastic cells. In both cases, the effect of oxidative stress seems to be bone loss. Indeed, ROS was shown to promote osteoclast resorption either directly, by mimicking RANK signalling and stimulating osteoclast differentiation, or indirectly, by stimulating osteoblast/osteoclast coupling and subsequent osteoclast differentiation supported by RANKL expression. Several groups have demonstrated the involvement of ROS in modulating redox-sensitive signalling pathways that play major roles in bone cell differentiation, including MAPK activation, β-catenin and NF-κB activity.

In this context, the design of antioxidant-based strategies to prevent bone loss would be of major interest. For the elderly, such strategies could strongly impact morbidity and mortality, as well as the social and economic burden associated with osteoporosis. In addition, the importance of bone resorption in the establishment of bone metastasis in breast and prostate cancers has been reported [73]. In this study, a vicious cycle contributing to tumour progression was described in which cancer cells promote RANKL expression and stimulate osteoclast resorption. It was suggested that the resulting release of growth factors from bone matrix degradation can create a favourable environment, leading to tumour cell establishment. Inhibition of osteoclastic activities using antioxidant approaches might prevent this vicious cycle and the establishment of subsequent bone metastasis.

It would be interesting to investigate whether antioxidant intervention could define a universal strategy for the treatment of different bone diseases. This is not a simple hypothesis to test. Most primary bone tumours such as osteosarcoma are highly malignant and current treatments rely on chemotherapies. Despite the involvement of oxidative stress in carcinogenesis, the aim of some chemotherapies is to induce ROS production in rapidly dividing cells and promote subsequent apoptosis of cancer cells [27,74,75]. Thus, an antioxidant strategy might not be relevant and might even be deleterious for the treatment of such diseases. However, several groups have recently studied the potential of micronutrients with antioxidant capabilities in reducing the side effects of chemotherapies [75,76]. Interestingly, they demonstrated in mouse models that astaxanthin and ebselen attenuate cyclophosphamide-induced genotoxicity in normal cells. Their results suggest that antioxidant approaches might be relevant to avoid the development secondary cancers associated with chemotherapy treatments.

Further studies will be needed to investigate whether antioxidant treatment strategies might be beneficial or detrimental to bone health. The answer might depend on the type of bone disease targeted, and the timing of such approaches might also be relevant, for instance, before bone tumour establishment or after chemotherapy. Current data suggest that antioxidant strategies might be beneficial to bone health if they are considered as approaches to prevent bone loss and its associated morbidity and mortality. In this

case, the long-term influence of an antioxidant-rich diet could be of great interest. Finally, manipulation of the redox balance in bone cells is clearly a potential approach that should be investigated for the treatment of bone disease. However, further studies of the cellular and molecular mechanisms underlying the relationship between oxidative stress and bone health will first be needed.

Acknowledgements

This work was supported by the Institut Français de Recherche en Odontologie (IFRO). Illustrations were produced with the assistance of Servier Medical Art.

References

1 Bogdan, C. (2001) Nitric oxide and the immune response. *Nat. Immunol.* 2 (10), 907–916
2 Gordon, S. (2003) Alternative activation of macrophages. *Nat. Rev. Immunol.* 3 (1), 23–35
3 Babior, B.M. (1978) Oxygen-dependent microbial killing by phagocytes (first of two parts). *N. Engl. J. Med.* 298 (12), 659–668
4 Reth, M. (2002) Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* 3 (12), 1129–1134
5 Chabannes, D. *et al.* (2007) A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 110 (10), 3691–3694
6 Chauveau, C. *et al.* (2005) Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* 106 (5), 1694–1702
7 Krause, K.H. (2007) Aging: a revisited theory based on free radicals generated by NOX family NADPH oxidases. *Exp. Gerontol.* 42 (4), 256–262
8 Valko, M. *et al.* (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell. Biol.* 39 (1), 44–84
9 Naka, K. *et al.* (2008) Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid. Redox. Signal* 10 (11), 1883–1894
10 Bai, X.C. *et al.* (2005) Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast. *J. Biol. Chem.* 280 (17), 17497–17506
11 Ha, H. *et al.* (2004) Reactive oxygen species mediate RANK signalling in osteoclasts. *Exp. Cell Res.* 301 (2), 119–127
12 Koh, J.M. *et al.* (2006) Homocysteine enhances bone resorption by stimulation of osteoclast formation and activity through increased intracellular ROS generation. *J. Bone Miner Res.* 21 (7), 1003–1011
13 Lee, N.K. *et al.* (2005) A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood* 106 (3), 852–859
14 Wittrant, Y. *et al.* (2008) High d(+)glucose concentration inhibits RANKL-induced osteoclastogenesis. *Bone* 42 (6), 1122–1130
15 Garrett, I.R. *et al.* (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone *in vitro* and *in vivo*. *J. Clin. Invest.* 85 (3), 632–639
16 Altindag, O. *et al.* (2008) Total oxidative/antioxidative status and relation to bone mineral density in osteoporosis. *Rheumatol. Int.* 28 (4), 317–321
17 Sendur, O.F. *et al.* (2009) Antioxidant status in patients with osteoporosis: a controlled study. *Joint Bone Spine*
18 Almeida, M. *et al.* (2007) Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. *J. Biol. Chem.* 282 (37), 27285–27297
19 Muthusami, S. *et al.* (2005) Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. *Clin. Chim. Acta* 360 (1–2), 81–86
20 Ozgocmen, S. *et al.* (2007) Role of antioxidant systems, lipid peroxidation and nitric oxide in postmenopausal osteoporosis. *Mol. Cell Biochem.* 295 (1–2), 45–52
21 Ozgocmen, S. *et al.* (2007) Effects of calcitonin, risedronate and raloxifene on erythrocyte antioxidant enzyme activity, lipid peroxidation and nitric oxide in postmenopausal osteoporosis. *Arch Med. Res.* 38 (2), 196–205
22 Lean, J.M. *et al.* (2005) Hydrogen peroxide is essential for oestrogen-deficiency bone loss and osteoclast formation. *Endocrinology* 146 (2), 728–735
23 Jagger, C.J. *et al.* (2005) Tumour necrosis factor-alpha mediates osteopenia caused by depletion of antioxidants. *Endocrinology* 146 (1), 113–118
24 Sanchez-Rodriguez, M.A. *et al.* (2007) Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterised by antioxidant enzymes. *BMC Musculoskelet Disord.* 8, 124
25 Yalin, S. *et al.* (2005) Is there a role of free oxygen radicals in primary male osteoporosis? *Clin. Exp. Rheumatol.* 23 (5), 689–692
26 Yossepowitch, O. *et al.* (2007) Advanced but not localised prostate cancer is associated with increased oxidative stress. *J. Urol.* 178 (4 Pt 1), 1238–1243 discussion 1243-1244
27 Magwere, T. *et al.* (2008) Manipulation of oxidative stress to induce cell death in Ewing's sarcoma family of tumours. *Eur. J. Cancer* 44 (15), 2276–2287
28 Roepke, M. *et al.* (2007) Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Cancer Biol. Ther.* 6 (2), 160–169
29 Strotmeyer, E.S. and Cauley, J.A. (2007) Diabetes mellitus, bone mineral density and fracture risk. *Curr. Opin. Endocrinol. Diabetes Obes.* 14 (6), 429–435
30 Hamada, Y. *et al.* (2007) Histomorphometric analysis of diabetic osteopenia in streptozotocin-induced diabetic mice: a possible role of oxidative stress. *Bone* 40 (5), 1408–1414
31 Schurman, L. *et al.* (2008) Metformin reverts deleterious effects of advanced glycation end-products (AGEs) on osteoblastic cells. *Exp. Clin. Endocrinol. Diabetes* 116 (6), 333–340
32 Kabuyama, Y. *et al.* (2008) Involvement of thioredoxin reductase 1 in the regulation of redox balance and viability of rheumatoid synovial cells. *Biochem. Biophys. Res. Commun.* 367 (2), 491–496
33 Fay, J. *et al.* (2006) Reactive oxygen species induce expression of vascular endothelial growth factor in chondrocytes and human articular cartilage explants. *Arthritis Res. Ther.* 8 (6), R189
34 Loeser, R.F. (2009) Aging and osteoarthritis: the role of chondrocyte senescence and aging changes in the cartilage matrix. *Osteoarthritis Cartilage*
35 Wei, X. *et al.* (2009) Titanium particles stimulate COX-2 expression in synovial fibroblasts through an oxidative stress-induced, calpain-dependent, NF-kappaB pathway. *Am. J. Physiol. Cell Physiol.*
36 Karakoc, M. *et al.* (2007) Serum oxidative-antioxidative status in patients with ankylosing spondylitis. *Rheumatol. Int.* 27 (12), 1131–1134
37 Yazici, C. *et al.* (2004) Protein oxidation status in patients with ankylosing spondylitis. *Rheumatology (Oxford)* 43 (10), 1235–1239
38 Ozgocmen, S. *et al.* (2004) Serum nitric oxide, catalase, superoxide dismutase and malondialdehyde status in patients with ankylosing spondylitis. *Rheumatol. Int.* 24 (2), 80–83
39 Lean, J.M. *et al.* (2003) A crucial role for thiol antioxidants in oestrogen-deficiency bone loss. *J. Clin. Invest.* 112 (6), 915–923
40 Hsu, H. *et al.* (1999) Tumour necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc. Natl. Acad. Sci. U. S. A.* 96 (7), 3540–3545
41 Lacey, D.L. *et al.* (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93 (2), 165–176
42 Grassi, F. *et al.* (2007) Oxidative stress causes bone loss in oestrogen-deficient mice through enhanced bone marrow dendritic cell activation. *Proc. Natl. Acad. Sci. U. S. A.* 104 (38), 15087–15092
43 Chen, J.R. *et al.* (2008) Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signalling cascade. *J. Pharmacol. Exp. Ther.* 324 (1), 50–59
44 Liu, A.L. *et al.* (2004) Metallothionein protects bone marrow stromal cells against hydrogen peroxide-induced inhibition of osteoblastic differentiation. *Cell Biol. Int.* 28 (12), 905–911
45 Bai, X.C. *et al.* (2004) Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. *Biochem. Biophys. Res. Commun.* 314 (1), 197–207

46 Arai, M. *et al.* (2007) Effects of reactive oxygen species (ROS) on antioxidant system and osteoblastic differentiation in MC3T3-E1 cells. *IUBMB Life* 59 (1), 27–33
47 Linares, G.R. *et al.* (2009) Glutaredoxin 5 regulates osteoblast apoptosis by protecting against oxidative stress. *Bone* 44 (5), 795–804
48 Morita, K. *et al.* (2007) Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. *J. Exp. Med.* 204 (7), 1613–1623
49 Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev.* 82 (1), 47–95
50 Bedard, K. and Krause, K.H. (2007) The Nox family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol. Rev.* 87 (1), 245–313
51 Shao, J.S. *et al.* (2007) Vascular bmp MSX2 wnt signalling and oxidative stress in arterial calcification. *Ann. N. Y. Acad. Sci.* 1117, 40–50
52 Mahadev, K. *et al.* (2001) Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b *in vivo* and enhances the early insulin action cascade. *J. Biol. Chem.* 276 (24), 21938–21942
53 Xu, D. *et al.* (2002) Oxidants painting the cysteine chapel: redox regulation of PTPs. *Dev. Cell* 2 (3), 251–252
54 Manolagas, S.C. and Almeida, M. (2007) Gone with the Wnts: beta-catenin, T-cell factor, forkhead box O and oxidative stress in age-dependent diseases of bone, lipid and glucose metabolism. *Mol. Endocrinol.* 21 (11), 2605–2614
55 Almeida, M. *et al.* (2007) Oxidative stress antagonises Wnt signalling in osteoblast precursors by diverting beta-catenin from T-cell factor- to forkhead box O-mediated transcription. *J. Biol. Chem.* 282 (37), 27298–27305
56 Kim, H.J. *et al.* (2006) Antioxidant alpha-lipoic acid inhibits osteoclast differentiation by reducing nuclear factor-kappaB DNA binding and prevents *in vivo* bone resorption induced by receptor activator of nuclear factor-kappaB ligand and tumour necrosis factor-alpha. *Free Radic. Biol. Med.* 40 (9), 1483–1493
57 Mann, V. *et al.* (2007) The antioxidant effect of oestrogen and Selective Oestrogen Receptor Modulators in the inhibition of osteocyte apoptosis *in vitro*. *Bone* 40 (3), 674–684
58 Korucuoglu, U. *et al.* (2006) Assessment of protein oxidation in women using raloxifene. *Mol. Cell Biochem.* 290 (1–2), 97–101
59 Valverde, P. (2008) Pharmacotherapies to manage bone loss-associated diseases: a quest for the perfect benefit-to-risk ratio. *Curr. Med. Chem.* 15 (3), 284–304
60 Deyhim, F. *et al.* (2008) Grapefruit pulp increases antioxidant status and improves bone quality in orchidectomised rats. *Nutrition* 24 (10), 1039–1104
61 Scalbert, A. *et al.* (2005) Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* 45 (4), 287–306
62 Shen, C.L. *et al.* (2008) Protective effect of green tea polyphenols on bone loss in middle-aged female rats. *Osteoporos Int.* 19 (7), 979–990
63 Lee, K.H. and Choi, E.M. (2008) Myricetin, a naturally occurring flavonoid, prevents 2-deoxy-D-ribose induced dysfunction and oxidative damage in osteoblastic MC3T3-E1 cells. *Eur. J. Pharmacol.* 591 (1–3), 1–6
64 Houde, V. *et al.* (2006) Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharides of periodontopathogens. *J. Periodontol.* 77 (8), 1371–1379
65 Prater, M.R. *et al.* (2008) Placental oxidative stress alters expression of murine osteogenic genes and impairs foetal skeletal formation. *Placenta* 29 (9), 802–808
66 Rao, L.G. *et al.* (2007) Lycopene consumption decreases oxidative stress and bone resorption markers in postmenopausal women. *Osteoporos Int.* 18 (1), 109–115
67 Sun, D. *et al.* (2003) Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomised mice. *J. Bone Miner Res.* 18 (7), 1206–1216
68 Schneider, S.M. *et al.* (2001) Activity of the leukocyte NADPH oxidase in whole neutrophils and cell-free neutrophil preparations stimulated with long-chain polyunsaturated fatty acids. *Inflammation* 25 (1), 17–23
69 Lee, E.Y. *et al.* (2007) Alpha-lipoic acid suppresses the development of collagen-induced arthritis and protects against bone destruction in mice. *Rheumatol. Int.* 27 (3), 225–233
70 Byun, C.H. *et al.* (2005) Alpha-lipoic acid inhibits TNF-alpha-induced apoptosis in human bone marrow stromal cells. *J. Bone Miner Res.* 20 (7), 1125–1135
71 Ostman, B. *et al.* (2009) Oxidative stress and bone mineral density in elderly men: antioxidant activity of alpha-tocopherol. *Free Radic. Biol. Med.*
72 Pattison, D.J. and Winyard, P.G. (2008) Dietary antioxidants in inflammatory arthritis: do they have any role in etiology or therapy? *Nat. Clin. Pract. Rheumatol.* 4 (11), 590–596
73 Wittrant, Y. *et al.* (2004) RANKL/RANK/OPG: new therapeutic targets in bone tumours and associated osteolysis. *Biochim. Biophys. Acta* 1704 (2), 49–57
74 Iwasaki, H. and Suda, T. (2009) Cancer stem cells and their niche. *Cancer Sci.* 100 (7), 1166–1172
75 Tripathi, D.N. and Jena, G.B. (2009) Intervention of astaxanthin against cyclophosphamide-induced oxidative stress and DNA damage: a study in mice. *Chem. Biol. Interact* 180 (3), 398–406
76 Tripathi, D.N. and Jena, G.B. (2008) Ebselen attenuates cyclophosphamide-induced oxidative stress and DNA damage in mice. *Free Radic. Res.* 42 (11–12), 966–977
77 Kadenbach, B. *et al.* (2009) Degenerative diseases, oxidative stress and cytochrome c oxidase function. *Trends Mol. Med.* 15 (4), 139–147
78 Maggio, D. *et al.* (2003) Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. *J. Clin. Endocrinol. Metab.* 88 (4), 1523–1527
79 Theoleyre, S. *et al.* (2004) The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodelling. *Cytokine Growth Factor Rev.* 15 (6), 457–475
80 Basu, S. *et al.* (2001) Association between oxidative stress and bone mineral density. *Biochem. Biophys. Res. Commun.* 288 (1), 275–279
81 Oh, B. *et al.* (2007) Associations of catalase gene polymorphisms with bone mineral density and bone turnover markers in postmenopausal women. *J. Med. Genet* 44 (1), e62

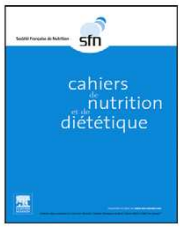
Annexe numéro 2:

Vitamine K et physiologie osseuse

Véronique Coxam, Marie-Jeanne Davicco, Fabien Wauquier, Yohann Wittrant

Cahiers de nutrition et de diététique, 2009

Cahiers de nutrition et de diététique (2009) 44, 163—172



PHYSIOLOGIE

Vitamine K et physiologie osseuse

Vitamin K and bone physiology

Véronique Coxam*, Marie-Jeanne Davicco, Fabien Wauquier, Yohann Wittrant

INRA Theix, unité de nutrition humaine (UMR1019), 63122 Saint-Genès Champanelle, France

Reçu le 10 mars 2009 ; accepté le 18 mars 2009
Disponible sur Internet le 29 avril 2009

MOTS CLÉS
Vitamine K ;
Santé osseuse ;
Supplémentation

Résumé La prévention nutritionnelle de l'ostéoporose associant classiquement calcithérapie et supplémentation en vitamine D doit évoluer vers de nouveaux concepts qui intègrent le potentiel exercé par certains nutriments et microconstituants, tout en respectant les grands équilibres. Parmi les nutriments d'intérêt pour la santé osseuse, la vitamine K occupe une place privilégiée. Le terme de vitamine K regroupe, en fait, plusieurs composés liposolubles dont la phylloquinone (vitamine K_1) présente dans les végétaux, les ménaquinones (vitamines K_2) d'origine bactérienne et donc retrouvée dans les produits fermentés. La vitamine K joue un rôle fondamental dans la conversion post-translationnelle des résidus glutamates en γ-carboxyglutamates d'un certain nombre de protéines dont l'ostéocalcine impliquée dans la régulation du processus de minéralisation et donc du capital osseux. Pourtant la carence en vitamine K est relativement fréquente, au moins dans certaines tranches de la population. Par conséquent, compte tenu de l'importance de la vitamine K pour le capital osseux, il convient d'être vigilant au respect des recommandations nutritionnelles. Toutefois, même si les risques induits par une consommation élevée de vitamine K apparaissent mineurs, compte tenu du nombre important de personnes sous AVK dans la population générale, une supplémentation systématique n'est pas recommandée.

KEYWORDS
Vitamin K;
Bone health;
Supplementation

Summary Nutritional prevention of osteoporosis based on calcium and vitamin D must implement new strategies which take into account the bone-building properties exhibited by some nutrients and micronutrients. Among those components, vitamin K is a source for putative innovative dietary health intervention, because it may have a potential promise for bone health. Vitamin K occurs in nature as a series of compounds among which. phylloquinone (vitamin K_1) originates from green leafy plants and green vegetables and the menaquinones (vitamin K_2) which are mainly from microbial origin and thus found in fermented food. Vitamin K plays an essential role in the post-translational conversion of specific glutamyl residues to γ-carboxyglutamyl residues in a limited number of proteins, including osteocalcin which plays a role in the orderly deposition of the hydroxyapatite matrix in bone, and thus in bone health. However, a significant

* Auteur correspondant. Véronique COXAM INRA Theix, unité de nutrition humaine (UMR1019), 63122 Saint-Genès Champanelle, France.
Adresse e-mail : coxam@clermont.inra.fr (V. Coxam).

0007-9960/$ — see front matter
doi:10.1016/j.cnd.2009.03.005

percentage of the population (a least some groups of age) fails to meet the recommended level of intake on a daily basis. Consequently, because there is an increasing body of evidence indicating that vitamin K is very important for bone health, it is clear that typical dietary intakes of vitamin K should be adequate to the recommandations. Nevertheless, it is premature to make population-based recommandations for routine supplementation, because of the interference with oral anticoagulant therapy which acts as vitamin K antagonists.

Introduction

La demande sociale du maintien d'une bonne santé et la nécessaire stabilisation des dépenses de santé conduisent à considérer l'alimentation dans la réduction des risques de survenue des maladies dégénératives liées à l'âge comme incontournable. Tel est notamment le cas pour l'ostéoporose, principale pathologie osseuse, pour laquelle la prise de conscience du rôle protecteur exercé par l'alimentation a vu l'émergence du concept de nutrition préventive. Dans ce contexte, l'étiologie des phénomènes de perte osseuse liée au vieillissement étant complexe et multifactorielle, la prévention nutritionnelle de l'ostéoporose associant classiquement calcithérapie et supplémentation en vitamine D doit évoluer vers de nouveaux concepts qui, outre le respect de l'adéquation aux besoins liés à chaque stade physiologique, intègrent le potentiel exercé par certains nutriments et microconstituants, tout en respectant les grands équilibres. Des recommandations nutritionnelles adaptées pourront ainsi permettre de pallier l'inadéquation de l'actuelle prophylaxie et, de toute évidence, d'optimiser toute thérapeutique médicamenteuse [1].

Parmi les nutriments d'intérêt pour la santé osseuse, la vitamine K occupe une place privilégiée. Le terme de vitamine K regroupe, en fait, plusieurs composés liposolubles qui dérivent du noyau 2-méthyl-1,4-naphtoquinone, dont : la phylloquinone (ou vitamine K_1, phytoménadione) présente dans les végétaux, les ménaquinones (ou vitamines K_2) d'origine bactérienne, et la ménadione synthétique (ou vitamine K_3) réservée à l'alimentation animale.

Rôle physiologique de la vitamine K

La vitamine K joue un rôle fondamental dans la conversion post-translationnelle des résidus glutamates en γ-carboxyglutamates d'un certain nombre de protéines impliquées dans le processus de coagulation (prothrombine, facteurs de coagulation VII, IX et X). Néanmoins, son rôle s'étend bien au-delà de cette cible initialement pressentie. En effet, elle est indispensable au métabolisme du cerveau car elle participe à la synthèse des sphingolipides, un des principaux constituants de la myéline des neurones et de leur membrane et certaines données laissent à penser qu'une carence en vitamine K pourrait être liée à la pathogenèse de la maladie d'Alzheimer.

Son implication dans la biologie du squelette fut découverte suite aux malformations fœtales (absence de membres supérieurs) engendrées par la prise maternelle d'antivitamines K. En fait, le tissu osseux synthétise également des protéines contenant de l'acide glutamique, telles que l'ostéocalcine (principale protéine non collagénique de l'os, d'ailleurs utilisée comme biomarqueur de l'activité ostéoblastique), la protéine Gla matricielle, et la protéine S. Toutefois, le rôle exact de l'ostéocalcine reste ambigu. En effet, chez la souris, l'invalidation du gène codant pour cette protéine se traduit par une augmentation de l'accrétion osseuse et une résistance à la castration. Néanmoins, il est admis qu'elle contrôle le processus de minéralisation par fixation sur les cristaux d'hydroxyapatite, grâce aux résidus glutamates dont l'affinité pour le minéral est acquise par la γ-carboxylation vitamine K dépendante, ce qui permet d'orienter le processus de nucléation et d'éviter une cristallisation anarchique. À l'inverse, les animaux invalidés pour le gène de la matrix-Gla protéine présentent une stature réduite (liée à l'accélération de la soudure des cartilages de conjugaison), ainsi que des images de calcification artérielle.

Il est probable que la vitamine K exerce aussi sur le squelette des effets indépendants de l'ostéocalcine. En effet, Ohsaki et al. [2] ont mis en évidence, chez le rat, des propriétés anti-inflammatoires de cette molécule. Par ailleurs, des études in vitro ont démontré que la ménaquinone pouvait inhiber la formation d'ostéoclastes et induire leur apoptose, via la modulation de la synthèse d'ARNm de protéines ostéoblastiques telles que la phosphatase alcaline osseuse, l'ostéoprotégérine, l'ostéopontine ou la matrix-Gla protéine. Toutefois, la vitamine K_1 est dénuée d'un tel effet. En outre, ces données n'ont pas été validées par des approches physiologiques in vivo.

Effets osseux de la vitamine K

Statut en vitamine K et capital osseux

La plupart des études d'observation qui ciblent les relations entre le statut en vitamine K et la santé osseuse (qu'elle soit appréciée par la mesure de la masse osseuse, ou par l'évaluation du risque de fracture), met en évidence le potentiel ostéoprotecteur de la vitamine K :

- une moindre acquisition de la masse osseuse chez l'enfant ayant un statut en vitamine K insuffisant [3] ;
- la mesure dans une cohorte de 1479 femmes et 112 hommes (étude Framingham) d'une densité minérale osseuse réduite, aussi bien au niveau vertébral que fémoral, dans le plus faible quartile de consommation de vitamine K (70,2 mg/j) pour la population féminine [4] ;
- outre cette incidence sur la masse osseuse, dans l'étude Epidos portant sur 7598 femmes âgées de plus de 75 ans, il est constaté que les concentrations systémiques en ostéocalcine décarboxylée permettent de prédire le risque fracturaire [5]. Ces données établissant une corrélation inverse entre le statut vitaminique K et le risque de fracture sont d'ailleurs corroborées par les résultats de la Nurse's Health study conduite chez 72 327 femmes âgées de 38 à 63 ans, le risque augmentant pour une consommation quotidienne inférieure à 109 µg [6]. Ces travaux

mis en place sur des cohortes importantes, sont tout à fait cohérents avec plusieurs autres investigations cliniques portant sur des effectifs plus réduits. Toutefois, les auteurs restent prudents dans l'interprétation des résultats en raison de l'existence de facteurs confondants (notamment un statut nutritionnel général qui n'est probablement pas suboptimal dans ces populations carencées en vitamine K), et parce que les taux circulants en vitamine K, sujets à des variations journalières, ne reflètent pas nécessairement la consommation à l'échelle de plusieurs années ;

- la mise en évidence de subcarences, décelées par des taux circulants faibles en vitamine K ou élevés en ostéocalcine décarboxylée, chez des patients ostéoporotiques ayant développé des épisodes fracturaires, par rapport à des volontaires sains du même âge ;
- les traitements à base d'AVK (tels que, notamment, la warfarine ou la coumarine) qui reposent sur le principe d'une liaison à la vitamine K époxyde réductase, enzyme impliquée dans le cycle de la vitamine K, et qui donc bloquent la carboxylation des résidus glutamates, empêchent, non seulement la coagulation, mais engendrent également à long terme des situations d'ostéopénie. La prescription d'une telle thérapeutique est donc considérée comme un facteur de risque d'ostéoporose.

À RETENIR

Une consommation de vitamine K basse est un facteur de risque d'ostéroporose.

Études de supplémentation en vitamine K

De façon générale, les études de supplémentation disponibles dans la littérature sont basées sur la prescription, soit de vitamine K_2 (ménaquinone 4 ; classiquement sur des populations asiatiques, et à la dose quotidienne de 45 mg), soit de vitamine K_1 (sur des cohortes d'origine caucasienne, et à des doses journalières allant de 200 μg à 10 mg), comme indiqué dans le Tableau 1. En fonction de la durée d'administration du traitement, les scientifiques fournissent des données relatives aux biomarqueurs du remodelage osseux, des paramètres de DMO, voire des critères de risque de fracture.

Cas de la vitamine K_2

Les différentes études cliniques ciblant l'utilisation de la vitamine K_2 (ménaquinone 4) sont relativement consensuelles et démontrent un bénéfice réel d'une telle supplémentation, qui passe notamment par le rétablissement du taux de γ carboxylation de l'ostéocalcine. Une méta-analyse, ainsi qu'une revue de la littérature ont d'ailleurs été publiées récemment. Ainsi, Cockayne et al. [7]. ont basé leur méta-analyse sur les études randomisées conduites sur plus de six mois. Treize d'entre elles fournissent des données relatives à la DMO, tandis que sept ont évalué l'impact sur le risque de fracture. Ces investigations permettant d'estimer les propriétés biomécaniques (de résistance) du squelette, comme indiqué précédemment, portaient uniquement sur des populations asiatiques et mettaient en jeu la vitamine K_2. Les auteurs ont calculé un *odd ratio* (OR) favorable pour la ménaquinone de 0,40 pour les fractures vertébrales (intervalle de confiance (IC 95 % ; 0,25—0,65), de 0,23 pour les fractures de la hanche (IC 95 % ; 0,12—0,47), et de 0,19 pour les fractures non vertébrales (IC 95 % ; 0,11—0,35). Les scientifiques concluent donc qu'une supplémentation à base de vitamine K_2 permet de réduire la perte osseuse chez les personnes à risque d'ostéoporose ou ostéoporotiques.

À RETENIR

La supplémentation en ménaquinone (vitamine K2) peut avoir des effets préventifs sur l'ostéoporose.

Plus récemment, Pearson et al. [8] ont realisé une revue bibliographique dans laquelle les huit études cliniques de court terme recensées démontrent une amélioration du statut en ostéocalcine carboxylée. Parmi les 23 investigations de plus long terme (6 mois—3 ans), la majorité a été conduite au Japon, avec de la vitamine K_2. À quelques exceptions près, ces travaux démontrent une suppression du processus d'ostéopénie chez les personnes à risque, que ce soit au niveau vertébral, fémoral ou des métacarpes, et même une amélioration des indices de propriétés de résistance osseuse. Les auteurs ont identifié uniquement cinq publications sur des populations européennes (dont quatre avec la vitamine K_1 et une avec la K_2).

En outre, il est important de mentionner le fait que l'Organisation mondiale de la Santé a attribué à l'efficacité de la vitamine K_2 sur la DMO et les fractures vertébrales la note de B (soit un avis positif à partir de données non définitives générées par des études cliniques randomisées et contrôlées de petite taille), les biphosphonates bénéficiant de la note A [9].

Il est toutefois important de nuancer ces résultats et d'éviter toute extrapolation immédiate à la population caucasienne. Effectivement, les habitudes de vie orientales sont différentes de la situation occidentale, de nombreux facteurs confondants pouvant être impliqués. Il ne faut notemment pas oublier que les aliments vecteurs de vitamine K_2 sont les produits fermentés, et en particulier ceux à base de soja qui constituent une source importante de phyto-estrogènes dont les vertus ostéoprotectrices sont reconnues chez les Asiatiques. En outre, les doses classiquement administrées sont toujours conséquentes (45 mg/j) et absolument pas nutritionnelles. Seuls Sasaki et al. [10] ont utilisé la dose quotidienne de 15 mg (et seulement sur 20 patients atteints d'une pathologie rénale). Ils ont mis en évidence une prévention de la perte osseuse vertébrale.

Cas de la vitamine K_1

Les études portant sur la vitamine K_1 sont plus conflictuelles.

En effet, les premiers travaux de Braam et al. [11] avaient mis en évidence un effet ostéoprotecteur de la consommation de phylloquinone (à la dose de 1 mg/j pendant trois ans), chez des femmes européennes ménopausées. Ces données ont été confirmées par Bolton-Smith et al. [12] qui démontrent qu'un apport quotidien de 200 μg de vitamine K_1 pendant deux ans, chez des femmes âgées, permet d'augmenter le contenu minéral osseux de l'extrémité du radius, toutefois uniquement si les personnes sont supplémentées en calcium (1 g) et vitamine D (10 μg). Dans l'étude de Bügel et al. [13], une supplémentation de 200 ou 500 μg par jour de vitamine K_1 pendant six semaines chez

Tableau 1 Tableau récapitulatif des études de supplémentation en vitamine K1 (K_1) et K2 (K_2). Impact sur le risque de fracture, la masse osseuse (densité minérale osseuse [DMO]) ou le remodelage osseux (biomarqueurs du métabolisme osseux).

	Références	Typologie de l'étude	Population	Principaux résultats
Risque de fracture				
[25]	Shiraki et al. 2000	2 ans de K_2 (45 mg/j) + Ca ou Ca (Japon)	241 femmes ostéoporotiques (âge moyen 65 ans)	Moindre ↘ de DMO vertébrale dans le groupe K_2 plus faible incidence de fractures dans le groupe K_2
[26]	Iwamoto et al. 2001	2 ans avec K_2 (45 mg/j) ou étidronate ou Ca (Japon)	72 femmes ménopausées ostéoporotiques (âge moyen 65 ans)	Moindre ↘ de DMO dans le groupe K_2 Moins de fractures vertébrales dans le groupe K_2
[27]	Sato et al. 2002	1 an avec K_2 (45 mg/j) ou absence de traitement (Japon)	120 femmes parkinsoniennes (âge moyen 71,9 ans)	DMO du métacarpe ↗ dans le groupe K_2 Moins de fractures dans le groupe K_2
[28]	Ishida et al. 2004	2 ans avec THS ou étidronate ou calcitonine ou 1αOHD3 ou K_2 (45 mg/j) ou placébo	396 femmes ménopausées ostéoporotiques (âge moyen 70 ans) (Japon)	Moindre ↘ de DMO dans les groupes K_2 et étidronate Plus faible incidence des fractures dans les groupes THS, étidronate, calcitonine et K_2
[29]	Sato et al. 2005	2 ans avec K_2 (45 mg/j) ou vitamine D2 ou Ca (Japon)	200 femmes avec Alzheimer et 100 témoins de même âge (âge moyen > 70 ans)	DMO du métacarpe ↗ dans le groupe K_2 Moins de fractures dans le groupe K_2
Masse osseuse				
[30]	Akiba et al. 1991	1 an avec K_2 (45 mg/j) (Japon)	17 patients en hémodialyse avec un faible remodelage osseux	K_2 ↗ ostéocalcine et prévient ↘ du contenu minéral osseux
[31]	Orimo et al. 1992	48 semaines avec K_2 (45 mg/j)/1αOHD3 (Japon)	546 patients ostéoporotiques	↗ 2,5 % de DMO du métacarpe par rapport à une ↘ de −2,4 % dans le groupe D. Pas de différence de la DMO vertébrale
[32]	Orimo et al. 1998	6 mois avec K_2 (90 mg/j) ou placébo (Japon)	80 patients ostéoporotiques (92 % femmes)	↗ 2,2 % de DMO du métacarpe, ↘ Ca urinaire
[33]	Sato et al. 1998	1 an avec K_2 (45 mg/j) ou absence de traitement (Japon)	108 hommes et femmes hémiplégiques	↗ DMO du métacarpe paralysé dans le groupe traité Perte osseuse du côté non attaint
[34]	Somekawa et al. 1999	6 mois avec K_2 (45 mg/j) ou 1,25 (OH)2D ou K_2 + vitamine D ou leuprolide	110 femmes sous leuprolide (Japon)	Moindre ↘ de DMO vertébrale dans les groupes K_2
[35]	Iwamoto et al. 1999	1 an THS ou 1αOHD ou K_2 (45 mg/j) ou placébo (Japon)	72 femmes ménopausées	DMO ↗ seulement dans le groupe sous THS
[36]	Iwamoto et al. 2000	2 ans avec 1αOHD ou K_2 (45 mg/j) ou K_2 + 1αOHD ou Ca (Japon)	92 femmes ostéoporotiques ménopausées	Les groupes D, K_2 et K_2 + D ont 1 DMO vertébrale > à celle mesurée dans les groupes D ou K_2

Tableau 1 (*Suite*)

	Références	Typologie de l'étude	Population	Principaux résultats
[37]	Yonemura et al. 2000	10 semaines avec prédnisolone ou prédnisolone + K_2 (45 mg/j) (Japon)	20 hommes et femmes avec une pathologie rénale (âge moyen 28 ans)	MK-4 prévient la ↘ de DMO induite par la prédnisolone
[12]	Bolton-Smith et al. 2001	2 ans avec K_1 (200 µg/j) ou Ca + vitamine D ou K_1 + Ca + D ou placébo	244 femmes ménopausées (Royaume-Uni) (âge moyen 55,5 ans)	↗ du CMO du radius dans le groupe K_1 + Ca + D
[38]	Nishiguchi et al. 2001	2 ans avec K_2 (45 mg/j) ou placebo (Japon)	30 femmes avec une cirrhose (âge moyen 55,5 ans)	Moindre ↘ de DMO vertébrale dans le groupe K_2 Ostéocalcine décarboxylée ↘ dans le groupe K_2
[20]	Ushiroyama et al. 2002	2 ans avec K_2 (45 mg/j) ou 1αOHD ou K_2 + D ou placébo (Japon)	172 femmes ostéoporotiques ou ostéopéniques (âge moyen 53,4 ans)	Le groupe K_2 + D a une ↗ de 4,92 % de la DMO vertébrale
[11]	Braam et al. 2003	3 ans avec minéraux + vitamine D ou minéraux + vitamine D + K_1 (1 mg/j) ou placébo	181 femmes ménopausées (âge moyen 50—60 ans) (Pays-Bas)	Moindre ↘ de DMO de la hanche dans le groupe K_1 + D
[16]	Braam et al. 2003	2 ans avec K_1 (10 mg/j) ou placébo dans chaque groupe (Europe) (âge moyen : 15—50 ans)	115 athlètes d'endurance (29 en aménorrhée, 49 en aménorrhée, 37 sous estrogènes	Pas d'effet significatif de K_1 sur perte osseuse ↘ de DMO dans tous les groupes
[39]	Iketani et al. 2003	K_2 (45 mg/j) ou absence de traitement (Japon)	21 femmes anorexiques, 12 témoins (âge moyen 16—27 ans)	Moindre ↘ de DMO vertébrale dans le groupe K_2 Ostéocalcine carboxylée ↗ dans le groupe K_2 et DPD ↘
[11]	Sasaki et al. 2005	1 an avec K_2 (15 mg/j) ou stéroides seuls (Japon)	20 patients sous stéroïdes pour pathologie rénale (âge moyen 40 ans)	Pas de ↘ de DMO vertébrale dans le groupe K_2 alors d'elle ↘ dans le groupe stéroïde
[40]	Yasui et al. 2006	2 ans avec K_2 (45 mg/j) ou MK-4 + 1αOHD3 (Japon)	34 femmes ménopausées ostéoporotiques ou ostéopéniques (âge moyen 53 ans)	% ostéocalcine décarboxylée ↘ DMO vertébrale ↘ dans le groupe MK-4 mais pas dans le groupe K_2 + D
[41]	Knapen et al. 2007	3 ans avec K_2 (45 mg/j) ou placébo (Pays-Bas)	325 femmes ménopausées (âge moyen 66 ans)	Marqueurs de formation (ostéocalcine et phosphatase alcaline osseuse) ↗ Ostéocalcine décarboxylée ↘ dans le groupe K_2. Pas d'effet significatif sur DMO
[17]	Binkley et al. 2008	12 mois avec K_2 (45 mg/j) ou K_1 (1 mg/j) ou placébo (États-Unis)	381 femmes ménopausées supplémentées en calcium et vitamine D	Pas d'effet des 2 traitements sur DMO vertébrale et fémorale
[15]	Volpe et al. 2008	6 mois avec 600 µg K_1 ou placébo (États-Unis)	14 femmes âgées de 25 à 50 ans	NTX augmente avec le traitement. Pas d'effet sur DMO

Tableau 1 (*Suite*)

	Références	Typologie de l'étude	Population	Principaux résultats
[18]	Cheung et al. 2008	2 ans avec 500 µg K_1 ou placébo, extension de 2 ans pour les premiers volontaires (États-Unis)	440 femmes ménopausées ostéopéniques supplémentées en vitamine D	Pas de différence significative en terme de DMO. Moins de fractures (9 vs 20) dans le groupe traité
Remodelage osseux				
[42]	Knapen et al. 1989	2 semaine avec 1 mg/j K_1 (Pays-Bas)	50 femmes ménopausées, 50 non ménopausées	Ostéocalcine carboxylée ↗ de 70—80 % chez les femmes ménopausées
[43]	Douglas et al. 1995	2 semaines avec 1 mg/j K_1 (Royaume-Uni)	20 femmes ménopausées avec fracture, 40 témoins de même âge, 10 femmes non ménopausées	Ostéocalcine carboxylée ↗ après K_1 ou K_1 + D
[44]	Sokoll et al. 1997	15 jours K_1 (100 mg/j ou 420 mg/j) (États-Unis)	9 hommes et femmes en bonne santé	Ostéocalcine décarboxylée ↗ dans le régime faible en K_1 et ↘ avec la forte consummation
[45]	Craciun et al. 1998	4 semaines avec 1 mg/j K_1 (Pays-bas)	8 athlètes de haut niveau	Ostéocalcine carboxylée ↗ marqueurs de résorption osseuse ↘ marqueurs de formation ↗
[46]	Caillot-Augusseau et al. 2000	Vitamine K pendant les jours 86—130 d'un vol spatial	2 cosmonautes	Ostéocalcine carboxylée ↗ marqueurs de résorption osseuse↘ après la supplémentation en vitamine K
[47]	Binkley et al. 2000	2 semaines avec K_1 (1 mg/j) (États-Unis)	219 hommes et femmes en bonne santé	% d'ostéocalcine totale et d'ostéocalcine décarboxylée 4/6/2009↘ dans tous les groupes supplémentés en K_1
[19]	Schaafsma et al. 2000	1 an (Pays-Bas) DMO normale : D3 (400 IU/j) + K_1 (80 mg/j) ou K_1 (80 mg/j ou placébo) Faible DMO : D3 + K_1 (80 mg/j) ou D3	96 femmes ménopausées avec une DMO normale et 45 avec une DMO faible (âge moyen 60 ans)	Faible DMO : l ostéocalcine décarboxylée < à celle chez ceux qui ont 1 DMO normale. % ostéocalcine carboxylée ↗ après supplementation DMO normale : % ostéocalcine carboxylée après supplémentation
[48]	Takahashi et al. 2001	4 semaines avec K_2 (45 mg/j) or 1αOHD3 ou K_2 + D (Japon)	89 femmes avec des fractures vertébrales	↘ ostéocalcine décarboxylée dans le groupe K_2 et K_2 + D
[49]	Miki et al. 2003	2 semaines avec K_2 (45 mg/j) + Ca ou Ca seul (Japon)	20 femmes avec des fractures vertébrales	↘ ostéocalcine décarboxylée dans le groupe K_2 + Ca

Tableau 1 (*Suite*)

	Références	Typologie de l'étude	Population	Principaux résultats
[50]	Shiraki et Itabashi (2009)	6 mois avec K_2 (45 mg/j)	109 femmes ménopausées	↘ ostéocalcine décarboxylée et ↗ ostéocalcine après 1 mois ↗ NTX après 6 mois

des femmes ménopausées est associée à une amélioration du taux d'ostéocalcine carboxylée, mais dépourvue d'effet sur les marqueurs de résorption osseuse. Cependant, une étude randomisée en double insu n'a pas montré de bénéfice osseux d'un apport quotidien de 500 µ par jour de vitamine K_1, pendant trois ans, à des femmes et hommes de 60—80 ans [14]. Il en est de même pour Volpe et al. (2008) [15], qui n'ont pu mettre en évidence une différence en termes de DMO, même si le NTX (biomarqueur d'accrétion) était augmenté, après supplémentation en vitamine K_1 à la dose de 600 µg pendant six mois chez 14 femmes en bonne santé âgées de 25 à 50 ans. L'administration de vitamine K_1 pendant deux ans à la dose de 10 mg par jour à des jeunes femmes sportives n'a pas non plus permis d'améliorer le statut osseux [16]. De plus, dans le cadre d'une étude portant sur une cohorte de 381 femmes ménopausées américaines (supplémentées en calcium et vitamine D), Binkley et al. [17] n'ont pas obtenu d'effet significatif de la consommation quotidienne, pendant 12 mois, de 1 mg de phylloquinone, ni d'ailleurs de 45 mg de vitamine K_2, sur les paramètres de DMO, que ce soit au niveau vertébral ou fémoral. Enfin, dans l'étude randomisée contre-placébo ECKO publiée par Cheung et al. [18], un apport pendant deux ans (et une extension de deux ans pour les premiers volontaires) de 5 mg par jour de vitamine K_1 ou d'un placébo, à 440 femmes ménopausées ostéopéniques supplémentées en vitamine D (taux basal normalisé à 77 nmol/l) ne permet pas d'améliorer significativement les paramètres de DMO. Cependant, moins de fractures ont été diagnostiquées dans le groupe traité.

À RETENIR

Les effets de la phylloquinone (vitamine K_1) sont plus contestés.

En résumé, les travaux portant sur l'utilisation la vitamine K_1 sont moins consensuels. Toutefois, comparativement à la vitamine K_2, les doses utilisées sont toujours plus faibles. La population cible est également différente. Ainsi, l'absence d'effet dans certaines études pourrait éventuellement s'expliquer par le fait que les volontaires sont parfois jeunes et non à risque en termes de capital osseux.

Interactions

Par ailleurs, de l'analyse de la littérature, il ressort que l'association de vitamine D (ainsi que probablement de minéraux tels que le magnésium ou le zinc) permet d'optimiser l'impact sur la DMO, qui s'explique au moins partiellement par une augmentation des concentrations plasmatiques en ostéocalcine carboxylée [19—20]. Ces osbservations sont tout à fait logiques dans la mesure où la vitamine D est capable de stimuler la synthèse d'ostéocalcine au niveau transcriptionel, via le récepteur de la vitamine D (VDR) situé dans la région promotrice du gène de la protéine.

En conclusion, il existe de fortes présomptions pour l'existence d'un effet protecteur de la vitamine K sur la qualité du squelette. Plusieurs éléments conduisent effectivement à émettre l'hypothèse d'une association entre le statut vitaminique et la DMO, et notamment entre l'ostéopénie consécutive à la ménopause et une carence à long terme en vitamine K. À ce titre, les concentrations plasmatiques en ostéocalcine décarboxylée sont considérées comme prédictives du risque de fractures ostéoporotiques. De plus, les études de supplémentation témoignent d'une réduction du risque fracturaire. Il convient toutefois de nuancer ces propos par le fait que seule la ménaquinone a, jusqu'à présent, fourni des résultats convaincants. Par ailleurs, l'effet santé est démontré avec la consommation de très fortes doses et uniquement sur des populations asiatiques.

La vitamine k en pratique

Les recommandations

Les apports nutritionnels conseillés sont de 10 µg par jour pour le nouveau-né et l'enfant et de 65 µg par jour pour les adolescents (16—19 ans). Un apport quotidien de 45 µg est préconisé pour les adultes et de 70 µg pour les personnes âgées (supérieur à 75 ans), soit environ 1 µg/kg par jour [21]. Aux États-Unis, compte tenu de la difficulté de déterminer le besoin (*average requirement*), les *adequate intakes* ont été définis sur la base des consommations usuelles des différentes tranches de la population. Ils sont respectivement de 120 µg par jour pour les hommes et de 90 µg par jour pour les femmes.

Néanmoins, l'estimation actuelle des besoins repose uniquement sur les propriétés relatives au maintien de la coagulation. Or, les marqueurs biologiques classiquement utilisés pour évaluer cette activité sont insufisamment sensibles pour révéler des carences marginales. De plus, puisque les travaux récents suggèrent que la vitamine K est un cofacteur indispensable à la γ-carboxylation enzymatique de l'acide glutamique, étape clef de l'activation et de la fonctionnalité des protéines dites vitamine K dépendantes, c'est-à-dire non seulement des facteurs de la coagulation II, VII, X, protéines C et S impliquées dans l'hémostase, mais également des protéines osseuses non collagéniques [22], il est probable que les besoins estimés uniquement au regard de l'activité de coagulation ne soient pas réellement pertinents pour la physiologie osseuse.

Les scientifiques envisagent donc de revoir à la hausse les recommandations, d'autant plus que, chez le sujet sain, la vitamine K ne pose pas de problème particulier de toxicité, comme le suggère l'utilisation de très

fortes doses de vitamine K_1 (1 mg/j) ou K_2 (45 mg/j) dans les études d'intervention, dénuées d'effets secondaires apparents [8]. Toutefois, ces molécules peuvent interagir avec des anticoagulants oraux, prescrits pour inhiber la gamma-carboxylation post-translationnelle des facteurs de coagulation-vitamine K dépendants (II, VII, IX et X) en situations de risque de thrombose comme, par exemple, après une intervention chirurgicale, un infarctus du myocarde ou une embolie pulmonaire.

En fait, l'analyse de la littérature démontre une absence d'interaction pour des doses quotidiennes inférieures à 100 μg. C'est pourquoi, dans son avis (saisine 2007-SA-0315) publié le 13 juin 2008, l'Agence française de sécurité sanitaire des aliments (Afssa) estime que la prévalence élevée de sujets suivant un traitement anticoagulant par AVK justifie une extrême prudence quant à l'enrichissement des aliments courants en vitamine K. C'est la raison pour laquelle elle a fixé à 25 μg par jour la dose maximale dans les compléments alimentaires, et refuse l'utilisation de doses supérieures, même encadrées par un étiquetage qui dissuaderait les personnes sous AVK de consommer ces compléments alimentaires. Par ailleurs, la législation européenne n'autorise pas les compléments alimentaires à base de vitamine K_2.

Consommation : statut, apports et sources

Le statut en vitamine K est actuellement appréhendé par la mesure de l'ostéocalcine décarboxylée, paramètre jugé plus pertinent que les classiques mesures de coagulation et qui représente également un marqueur prédictif de la DMO et du risque de fracture. De fait, il est évident que les apports alimentaires en vitamine K sont insuffisants à la carboxylation complète de l'ostéocalcine, dont 10 à 30 % circulent sous forme décarboxylée chez l'adulte sain. Une valeur seuil de subcarence de 4 ng/ml a ainsi été proposée.

Les apports en vitamine K sont, en fait, extrêmement variables d'un individu à un autre. Sur la base d'une enquête alimentaire pratiquée sur 14 jours chez 4741 volontaires américains, Booth et al. [23] ont estimé la consommation moyenne à 90,1, 81,8 et 65,5 μg par jour, respectivement, pour les tranches d'âge de 65 ans et plus, 45—64 ans et 18—44 ans. Même si ces valeurs sont proches des préconisations, 5 à 15 % des sujets ont des concentrations plasmatiques indétectables en phylloquinone. En outre, plus de la moitié des enfants et adolescents ont des apports inférieurs aux recommandations. Enfin, les nourrissons sont particulièrement vulnérables en raison d'une alimentation lactée (déficiente en vitamine K), de leurs faibles réserves et d'une colonisation bactérienne restreinte de leur tractus intestinal [24]. Par ailleurs, il faut noter que la prise d'antibiotiques, parce qu'ils détruisent la microflore intestinale, peut altérer le statut en vitamine K. Tel est d'ailleurs le cas des patients souffrant de la maladie de Crohn. En outre, certains traitements de l'hypercholestérolémie ou de l'obésité, basés sur le piégeage des graisses, comme l'Orlistat par exemple, peuvent inhiber l'absorption intestinale de la vitamine K, et par conséquent le statut vitaminique.

Il est admis qu'un repas classique peut fournir 300 à 400 μg de vitamine K. Les produits végétaux tels que les légumes verts à feuilles, ainsi que certaines huiles végétales et margarines, constituent les principales sources de vitamine K_1 (70 % des apports totaux en vitamine K ; épinards : 380 μg/100 g ; salade verte : 315 μg/100 g ; brocolis : 180 μg/100 g ; huile de soja : 193 μg/100 g). Quant aux ménaquinones, le foie des ruminants en contient des quantités relativement importantes (10—20 μg/100 g ; formes 7, 11, 12 et 13). Les fromages (5 à 10 μg/100 g de ménaquinones 8 et 10 à 20 μg/100 g de ménaquinone 9), ainsi que les produits fermentés à base de soja, représentent également une excellente contribution aux apports. Il faut aussi considérer la production par les bactéries lactiques qui atteint entre 29 et 123 μg/l de milieu fermenté, et donc ne pas exclure la source endogène de ménaquinones à partir de la flore bactérienne. Toutefois, la biodisponibilité de la phylloquinone est extrêmement faible (inférieure à 15 %). En revanche, les ménaquinones sont bien absorbées, ce qui explique l'équivalence de la contribution des deux catégories de molécules au statut en vitamine K, malgré les différences d'apports. Les différentes formes de la vitamine K étant liposolubles, la molécule doit être incorporée dans des micelles mixtes, processus qui requiert la présence de graisses. De ce fait, les concentrations plasmatiques en vitamine K sont étroitement corrélées aux triglycérides, ainsi d'ailleurs qu'au phénotype ApoE. Les ménaquinones représentent 90 % des réserves hépatiques, alors qu'au niveau sanguin, la forme circulante majeure est la phylloquinone. La phylloquinone et les ménaquinones 6, 7 et 8 sont également détectées dans les tissus cibles, tels que l'os, à des concentrations équivalentes à celles retrouvées dans le foie. Toutefois, le foie étant le principal organe cible de la vitamine K, les autres tissus ne bénéficient de la molécule que lorsqu'elle est apportée à forte dose.

Conclusion

En conclusion, la vitamine K est indispensable à la fonctionnalité de l'ostéocalcine, protéine non collagénique majeure du tissu osseux, impliquée dans la régulation des processus de minéralisation. Elle apporte ainsi une contribution essentielle au maintien de la qualité de notre squelette. Cependant, une étude de statut révèle une carence en vitamine K relativement fréquente, au moins dans certaines tranches de la population. Or, il ressort des études épidémiologiques que le risque de fracture est augmenté pour des consommations inférieures à 109 μg par jour (étude de Framingham). Les investigations cliniques d'intervention corroborent cette constatation puisqu'elles démontrent qu'une supplémentation (en vitamine K_2 et à des doses élevées (mg/j) permet de réduire le risque fracturaire. Par conséquent, compte tenu de l'importance de la vitamine K pour le capital osseux, il convient d'être vigilent au respect des recommandations nutritionnelles, d'autant plus qu'elles n'ont été élaborées que sur la base de l'implication de la molécule dans les processus de coagulation (et sont donc probablement sous-estimées). Toutefois, même si les risques induits par une consommation élevée de vitamine K apparaissent mineurs, compte tenu du nombre important de personnes sous AVK dans la population générale, une supplémentation systématique n'est pas recommandée. Quant à la vitamine K_2, la supplémentation n'est pas autorisée en Europe.

Conflits d'intérêts

Aucun.

Références

[1] Coxam V, Davicco MJ, Demigné C. Nutriments et métabolisme osseux. In: Roberfroid M, Coxam V, Delzenne N, editors. Aliments fonctionnels. 2e édition Lavoisier; 2008.

[2] Ohsaki Y, Shirakawa H, Hiwatashi K, Furukawa Y, Mizutani T, Komai M. Vitamin K suppresses liposaccharide-induced inflammation in the rat. Biosci Biotechnol Biochem 2006;70: 926—32.

[3] van Summeren MJH, van Coeverden SCCM, Schurgers LJ, et al. Vitamin K status is associated with childhood bone mineral content Br. J Nutr 2008;100:852—8.

[4] Booth SL, Broe KE, Gagnon DR, et al. Vitamin K intake and bone mineral density in women and men. Am J Clin Nutr 2003;77:512—6.

[5] Vergnaud P, Garnero P, Meunier PJ, Bréart G, Kamihagi K, Delmas PD. Undercarboxylated osteocalcin measured with a specific immunoassay predicts hip fracture in elderly women: The EPIDOS Study. J Clin Endocrinol Metab 1997;82:719—24.

[6] Feskanich D, Weber P, Willett WC, Rockett H, Booth SL, Colditz GA. Vitamin K intake and hip fractures in women: A prospective study. Am J Clin Nutr 1999;69:74—9.

[7] Cockayne S, Adamson J, Lanham-New S, Shearer MJ, Gilbody S, Torgerson DJ. Vitamin K and the prevention of fractures: Systematic review and meta-analysis of randomized controlled trials. Arch Intern Med 2006;166:1256—61.

[8] Pearson DA. Bone health and osteoporosis: The role of vitamin K and potential antagonism by anticoagulants. Nutr Clin Pract 2007;22:517—44.

[9] WHO Scientific group on the preview and management of osteoporosis. Prevention and management of osteoporosis, report of a WHO scientific Group. Geneva (2003).

[10] Sasaki N, Kusano E, Takahashi H, et al. Vitamin K_2 inhibits glucocorticoid-induced bone loss partly by preventing the reduction of osteoprotegerin (OPG). J Bone Miner Metab 2005;23:41—7.

[11] Braam LA, Knapen MH, Geusens P, et al. Vitamin K_1 supplementation retards bone loss in postmenopausal women between 50 and 60 years of age. Calcif Tissue Int 2003;73:21—6.

[12] Bolton-Smith C, Mole PA, McMurdo M, Paterson CR, Shearer MJ. Two-year intervention with phylloquinone (vitamin K_1), vitamin D and calcium: Effect on bone mineral content of older women. Ann Nutr Metab 2001;45:46.

[13] Bügel S, Sørensen AD, Hels 0, et al. Effect of phylloquinone supplementation on biochemical markers of vitamin K status and bone turnover in postmenopausal women. Br J Nutr 2007;97:373—80.

[14] Booth SL, Dallal G, Shea MK, et al. Effect of vitamin K supplementation on bone loss in elderly men and women. J Clin Endocrinol Metab 2008;93:1217—23.

[15] Volpe SL, Leung MM, Giordano H. Vitamin K supplementation does not significantly impact bone mineral density and biochemical markers of bone in pre- and perimenopausal women. Nutr Res 2008;28:577—82.

[16] Braam LA, Knapen MH, Geusens P, Brouns F, Vermeer C. Factors affecting bone loss in female endurance athletes. Am J Sports Med 2003;31:889—95.

[17] Binkley N, Harke J, Krueger D, et al. Vitamin K treatment reduces undercarboxylated osteocalcin but does not alter bone turnover, density or geometry in healthy postmenopausal North American Women. J Bone Miner Res 2008, ahead from publication.

[18] Cheung AM, Tile L, Lee Y, et al. Vitamin K supplementation in postmenopausal women with osteopenia (ECKO trial): A randomized controlled trial. PLoS Med 2008;5:e196.

[19] Schaafsma A, Muskiet FA, Storm H, Hofstede GJ, Pakan I, Van der Veer E. Vitamin D(3) and vitamin K(1) supplementation of Dutch postmenopausal women with normal and low bone mineral densities: Effects on serum 25-hydroxyvitamin D and carboxylated osteocalcin. Eur J Clin Nutr 2000;54:626—31.

[20] Ushiroyama T, Ikeda A, Ueki M. Effect of continuous combined therapy with vitamin K(2) and vitamin D(3) on bone mineral density and coagulofibrinolysis function in postmenopausal women. Maturitas 2002;41:211—21.

[21] Guillaumont M. Vitamines liposolubles : Vitamine K. In: Martin A, editor. Apports nutritionnels conseillés pour la population française. Londres—Paris—New York: Tec & Doc; 2001. p. 244—8.

[22] Binkley NC, Suttie JW. Vitamin K nutrition and osteoporosis. J Nutr 1995;125:1812—21.

[23] Booth SL, Webb DR, Peters JC. Assessment of phylloquinone and dihydrophylloquinone dietary intakes among a nationally representative sample of US consumers using 14-day food diaries. J Am Diet Assoc 1999;99:1072—6.

[24] Prynne CJ, Thane CW, Prentice A, Wadsworth ME. Intake and sources of phylloquinone (vitamin K[1]) in 4-year-old British children: Comparison between 1950 and the 1990s. Public Health Nutr 2005;8:171—80.

[25] Shiraki M, Shiraki Y, Aoki C, Miura M. Vitamin K_2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. J Bone Miner Res 2000;15:515—21.

[26] Iwamoto J, Takeda T, Ichimura S. Effect of menatetrenone on bone mineral density and incidence of vertebral fractures in postmenopausal women with osteoporosis: A comparison with the effect of etidronate. J Orthop Sci 2001;6:487—92.

[27] Sato Y, Honda Y, Kaji M, et al. Amelioration of osteoporosis by menatetrenone in elderly female Parkinson's disease patients with vitamin D deficiency. Bone 2002;31:114—8.

[28] Ishida Y, Kawai S. Comparative efficacy of hormone replacement therapy, etidronate, calcitonin, alfacalcidol, and vitamin K in postmenopausal women with osteoporosis: The Yamaguchi Osteoporosis Prevention Study. Am J Med 2004;117:549—55.

[29] Sato Y, Kanoko T, Satoh K, Iwamoto J. Menatetrenone and vitamin D2 with calcium supplements prevent nonvertebral fracture in elderly women with Alzheimer's disease. Bone 2005;36:61—8.

[30] Akiba T, Kurihara S, Tachibana K, et al. Vitamin K (K) increased bone mass (BM) in hemodialysis patients (Pts) with low-turnover bone disease (LTOBD). J Am Soc Nephrol 1991;608:42P.

[31] Orimo M, Shiraki M, Fujita T, Onomura T, Ioune T, Kushida K. Clinical evaluation of meatetrenone in the treatment of involutional osteoporosis—a double blind multicenter comparative study with 1-alpha-hydroxyvitamin D3. J Bone Miner Res 1992;7:S122.

[32] Orimo M, Shiraki M, Tomita A, Morii H, Fujita T, Ohata M. Effects of menatetrenone on the bone and calcium metabolism in osteoporosis: A double-blind placebo-controlled study. J Bone Miner Res 1998;16:106—12.

[33] Sato Y, Honda Y, Kuno H, Oizumi K. Menatetrenone ameliorates osteopenia in disuse-affected limbs of vitamin D- and K-deficient stroke patients. Bone 1998;23:291—6.

[34] Somekawa Y, Chigughi M, Harada M, Ishibashi T. Use of vitamin K_2 (menatetrenone) and 1,25-dihydroxyvitamin D3 in the prevention of bone loss induced by leuprolide. J Clin Endocrinol Metab 1999;84:2700—4.

[35] Iwamoto I, Kosha S, Noguchi S, et al. A longitudinal study of the effect of vitamin K_2 on bone mineral density in postmenopausal women a comparative study with vitamin D3 and estrogen-progestin therapy. Maturitas 1999;31:161—4.

[36] Iwamoto J, Takeda T, Ichimura S. Effect of combined administration of vitamin D3 and vitamin K_2 on bone mineral density of the lumbar spine in postmenopausal women with osteoporosis. J Orthop Sci 2000;5:546—51.

[37] Yonemura K, Kimura M, Miyaji T, Hishida A. Short-term effect of vitamin K administration on prednisolone-induced loss of bone mineral density in patients with chronic glomerulonephritis. Calcif Tissue Int 2000;66:123—8.

[38] Nishiguchi S, Shimoi S, Kurooka H, et al. Randomized pilot trial of vitamin K_2 for bone loss in patients with primary biliary cirrhosis. J Hepatol 2001;35:543—5.

[39] Iketani T, Kiriike N, Murray S, et al. Effect of menatetrenone (vitamin K_2) treatment on bone loss in patients with anorexia nervosa. Psychiatry Res 2003;117:259—69.

[40] Yasui T, Miyatani Y, Tomita J, et al. Effect of vitamin K_2 treatment on carboxylation of osteocalcin in early postmenopausal women. Gynecol Endocrinol 2006;22: 455—9.
[41] Knapen MH, Schurgers LJ, Vermeer C. Vitamin K_2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. Osteoporos Int 2007;18:963—72.
[42] Knapen MH, Hamulyák K, Vermeer C. The effect of vitamin K supplementation on circulating osteocalcin (bone Gla protein) and urinary calcium excretion. Ann Intern Med 1989;111:1001—5.
[43] Douglas AS, Robins SP, Hutchison JD, Porter RW, Stewart A, Reid DM. Carboxylation of osteocalcin in post-menopausal osteoporotic women following vitamin K and D supplementation. Bone 1995;17:15—20.
[44] Sokoll LJ, Sadowski JA. Comparison of biochemical indexes for assessing vitamin K nutritional status in a healthy adult population. Am J Clin Nutr 1996;63:566—73.
[45] Craciun AM, Wolf J, Knapen MH, Brouns F, Vermeer C. Improved bone metabolism in female elite athletes after vitamin K supplementation. Int J Sports Med 1998;19: 479—84.
[46] Caillot-Augusseau A, Vico L, Heer M, et al. Space flight is associated with rapid decreases of undercarboxylated osteocalcin and increases of markers of bone resorption without changes in their circadian variation: Observations in two cosmonauts. Clin Chem 2000;46:1136—43.
[47] Binkley NC, Krueger DC, Engelke JA, Foley AL, Suttie JW. Vitamin K supplementation reduces serum concentrations of under-gamma-carboxylated osteocalcin in healthy young and elderly adults. Am J Clin Nutr 2000;72:1523—8.
[48] Takahashi M, Naitou K, Ohishi T, Kushida K, Miura M. Effect of vitamin K and/or D on undercarboxylated and intact osteocalcin in osteoporotic patients with vertebral or hip fractures. Clin Endocrinol 2001;54:219—24.
[49] Miki T, Nakatsuka K, Naka H, et al. Vitamin K(2) (menaquinone 4) reduces serum undercarboxylated osteocalcin level as early as two weeks in elderly women with established osteoporosis. J Bone Miner Metab 2003;21:161—5.
[50] Shiraki M, Itabashi A. Short-term menatetrenone therapy increases gamma-carboxylation of osteocalcin with a moderate increase of bone turnover in postmenopausal osteoporosis: A randomized prospective study. *J. Bone Miner. Metab*. 2009, online first.

Annexe numéro 3:

Inorganic phosphate stimulates apoptosis in murine MO6-G3 odontoblast-like cells

A. Bourgine, L. Beck, S. Khoshniat, F. Wauquier, L. Oliver, E. Hue, B. Alliot-Licht, P. Weiss, J. Gucheux, Y. Wittrant

Archives of oral biology, 2011

ARCHIVES OF ORAL BIOLOGY 56 (2011) 977–983

available at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Inorganic phosphate stimulates apoptosis in murine MO6-G3 odontoblast-like cells

A. Bourgine[a,b], L. Beck[a,b], S. Khoshniat[a,b], F. Wauquier[c,d], L. Oliver[b,e], E. Hue[b,e], B. Alliot-Licht[a,b], P. Weiss[a,b], J. Guicheux[a,b,*], Y. Wittrant[a,c,d]

[a] INSERM, U791, Centre for Osteoarticular and Dental Tissue Engineering (LIOAD), Group STEP "Skeletal Tissue Engineering and Physiopathology", Nantes F-44042, France
[b] PRES UNAM, UFR Odontologie, Nantes F-44042, France
[c] INRA, UMR1019, Human Nutrition Unit, group "Bone, nutrition and metabolism", F-63000 Clermont-Ferrand, France
[d] Université de Clermont, UMR 1019, UFR Médecine, F-63000 Clermont-Ferrand, France
[e] Institut de Recherche Thérapeutique de l'Université de Nantes, CRCNA INSERM UMR892, France

ARTICLE INFO

Article history:
Accepted 1 March 2011

Keywords:
Tooth
Odontoblast
Inorganic phosphate
Apoptosis
Caries formation

ABSTRACT

Objective: Dental pathologies such as caries are the most prevalent disease worldwide with infectious and social complications. During the process of caries formation, the tooth is degraded and demineralization of enamel and dentine leads to the release of large amounts of inorganic phosphate (Pi) within dental tubuli. As Pi has been shown to induce apoptosis in skeletal cells, including osteoblasts and chondrocytes, we questioned whether high concentrations of Pi could affect odontoblast viability, proliferation and apoptosis.

Design: Using the odontoblast-like MO6-G3 cell line as a model, we used cell counting and MTS-based colorimetric assays to measure cell viability and proliferation. Apoptosis was assessed using Hoechst nuclei staining and detection of the early apoptotic markers annexin V and Apo2.7.

Results: We show for the first time that a high Pi concentration (7 mM) induced a decrease in odontoblast viability and proliferation together with a large increase in apoptosis. These effects were blunted in calcium-free medium, possibly due to the formation of calcium-phosphate crystals in the presence of high Pi concentrations.

Conclusion: This study contributes to clarifying the effect of Pi on odontoblast viability and apoptosis, which may improve our understanding of the role of Pi during caries formation.

1. Introduction

Dental caries is probably the most prevalent disease worldwide. It can cause a great deal of pain and distress, and the loss of teeth has profound consequences in terms of eating, speaking, sleeping, quality of life, intellectual development and social interaction.[1–7] Its high prevalence also means that the financial cost is enormous. Dental caries are the leading cause of tooth extraction and the secondary cause for respiratory infections that cost over 60 billion dollars in 2006 in the United States.[8,9] When uncured, dental caries lead to local and systemic complications such as pulpitis, cellulitis and septis.[10–13]

* *Corresponding author at*: INSERM, U791, Centre for Osteoarticular and Dental Tissue Engineering (LIOAD), Group STEP "Skeletal Tissue Engineering and Physiopathology", 1 Place Alexis ricordeau, Nantes F-44042, France. Tel.: +33 0 2 40412919/16; fax: +33 0 2 40083712.
E-mail address: jerome.guicheux@inserm.fr (J. Guicheux).
0003–9969/$ – see front matter
doi:10.1016/j.archoralbio.2011.03.001

Dentine, which is produced throughout life, is composed of 70% hydroxyapatite crystals and represents the major part of the tooth. Dentine is secreted by odontoblasts, which are specialized cells located at the pulp–dentine junction. Although apoptosis is known to be a key phenomenon in the regulation of the life span of odontoblasts, the mechanisms controlling odontoblast loss in injured teeth are largely unknown.[14] Odontoblast apoptosis can be induced by bacteria and endotoxins related to the caries lesion[15,16] and a recent study illustrated the correlation between apoptosis and dental pulp volume reduction, suggesting that apoptosis could be part of the mechanisms that regulate human dental pulp chamber remodelling during tooth pathology.[14] Of interest, during the demineralization process due to caries lesions, the tooth is degraded and demineralization of enamel and dentine leads to the local release of large amounts of inorganic phosphate (Pi) within dental tubuli.[17,18] Since Pi has been shown to induce osteoblast and chondrocyte apoptosis,[19–23] and given that odontoblasts represent the first cells exposed to dentinal fluid, Pi could provide a signal that modulates odontoblast cell viability and apoptosis.

2. Materials and methods

2.1. Cell culture and inorganic phosphate treatments

The odontoblast-like cell line MO6-G3, expressing the major proteins associated with the odontoblast phenotype including dentine sialophosphoprotein,[24–26] was kindly obtained from Dr. Mary MacDougall (Department of Oral/Maxillofacial Surgery, University of Alabama, Birmingham). Cells were seeded at 10,000 cells/cm^2 and grown in alpha-MEM medium (Invitrogen) supplemented with 10% FCS (Hyclone) at 33 °C in a 5% CO_2 humidified atmosphere. When the cells reached 80% confluence, they were serum starved for 24 h before incubation with inorganic phosphate (Pi). Cultures were treated with Pi at concentrations ranging from 1 mM (normal control conditions) to 10 mM for increasing periods of time (0–72 h). In order to test the involvement of calcium in mediating the Pi effects, cells were additionally cultured in calcium-free DMEM (Invitrogen, ref 21068-028) in the presence of 1 mM or 10 mM Pi.

2.2. Cell viability and proliferation

The colorimetric MTS CellTiter 96® AQ_{ueous} Assay (Promega) was used as per the manufacturer's instructions to determine the effect of Pi on cell proliferation and viability. The number of viable cells is proportional to the absorbance at 492 nm. To confirm the results from the MTS assay, cells were counted in a Malassez chamber using the Trypan blue (Sigma–Aldrich) exclusion method to distinguish between live and dead cells. Cell nuclei staining was performed with Hoechst 33258 (Sigma) at a final concentration of 1 μM for 30 min in culture media. After three washes in pre-warmed PBS (33 °C), nuclear morphology was observed using an inverted fluorescent microscope (Nikon, Düsseldorf, Germany) and pictures of nuclei were taken (100× magnification).

2.3. Flow cytometry

Cells were trypsinized, washed with PBS and incubated for 15 min at RT in a buffer (10 mM HEPES, 135 mM NaCl, 5 mM $CaCl_2$) containing APO2.7-PE conjugate (Beckman Coulter) or annexin V-PE (BD Biosciences), as per the manufacturer's instructions. Cells were analysed by flow cytometry on a BD Biosciences FACSCalibur flow cytometer with CELLQuest Pro software.

2.4. Statistical analysis

All data were collected from at least three separate experiments. Bars on the graphs represent means ± SD Statistics were carried out with GraphPad InStat software using one-way analysis of variance and the Student–Newman–Keuls post hoc test. Where the p value was <0.05, groups were considered to be significantly different.

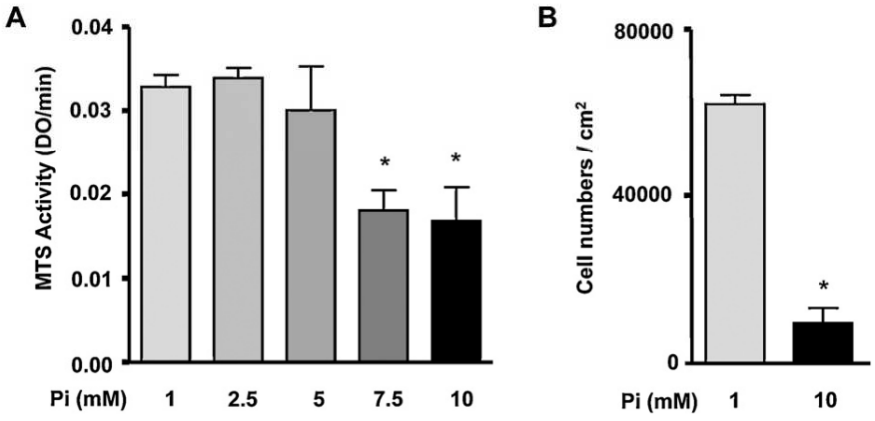


Fig. 1 – Effect of Pi on MO6-G3 cell proliferation and viability. MO6-G3 cells were incubated with 1–10 mM Pi for 24 h. (A) MTS activity was determined by measuring optical density at 492 nm. (B) Cells were trypsinized, incubated with Trypan blue exclusion dye and the percentage of live cells was determined under a microscope in a Malassez chamber. Values are mean ± SD; * indicates $p < 0.05$ versus the control condition (1 mM Pi).

3. Results

3.1. Pi alters cell viability and proliferation

The effect of high Pi concentration on MO6-G3 cell viability was assessed by measuring the mitochondrial conversion of MTS into formazan. Fig. 1 shows that increasing concentration of Pi decreased cell viability. Incubating cells with ≥7.5 mM Pi reduced cell viability 2-fold (Fig. 1A). The data obtained from cell counting confirmed the MTS analysis and showed that after a 24-h incubation of MO6-G3 cells with 10 mM Pi, the number of cells represented only 15% of the control condition (1 mM Pi) (Fig. 1B). Hence, a Pi concentration similar to the

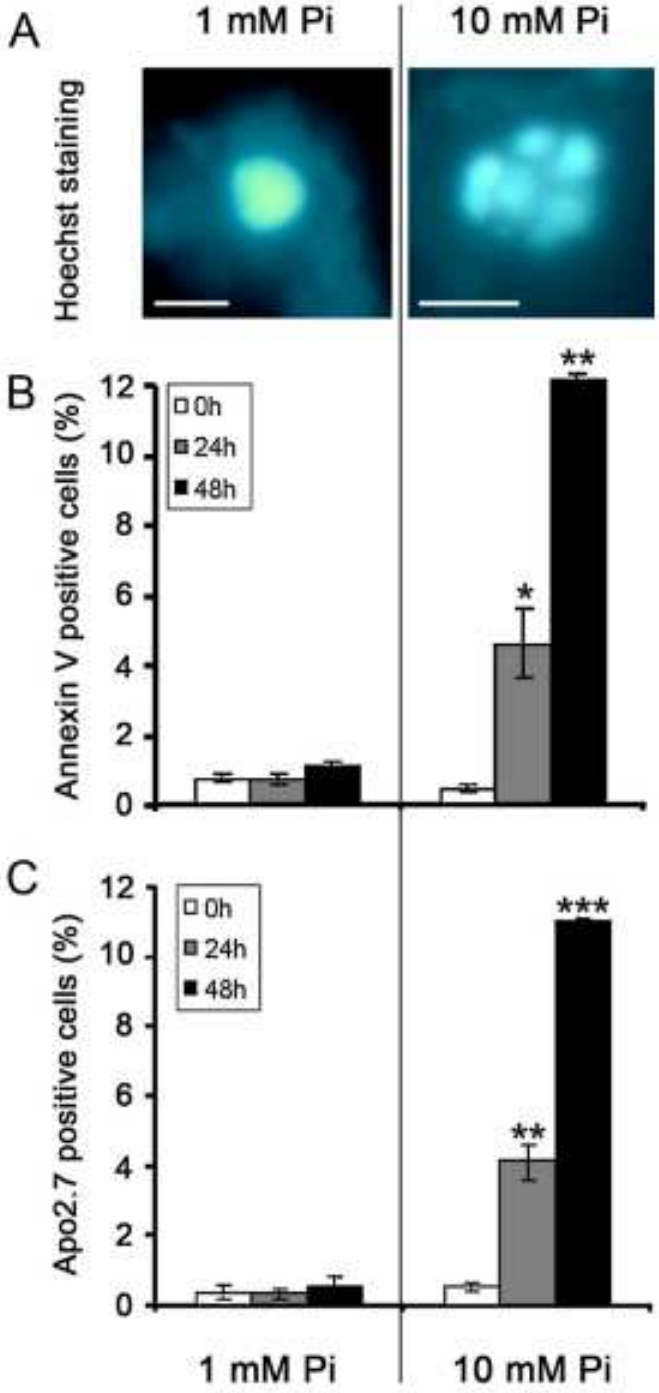


Fig. 2 – Effect of Pi on MO6-G3 cell apoptosis. MO6-G3 cells were incubated with 1 and 10 mM Pi for the indicated times. (A) After a 24 h incubation, odontoblast nuclei were stained with Hoechst dye at a final concentration of 1 μg/ml and observed under an inverted fluorescent microscope. Apoptotic nuclei (right panel) were more often found in high Pi-treated cells than in control conditions (left). *Bar*: 100 μm. The percentage of apoptotic cells was determined by FACS analysis using annexin V-PE (B) and APO2.7-PE (C) staining, as described in Section 2. Values are means ± SD; * indicates $p < 0.05$ versus control condition (1 mM Pi).

physiological concentration of Pi found in serum (1 mM) is necessary for normal proliferation of MO6-G3 cells.

3.2. High Pi concentration stimulates pro-apoptotic mechanisms

To determine whether the Pi-induced decrease in cell viability involved apoptotic mechanisms, MO6-G3 cells treated for 24 h with either 1 mM or 10 mM Pi were assessed for DNA fragmentation, and annexin V and APO2.7 immunostainings. Staining with the fluorescent dye Hoechst 33258 revealed that numerous nuclei of cells treated with a high Pi concentration (10 mM) displayed irregular structures with some condensations that are characteristic of apoptotic cells, whilst nuclei from control cells (1 mM Pi) showed a homogeneous structure with intact contours (Fig. 2A). The involvement of early apoptotic mechanisms in the Pi-induced decrease in cell viability was further investigated by assessing annexin V and APO2.7 staining in MO6-G3 cells. Cells were treated with either 1 mM or 10 mM Pi for 0, 24 and 48 h, collected and analysed by flow cytometry. The results demonstrate that 10 mM Pi dramatically increased the percentage of cells positive for annexin V and APO2.7, and that this effect increased with time (Fig. 2B and C). The dramatic increase in the expression of early apoptotic markers shows that Pi-induced odontoblast cell death involves pro-apoptotic mechanisms.

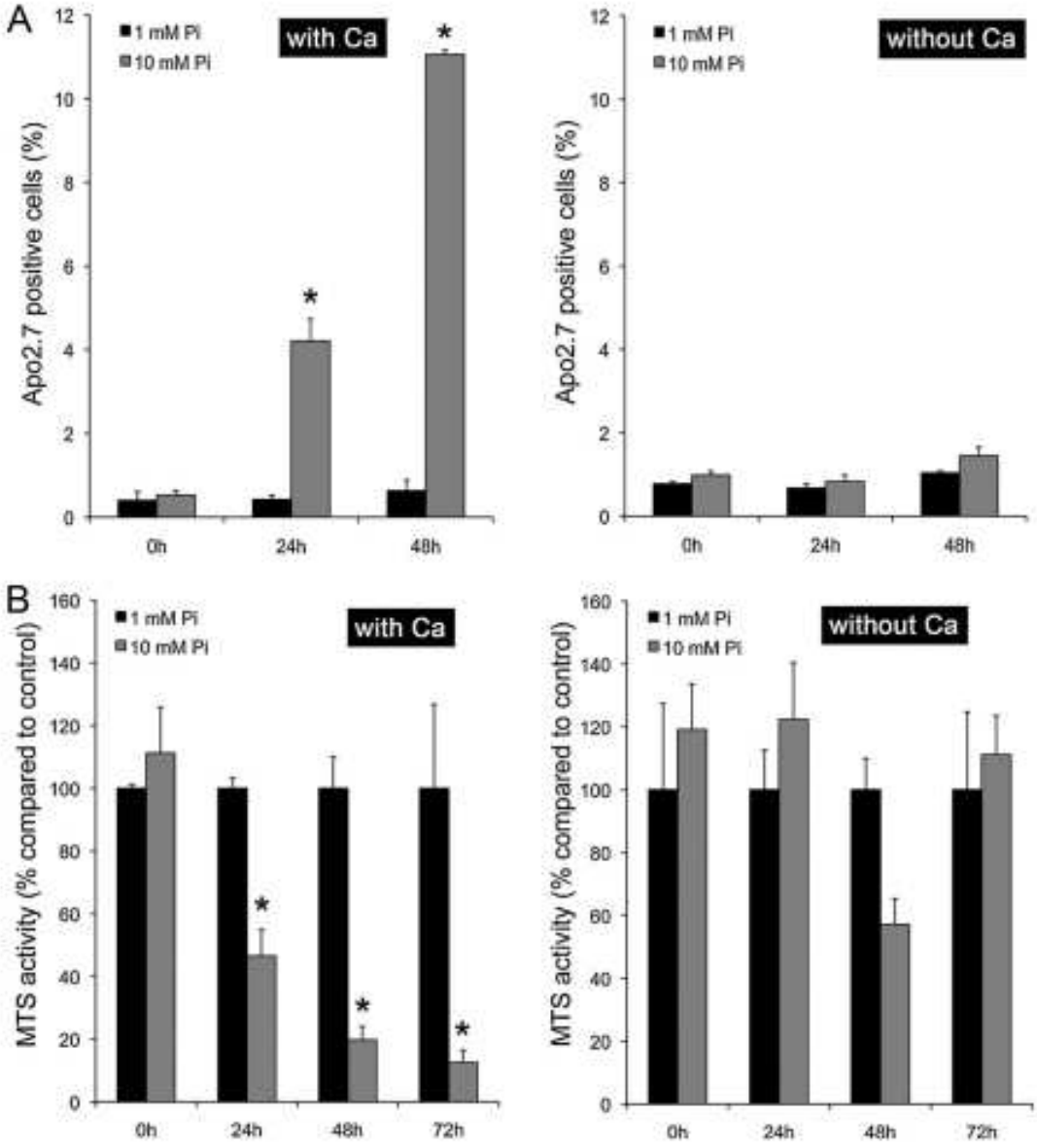


Fig. 3 – Role of calcium in Pi-mediated modulation of MO6-G3 cell viability and apoptosis. MO6-G3 cells were incubated with 1 or 10 mM Pi for different periods of time in calcium-containing or calcium-free media, as indicated. (A) The percentage of apoptotic cells was determined by FACS analysis using APO2.7-PE staining as described in Section 2. (B) Cell viability was determined using the MTS assay. After treatment, optical density was measured at 492 nm. MTS activity was arbitrarily given as a percentage of the control condition for each incubation period (1 mM Pi). Values are means ± SD; * indicates $p < 0.05$ versus control condition (1 mM Pi).

3.3. *Calcium is involved in Pi-induced apoptosis and viability*

MO6-G3 cells were incubated with 1 mM or 10 mM Pi and monitored every 6 h for 24 h under an inverted microscope. In the presence of 10 mM Pi, chemical structures resembling crystals were readily visible after 12 h (data not shown). Together with the apparition of crystal-like structures at 12 h, the morphology of the cells was modified, showing signs of shrinking. The effects were dramatic after a 24-h incubation period (data not shown). These results confirmed previous observations obtained in our lab,[27,28] which demonstrated the presence of amorphous calcium phosphate or apatite-like crystals. Since these crystals are made of phosphate and calcium, they may be important in mediating the Pi effects. To test this hypothesis, we incubated MO6-G3 cells for 0–48 h in a calcium-free medium containing either 1 mM or 10 mM Pi. Under these conditions, Pi failed to enhance APO2.7 staining (Fig. 3A), suggesting that calcium is required for Pi-dependent MO6-G3 apoptosis. Next, we assessed whether Pi could also permanently alter cell viability. When MO6-G3 cells were incubated with a high Pi concentration (10 mM) for up to 72 h, cell viability was dramatically and durably decreased (Fig. 3B). In the absence of calcium in the medium, the effect of Pi was blunted (Fig. 3B), together with the formation of crystals (data not shown). Taken together, these results show that the effects of Pi on cell viability, proliferation and apoptosis are determined by the presence of both calcium and phosphate in the culture medium.

4. Discussion

During the demineralization process due to caries lesions, massive amounts of Pi are released locally into the dentinal fluid. As odontoblasts are directly exposed to dentinal fluid, we investigated in this study the biological influence of Pi on the odontoblast-like MO6-G3 cell line. Our data demonstrate that a high Pi concentration decreases MO6-G3 viability and promotes apoptosis. The Pi-mediated increase in early apoptotic markers in calcium-free medium was blunted, which revealed the role of calcium in mediating Pi effects on odontoblast apoptosis. Combined data from microscopy and spectroscopy[27] suggested that the decrease in MO6-G3 viability is concomitant to calcium/phosphate crystal formation.

Calcium and Pi are required for the formation of biological apatites. In the absence of these ions, or in the case of abnormal absorption or reabsorption, mineralization is impaired, resulting in the formation of poorly mineralized bone. During the last decade, increasing evidence indicates that Pi is also required in events other than mineralization, proposing Pi itself as a signalling molecule capable of modulating multiple cellular functions in bone.[29] Multiple studies now illustrate the important biological role of Pi in regulating osteoblast and chondrocyte differentiation and proliferation.[20,30–34] In contrast to the intense research efforts to decipher the biological role of Pi in bone and cartilage, very little is known about the role of Pi in teeth.

In this study we show that a high Pi concentration stimulates odontoblast apoptosis, confirming previous observations.[35] The modulation of odontoblast apoptosis is important for at least two reasons. First, as odontoblasts must survive for the entire life span of the animal in spite of the continuous decrease in the dental pulp chamber volume, an understanding of the regulatory mechanisms that control cell death in dental pulp may provide new insights into the process of tooth homeostasis. Recently, Mitsiadis demonstrated that apoptosis is a part of the mechanism that regulates human dental pulp chamber remodelling during tooth development and pathology.[14] Second, conditions or agents that initiate apoptosis are likely to trigger dental disorders accompanied by dentine remodelling.[36,37] Although our work identifies Pi as an important trigger for apoptosis, many questions regarding its mode of action still remain to be deciphered. In particular, the role of calcium in the Pi-mediated biological effects deserves further consideration. Recent experiments conducted in osteoblasts showed that the Pi-mediated cellular effects were related to the formation of Ca–P precipitates.[28] At the Ca and Pi concentrations used in the present study, the Ca–P crystal formation occurred within the first 30 min of incubation,[27] suggesting a possible role for Ca–P crystals in the mediation of ion pair effects. However, more detailed studies are necessary to determine whether Ca–P crystals are indeed implicated in this process as well as the possible mechanisms of action. Cellular recognition of monosodium urate (MSU) monohydrate crystals is likely to be due to the presence of specific Toll-like receptors 2 and 4,[38,39] whereas annexin 2 has been identified as a potential calcium oxalate monohydrate crystal receptor.[40] More recently, Ea et al. showed that overexpression of annexin 5 could mediate the binding of basic calcium phosphate (BCP; a term including carbonate-substitute apatite, octacalcium phosphate, and tricalcium phosphate) crystals to chondrocyte membranes, leading to increased apoptosis.[41] Whether a BCP crystal-binding protein such as annexin 5 or another unidentified protein could be involved in the response of odontoblasts to Ca–P precipitates has never been shown and requires further investigation. Alternatively, it remains possible that the formation of Ca–P crystals is not instrumental to the apoptotic response of odontoblasts. Although Ca–P crystal formation did occur at the Ca and Pi concentrations used in the present study, it remains possible that an increase in free Ca and/or Pi extracellular concentration could account for the stimulation of pro-apoptotic mechanisms in MO6-G3 cells. Particularly, the role of Ca or Pi transport into the cell and of CaSR, remain to be determined. An extracellular Ca- and Pi-mediated effect on apoptotic mechanisms that would be independent of Ca–P crystals may be of physiological relevance during the process or caries formation, during which large amounts of Ca and Pi are release into the dentinal fluid. This hypothesis may also be more consistent with the fact that the normal mineralized environment of odontoblasts does not normally induce apoptosis.

In this study we showed that a physiological Pi concentration is necessary for normal proliferation of MO6-G3 cells, whereas pro-apoptosis mechanisms are induced by high Pi concentration. We showed that the effect of Pi on cell viability, proliferation and apoptosis are determined by the presence of both calcium and phosphate in the culture medium. This study contributes to clarifying the role of Pi on odontoblast activities during caries formation.

Conflict of interest

No conflict of interest.

Funding source

This work was supported by grants from "Institut Français de Recherche en Odontologie", "Fondation pour la Recherche Médicale", "Société Française de Rhumatologie", "Région Pays de la Loire" and INSERM. Annabelle Bourgine received a fellowship from INSERM/Région Pays de la Loire. Solmaz Khoshniat received a fellowship from the French Ministry of Research.

Ethical approval

Not relevant.

Acknowledgments

We thank Dr. Mary MacDougall for kindly providing the MO6-G3 cell line and C. Merceron and E. Rederstorff for their help with MTS analysis.

REFERENCES

1. Ayhan H, Suskan E, Yildirim S. The effect of nursing or rampant caries on height, body weight and head circumference. *J Clin Pediatr Dent* 1996;**20**(3):209–12.
2. Acs G, Shulman R, Ng MW, Chussid S. The effect of dental rehabilitation on the body weight of children with early childhood caries. *Pediatr Dent* 1999;**21**(2):109–13.
3. Ratnayake N, Ekanayake L. Prevalence and impact of oral pain in 8-year-old children in Sri Lanka. *Int J Paediatr Dent* 2005;**15**(2):105–12.
4. Miller J, Vaughan-Williams E, Furlong R, Harrison L. Dental caries and children's weights. *J Epidemiol Community Health* 1982;**36**(1):49–52.
5. Anderson HK, Drummond BK, Thomson WM. Changes in aspects of children's oral-health-related quality of life following dental treatment under general anaesthesia. *Int J Paediatr Dent* 2004;**14**(5):317–25.
6. Low W, Tan S, Schwartz S. The effect of severe caries on the quality of life in young children. *Pediatr Dent* 1999;**21**(6):325–6.
7. White H, Lee JY, Vann Jr WF. Parental evaluation of quality of life measures following pediatric dental treatment using general anesthesia. *Anesth Prog* 2003;**50**(3):105–10.
8. Azarpazhooh A, Leake JL. Systematic review of the association between respiratory diseases and oral health. *J Periodontol* 2006;**77**(9):1465–82.
9. Garcia-Godoy F, Murray PE. Status and potential commercial impact of stem cell-based treatments on dental and craniofacial regeneration. *Stem Cells Dev* 2006;**15**(6):881–7.
10. Hahn CL, Liewehr FR. Relationships between caries bacteria, host responses, and clinical signs and symptoms of pulpitis. *J Endod* 2007;**33**(3):213–9.
11. Douglass AB, Douglass JM. Common dental emergencies. *Am Fam Physician* 2003;**67**(3):511–6.
12. Lygidakis NA, Marinou D, Katsaris N. Analysis of dental emergencies presenting to a community paediatric dentistry centre. *Int J Paediatr Dent* 1998;**8**(3):181–90.
13. Loesche WJ. Association of the oral flora with important medical diseases. *Curr Opin Periodontol* 1997;**4**:21–8.
14. Mitsiadis TA, De Bari C, About I. Apoptosis in developmental and repair-related human tooth remodeling: a view from the inside. *Exp Cell Res* 2008;**314**(4):869–77.
15. Tonetti MS, Cortellini D, Lang NP. In situ detection of apoptosis at sites of chronic bacterially induced inflammation in human gingiva. *Infect Immun* 1998;**66**(11):5190–5.
16. Wang PL, Shirasu S, Daito M, Ohura K. Streptococcus mutans lipoteichoic acid-induced apoptosis in cultured dental pulp cells from human deciduous teeth. *Biochem Biophys Res Commun* 2001;**281**(4):957–61.
17. Larmas M. Odontoblast function seen as the response of dentinal tissue to dental caries. *Adv Dent Res* 2001;**15**:68–71.
18. Smith AJ, Murray PE, Sloan AJ, Matthews JB, Zhao S. Trans-dentinal stimulation of tertiary dentinogenesis. *Adv Dent Res* 2001;**15**:51–4.
19. Mansfield K, Pucci B, Adams C, Shapiro I. Induction of apoptosis in skeletal tissues: phosphate-mediated chick chondrocyte apoptosis is calcium dependent. *Calcif Tissue Int* 2003;**73**(2):161–72.
20. Mansfield K, Rajpurohit R, Shapiro I. Extracellular phosphate ions cause apoptosis of terminally differentiated epiphyseal chondrocytes. *J Cell Physiol* 1999;**179**(3):276–86.
21. Mansfield K, Teixeira CC, Adams CS, Shapiro IM. Phosphate ions mediate chondrocyte apoptosis through a plasma membrane transporter mechanism. *Bone* 2001;**28**(1):1–8.
22. Meleti Z, Shapiro I, Adams C. Inorganic phosphate induces apoptosis of osteoblast-like cells in culture. *Bone* 2000;**27**(3):359–66.
23. Teixeira C, Mansfield K, Hertkorn C, Ischiropoulos H, Shapiro I. Phosphate-induced chondrocyte apoptosis is linked to nitric oxide generation. *Am J Physiol Cell Physiol* 2001;**281**(3):C833–9.
24. MacDougall M, Selden JK, Nydegger JR, Carnes DL. Immortalized mouse odontoblast cell line MO6-G3 application for in vitro biocompatibility testing. *Am J Dent* 1998;**11**. [Spec No: S11–S16].
25. MacDougall M, Unterbrink A, Carnes D, Rani S, Luan X, Chen S. Utilization of MO6-G3 immortalized odontoblast cells in studies regarding dentinogenesis. *Adv Dent Res* 2001;**15**:25–9.
26. Mesgouez C, Oboeuf M, Mauro N, Colon P, MacDougall M, Machtou P, et al. Ultrastructural and immunocytochemical characterization of immortalized odontoblast MO6-G3. *Int Endod J* 2006;**39**(6):453–63.
27. Wittrant Y, Bourgine A, Khoshniat S, Alliot-Licht B, Masson M, Gatius M, et al. Inorganic phosphate regulates Glvr-1 and -2 expression: role of calcium and ERK1/2. *Biochem Biophys Res Commun* 2009;**381**(2):259–63.
28. Khoshniat S, Bourgine A, Julien M, Petit M, Pilet P, Rouillon T, et al. Phosphate-dependent stimulation of MGP and OPN expression in osteoblasts via the ERK1/2 pathway is modulated by calcium. *Bone* 2010. [Epub ahead of print].
29. Khoshniat S, Bourgine A, Julien M, Weiss P, Guicheux J, Beck L. The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals. *Cell Mol Life Sci* 2010. [Epub ahead of print].
30. Conrads K, Yi M, Simpson K, Lucas D, Camalier C, Yu L, et al. A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. *Mol Cell Proteomics* 2005;**4**(9):1284–96.
31. Naviglio S, Spina A, Chiosi E, Fusco A, Illiano F, Pagano M, et al. Inorganic phosphate inhibits growth of human osteosarcoma U2OS cells via adenylate cyclase/cAMP pathway. *J Cell Biochem* 2006;**98**(6):1584–96.

32. Bellows CG, Heersche JN, Aubin JE. Inorganic phosphate added exogenously or released from beta-glycerophosphate initiates mineralization of osteoid nodules in vitro. *Bone Miner* 1992;**17**(1):15–29.
33. Adams CS, Mansfield K, Perlot RL, Shapiro IM. Matrix regulation of skeletal cell apoptosis. Role of calcium and phosphate ions. *J Biol Chem* 2001;**276**(23):20316–22.
34. Magne D, Bluteau G, Faucheux C, Palmer G, Vignes-Colombeix C, Pilet P, et al. Phosphate is a specific signal for ATDC5 chondrocyte maturation and apoptosis-associated mineralization: possible implication of apoptosis in the regulation of endochondral ossification. *J Bone Miner Res* 2003;**18**(8):1430–42.
35. Fujisawa R, Mizuno M, Tamura M. Effect of dentin phosphoprotein on phosphate-induced apoptosis of odontoblast-like cells. *Cells Tissues Organs* 2009;**189**(1–4):60–4.
36. Mitsiadis TA, Rahiotis C. Parallels between tooth development and repair: conserved molecular mechanisms following carious and dental injury. *J Dent Res* 2004;**83**(12):896–902.
37. Tziafas D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. *J Dent* 2000;**28**(2): 77–92.
38. Liu-Bryan R, Pritzker K, Firestein GS, Terkeltaub R. TLR2 signaling in chondrocytes drives calcium pyrophosphate dihydrate and monosodium urate crystal-induced nitric oxide generation. *J Immunol* 2005;**174**(8):5016–23.
39. Liu-Bryan R, Scott P, Sydlaske A, Rose DM, Terkeltaub R. Innate immunity conferred by Toll-like receptors 2 and 4 and myeloid differentiation factor 88 expression is pivotal to monosodium urate monohydrate crystal-induced inflammation. *Arthritis Rheum* 2005;**52**(9):2936–46.
40. Kumar V, Farell G, Deganello S, Lieske JC. Annexin II is present on renal epithelial cells and binds calcium oxalate monohydrate crystals. *J Am Soc Nephrol* 2003;**14**(2): 289–97.
41. Ea HK, Monceau V, Camors E, Cohen-Solal M, Charlemagne D, Liote F. Annexin 5 overexpression increased articular chondrocyte apoptosis induced by basic calcium phosphate crystals. *Ann Rheum Dis* 2008;**67**(11):1617–25.

Cinquième partie :

Références bibliographiques

Les références présentes dans cette section n'incluent pas celles utilisées dans la rédaction des différents articles pour lesquels ce listing existe déjà.

(1991). "Consensus development conference : Diagnosis, prophylaxis and treatment of osteoporosis." Am J Med **90**: C170-210.

(2006). "The role of calcium in peri- and postmenopausal women: 2006 position statement of the North American Menopause Society." Menopause **13**(6): 862-77; quiz 878-80.

Abelow, B. J., T. R. Holford, et al. (1992). "Cross-cultural association between dietary animal protein and hip fracture: a hypothesis." Calcif Tissue Int **50**(1): 14-8.

Banu, J., A. Bhattacharya, et al. (2010). "Endogenously produced n-3 fatty acids protect against ovariectomy induced bone loss in fat-1 transgenic mice." J Bone Miner Metab **28**(6): 617-26.

Barak, L. S., S. S. Ferguson, et al. (1997). "A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation." J Biol Chem **272**(44): 27497-500.

Baron, R. (2008). Anatomy and Ultrastructure of Bone - Histogenesis, Growth and Remodeling. Boston, Andrew Arnold.

Barrett-Connor, E., L. Mosca, et al. (2006). "Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women." N Engl J Med **355**(2): 125-37.

Bartl, R. and B. Frisch (2009). Bone Density in Osteoporosis. Osteoporosis : Diagnosis, Prevention, Therapy. Berlin, Springer**:** C63-73.

Behre, H. M., S. Kliesch, et al. (1997). "Long-term effect of testosterone therapy on bone mineral density in hypogonadal men." J Clin Endocrinol Metab **82**(8): 2386-90.

Bellenger, J., S. Bellenger, et al. (2011). "High pancreatic n-3 fatty acids prevent STZ-induced diabetes in fat-1 mice: inflammatory pathway inhibition." Diabetes **60**(4): 1090-9.

Bikle, D. (2008). "Hormonal Regulation of Bone Mineral Homeostasis." US Musculoskletal Reviews: C71-74.

Bischoff-Ferrari, H. A., E. Giovannucci, et al. (2006). "Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes." Am J Clin Nutr **84**(1): 18-28.

Bischoff-Ferrari, H. A., W. C. Willett, et al. (2005). "Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials." Jama **293**(18): 2257-64.

Bjarnason, N. H., E. E. Henriksen, et al. (2002). "Mechanism of circadian variation in bone resorption." Bone **30**(1): 307-13.

Black, D. M., S. R. Cummings, et al. (1996). "Randomised trial of effect of alendronate on risk of fracture in women with existing vertebral fractures. Fracture Intervention Trial Research Group." Lancet **348**(9041): 1535-41.

Black, D. M., P. D. Delmas, et al. (2007). "Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis." N Engl J Med **356**(18): 1809-22.

Black, D. M., S. L. Greenspan, et al. (2003). "The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis." N Engl J Med **349**(13): 1207-15.

Bolland, M. J., P. A. Barber, et al. (2008). "Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial." Bmj **336**(7638): 262-6.

Bonjour, J. P., M. A. Schurch, et al. (1997). "Protein intake, IGF-1 and osteoporosis." Osteoporos Int **7 Suppl 3**: S36-42.

Boskey, A. L. (1998). "Biomineralization: conflicts, challenges, and opportunities." J Cell Biochem Suppl **30-31**: 83-91.

Branca, F. (2003). "Dietary phyto-oestrogens and bone health." Proc Nutr Soc **62**(4): 877-87.

Bringhurst, F., M. Demay, et al. (2005). Bone and mineral metabolism in health and disease. Harrison's Principles of Internal Medicine. New York, Mc Graw-Hill**:** C2238-2249.

Briscoe, C. P., M. Tadayyon, et al. (2003). "The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids." J Biol Chem **278**(13): 11303-11.

Brownlie, R., R. M. Mayers, et al. (2008). "The long-chain fatty acid receptor, GPR40, and glucolipotoxicity: investigations using GPR40-knockout mice." Biochem Soc Trans **36**(Pt 5): 950-4.

Burge, R., B. Dawson-Hughes, et al. (2007). "Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025." J Bone Miner Res **22**(3): 465-75.

Burgess, T. L., Y. Qian, et al. (1999). "The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts." J Cell Biol **145**(3): 527-38.

Burghardt, P. R., E. S. Kemmerer, et al. (2010). "Dietary n-3:n-6 fatty acid ratios differentially influence hormonal signature in a rodent model of metabolic syndrome relative to healthy controls." Nutr Metab (Lond) **7**: 53.

Bushinsky, D. A. (1995). "Stimulated osteoclastic and suppressed osteoblastic activity in metabolic but not respiratory acidosis." Am J Physiol **268**(1 Pt 1): C80-8.

Callewaert, F., S. Boonen, et al. (2009). "Sex steroids and the male skeleton: a tale of two hormones." Trends Endocrinol Metab **21**(2): 89-95.

Cappelli, P., L. Di Liberato, et al. (1997). "N-3 polyunsaturated fatty acid supplementation in chronic progressive renal disease." J Nephrol **10**(3): 157-62.

Cashman, K. D. (2002). "Calcium intake, calcium bioavailability and bone health." Br J Nutr **87 Suppl 2**: S169-77.

Cauley, J. A., J. Robbins, et al. (2003). "Effects of estrogen plus progestin on risk of fracture and bone mineral density: the Women's Health Initiative randomized trial." Jama **290**(13): 1729-38.

Cavaghan, M. K., D. A. Ehrmann, et al. (1997). "Treatment with the oral antidiabetic agent troglitazone improves beta cell responses to glucose in subjects with impaired glucose tolerance." J Clin Invest **100**(3): 530-7.

Center, J. R., T. V. Nguyen, et al. (1999). "Mortality after all major types of osteoporotic fracture in men and women: an observational study." Lancet **353**(9156): 878-82.

Chan, B. Y., A. Gartland, et al. (2007). "PPAR agonists modulate human osteoclast formation and activity in vitro." Bone **40**(1): 149-59.

Chesnut, I. C., A. Skag, et al. (2004). "Effects of oral ibandronate administered daily or intermittently on fracture risk in postmenopausal osteoporosis." J Bone Miner Res **19**(8): 1241-9.

Chiang, C., M. Chiu, et al. (2009). "Mineralization and bone resorption are regulated by the androgen receptor in male mice." J Bone Miner Res **24**(4): 621-31.

Chin, H. J., Y. Y. Fu, et al. (2010). "Omacor, n-3 polyunsaturated fatty acid, attenuated albuminuria and renal dysfunction with decrease of SREBP-1 expression and triglyceride amount in the kidney of type II diabetic animals." Nephrol Dial Transplant **25**(5): 1450-7.

Clowes, J. A., R. A. Hannon, et al. (2002). "Effect of feeding on bone turnover markers and its impact on biological variability of measurements." Bone **30**(6): 886-90.

Cornish, J., A. MacGibbon, et al. (2008). "Modulation of osteoclastogenesis by fatty acids." Endocrinology **149**(11): 5688-95.

Corral, D. A., M. Amling, et al. (1998). "Dissociation between bone resorption and bone formation in osteopenic transgenic mice." Proc Natl Acad Sci U S A **95**(23): 13835-40.

Coxam, V., M.-J. Davicco, et al. (2009). "Vitamine K et physiologie osseuse." Cahiers de nutrition et de diététique **44**: C163-172.

Darnay, B. G., V. Haridas, et al. (1998). "Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappab and c-Jun N-terminal kinase." J Biol Chem **273**(32): 20551-5.

David, J. P., K. Sabapathy, et al. (2002). "JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and -independent mechanisms." J Cell Sci **115**(Pt 22): 4317-25.

Dawson-Hughes, B., S. S. Harris, et al. (2004). "Effect of dietary protein supplements on calcium excretion in healthy older men and women." J Clin Endocrinol Metab **89**(3): 1169-73.

Del Fattore, A., A. Teti, et al. (2008). "Osteoclast receptors and signaling." Arch Biochem Biophys **473**(2): 147-60.

Dennehy, C. and C. Tsourounis (2010). "A review of select vitamins and minerals used by postmenopausal women." Maturitas **66**(4): 370-80.

Devescovi, V., E. Leonardi, et al. (2008). "Growth factors in bone repair." Chir Organi Mov **92**(3): 161-8.

Doffinger, R., A. Smahi, et al. (2001). "X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling." Nat Genet **27**(3): 277-85.

Doshi, L. S., M. K. Brahma, et al. (2009). "Acute administration of GPR40 receptor agonist potentiates glucose-stimulated insulin secretion in vivo in the rat." Metabolism **58**(3): 333-43.

Draper, M. W. (2003). "The role of selective estrogen receptor modulators (SERMs) in postmenopausal health." Ann N Y Acad Sci **997**: 373-7.

Edfalk, S., P. Steneberg, et al. (2008). "Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion." Diabetes **57**(9): 2280-7.

Ernst, M., J. K. Heath, et al. (1989). "Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones." Endocrinology **125**(2): 825-33.

Ettinger, B., D. M. Black, et al. (1999). "Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators." Jama **282**(7): 637-45.

Européenne, R. d. l. C. (1999). Building strong bones and preventing fractures. Bruxelles, Communauté Européenne.

Everts, V., J. M. Delaisse, et al. (2002). "The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation." J Bone Miner Res **17**(1): 77-90.

Fink, H. A., S. K. Ewing, et al. (2006). "Association of testosterone and estradiol deficiency with osteoporosis and rapid bone loss in older men." J Clin Endocrinol Metab **91**(10): 3908-15.

Foundation, I. O. (2006). "The role of food and nutrition in building and maintaining strong bones." from http://www.iof-bonehealth.org/publications/bone-appetit.html.

Foundation, N. O. (2008). from http://nof.org/osteoporosis/diseasefacts.html.

Fu, Q., R. L. Jilka, et al. (2002). "Parathyroid hormone stimulates receptor activator of NFkappa B ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein." J Biol Chem **277**(50): 48868-75.

Fujiwara, K., F. Maekawa, et al. (2005). "Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release." Am J Physiol Endocrinol Metab **289**(4): E670-7.

Fukumoto, S. and T. J. Martin (2009). "Bone as an endocrine organ." Trends Endocrinol Metab **20**(5): 230-6.

Galli, C. and P. C. Calder (2009). "Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review." Ann Nutr Metab **55**(1-3): 123-39.

Gaugris, S., R. P. Heaney, et al. (2005). "Vitamin D inadequacy among post-menopausal women: a systematic review." Qjm **98**(9): 667-76.

Gilman, J. and K. D. Cashman (2007). "The effect of marine oil-derived n-3 fatty acids on transepithelial calcium transport in Caco-2 cell models of healthy and inflamed intestines." Br J Nutr **97**(2): 281-8.

Gras, D., P. Chanez, et al. (2009). "Thiazolidinediones induce proliferation of human bronchial epithelial cells through the GPR40 receptor." Am J Physiol Lung Cell Mol Physiol **296**(6): L970-8.

Gu, G., M. Mulari, et al. (2005). "Death of osteocytes turns off the inhibition of osteoclasts and triggers local bone resorption." Biochem Biophys Res Commun **335**(4): 1095-101.

Hall, S. L. and G. A. Greendale (1998). "The relation of dietary vitamin C intake to bone mineral density: results from the PEPI study." Calcif Tissue Int **63**(3): 183-9.

Hannan, M. T., K. L. Tucker, et al. (2000). "Effect of dietary protein on bone loss in elderly men and women: the Framingham Osteoporosis Study." J Bone Miner Res **15**(12): 2504-12.

Hardy, S., G. G. St-Onge, et al. (2005). "Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40." J Biol Chem **280**(14): 13285-91.

Harris, S. T., N. B. Watts, et al. (1999). "Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy With Risedronate Therapy (VERT) Study Group." Jama **282**(14): 1344-52.

Hayden, J. M., S. Mohan, et al. (1995). "The insulin-like growth factor system and the coupling of formation to resorption." Bone **17**(2 Suppl): 93S-98S.

Heaney, R. P. (1992). "Calcium in the prevention and treatment of osteoporosis." J Intern Med **231**(2): 169-80.

Heaney, R. P. (2000). "Calcium, dairy products and osteoporosis." J Am Coll Nutr **19**(2 Suppl): 83S-99S.

Heino, T. J., T. A. Hentunen, et al. (2002). "Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen." J Cell Biochem **85**(1): 185-97.

Henriksen, K., D. J. Leeming, et al. (2007). "Osteoclasts prefer aged bone." Osteoporos Int **18**(6): 751-9.

Hodsman, A. B., D. A. Hanley, et al. (2003). "Efficacy and safety of human parathyroid hormone-(1-84) in increasing bone mineral density in postmenopausal osteoporosis." J Clin Endocrinol Metab **88**(11): 5212-20.

Hoebertz, A., T. R. Arnett, et al. (2003). "Regulation of bone resorption and formation by purines and pyrimidines." Trends Pharmacol Sci **24**(6): 290-7.

Hofbauer, L. C., S. Khosla, et al. (2000). "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption." J Bone Miner Res **15**(1): 2-12.

Hofbauer, L. C., S. Khosla, et al. (1999). "Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells." Endocrinology **140**(9): 4367-70.

Hong, D., H. X. Chen, et al. (2008). "The biological roles of extracellular and intracytoplasmic glucocorticoids in skeletal cells." J Steroid Biochem Mol Biol **111**(3-5): 164-70.

Horcajada-Molteni, M. and V. Coxam (2001). Flavonols and iodoflavones prevent bone loss in the ovariectomized rat, a model for postmenopausal osteoporosis. Nutritional aspects of osteoporosis. San Diego, CA, Academic Press**:** C325-340.

Hoshi, K., T. Komori, et al. (1999). "Morphological characterization of skeletal cells in Cbfa1-deficient mice." Bone **25**(6): 639-51.

Hunt, J. R., L. K. Johnson, et al. (2009). "Dietary protein and calcium interact to influence calcium retention: a controlled feeding study." Am J Clin Nutr **89**(5): 1357-65.

Hwang, S. Y. and J. W. Putney, Jr. (2011). "Calcium signaling in osteoclasts." Biochim Biophys Acta **1813**(5): 979-83.

Ihle, R. and A. B. Loucks (2004). "Dose-response relationships between energy availability and bone turnover in young exercising women." J Bone Miner Res **19**(8): 1231-40.

Imai, Y., S. Kondoh, et al. (2010). "Minireview: osteoprotective action of estrogens is mediated by osteoclastic estrogen receptor-alpha." Mol Endocrinol **24**(5): 877-85.

Insogna, K. L., M. Sahni, et al. (1997). "Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts." J Clin Invest **100**(10): 2476-85.

Iotsova, V., J. Caamano, et al. (1997). "Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2." Nat Med **3**(11): 1285-9.

Ishizuya, T., S. Yokose, et al. (1997). "Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells." J Clin Invest **99**(12): 2961-70.

Itoh, Y., Y. Kawamata, et al. (2003). "Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40." Nature **422**(6928): 173-6.

John, M. R., W. G. Goodman, et al. (1999). "A novel immunoradiometric assay detects full-length human PTH but not amino-terminally truncated fragments: implications for PTH measurements in renal failure." J Clin Endocrinol Metab **84**(11): 4287-90.

Johnell, O., W. H. Scheele, et al. (2002). "Additive effects of raloxifene and alendronate on bone density and biochemical markers of bone remodeling in postmenopausal women with osteoporosis." J Clin Endocrinol Metab **87**(3): 985-92.

Jones, M. E., W. C. Boon, et al. (2006). "Of mice and men: the evolving phenotype of aromatase deficiency." Trends Endocrinol Metab **17**(2): 55-64.

Jones, S. J., C. Gray, et al. (1994). "Simulation of bone resorption-repair coupling in vitro." Anat Embryol (Berl) **190**(4): 339-49.

Kantham, L., S. J. Quinn, et al. (2009). "The Calcium-Sensing Receptor (CaSR) Defends Against Hypercalcemia Independent of its Regulation of Parathyroid Hormone Secretion." Am J Physiol Endocrinol Metab.

Kaplamadzhiev, D. B., H. Hisha, et al. (2010). "Bone marrow-derived stromal cells can express neuronal markers by DHA/GPR40 signaling." Biosci Trends **4**(3): 119-29.

Kapoor, R. and Y. S. Huang (2006). "Gamma linolenic acid: an antiinflammatory omega-6 fatty acid." Curr Pharm Biotechnol **7**(6): 531-4.

Karsdal, M. A., M. S. Fjording, et al. (2001). "Transforming growth factor-beta-induced osteoblast elongation regulates osteoclastic bone resorption through a p38 mitogen-activated protein kinase- and matrix metalloproteinase-dependent pathway." J Biol Chem **276**(42): 39350-8.

Katznelson, L., J. S. Finkelstein, et al. (1996). "Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism." J Clin Endocrinol Metab **81**(12): 4358-65.

Keen, R. W. (2003). "Burden of osteoporosis and fractures." Curr Osteoporos Rep **1**(2): 66-70.

Kerstetter, J. E., A. C. Looker, et al. (2000). "Low dietary protein and low bone density." Calcif Tissue Int **66**(4): 313.

Kerstetter, J. E., K. O. O'Brien, et al. (2005). "The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women." J Clin Endocrinol Metab **90**(1): 26-31.

Khosla, S. (2001). "Minireview: the OPG/RANKL/RANK system." Endocrinology **142**(12): 5050-5.

Khosla, S., D. Burr, et al. (2007). "Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research." J Bone Miner Res **22**(10): 1479-91.

Kim, H. J., K. T. Lee, et al. (2008). "Dietary docosahexaenoic acid-rich diacylglycerols ameliorate hepatic steatosis and alter hepatic gene expressions in C57BL/6J-Lep(ob/ob) mice." Mol Nutr Food Res **52**(8): 965-73.

Kim, J. H., H. M. Jin, et al. (2009). "The mechanism of osteoclast differentiation induced by IL-1." J Immunol **183**(3): 1862-70.

Kurata, K., T. J. Heino, et al. (2006). "Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture." J Bone Miner Res **21**(4): 616-25.

Lacey, D. L., E. Timms, et al. (1998). "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation." Cell **93**(2): 165-76.

Lambers, T. T., R. J. Bindels, et al. (2006). "Coordinated control of renal Ca2+ handling." Kidney Int **69**(4): 650-4.

Latour, M. G., T. Alquier, et al. (2007). "GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo." Diabetes **56**(4): 1087-94.

Lazowski, D. A., L. J. Fraher, et al. (1994). "Regional variation of insulin-like growth factor-I gene expression in mature rat bone and cartilage." Bone **15**(5): 563-76.

Lenart, B. A., A. S. Neviaser, et al. (2009). "Association of low-energy femoral fractures with prolonged bisphosphonate use: a case control study." Osteoporos Int **20**(8): 1353-62.

Leveille, S. G., A. Z. LaCroix, et al. (1997). "Dietary vitamin C and bone mineral density in postmenopausal women in Washington State, USA." J Epidemiol Community Health **51**(5): 479-85.

Lomaga, M. A., W. C. Yeh, et al. (1999). "TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling." Genes Dev **13**(8): 1015-24.

Lorget, F., S. Kamel, et al. (2000). "High extracellular calcium concentrations directly stimulate osteoclast apoptosis." Biochem Biophys Res Commun **268**(3): 899-903.

Lyles, K. W., C. S. Colon-Emeric, et al. (2007). "Zoledronic Acid in Reducing Clinical Fracture and Mortality after Hip Fracture." N Engl J Med **357**: nihpa40967.

Ma, D., B. Tao, et al. (2007). "Expression of free fatty acid receptor GPR40 in the central nervous system of adult monkeys." Neurosci Res **58**(4): 394-401.

Ma, D., M. Zhang, et al. (2010). "DHA promotes the neuronal differentiation of rat neural stem cells transfected with GPR40 gene." Brain Res **1330**: 1-8.

MacLean, C., S. Newberry, et al. (2008). "Systematic review: comparative effectiveness of treatments to prevent fractures in men and women with low bone density or osteoporosis." Ann Intern Med **148**(3): 197-213.

Manolagas, S. C. (2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis." Endocr Rev **21**(2): 115-37.

Marie, P. (2001). "Différenciation, fonction et contrôle de l'ostéoblaste." Med Sci (Paris) **17**(12): C1252-1259.

Marie, P. and P. Halbout (2008). "OPG/RANKL: role and therapeutic target in osteoporosis." Med Sci (Paris) **24**(1): C105-110.

Marx, S. J. (2000). "Hyperparathyroid and hypoparathyroid disorders." N Engl J Med **343**(25): 1863-75.

Matsumoto, M., T. Sudo, et al. (2000). "Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL)." J Biol Chem **275**(40): 31155-61.

Matsuo, K., D. L. Galson, et al. (2004). "Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos." J Biol Chem **279**(25): 26475-80.

Melton, L. J., 3rd, E. A. Chrischilles, et al. (1992). "Perspective. How many women have osteoporosis?" J Bone Miner Res **7**(9): 1005-10.

Meunier, P., J.-F. Brantus, et al. (2005). Circonstances du diagnostic et diagnostic de l'ostéoporose : les facteurs de risques. L'ostéoporose. Paris, Masson**:** C21-25.

Morabito, N., A. Crisafulli, et al. (2002). "Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study." J Bone Miner Res **17**(10): 1904-12.

Morton, D. J., E. L. Barrett-Connor, et al. (2001). "Vitamin C supplement use and bone mineral density in postmenopausal women." J Bone Miner Res **16**(1): 135-40.

Mosekilde, L. (2008). "Vitamin D requirement and setting recommendation levels: long-term perspectives." Nutr Rev **66**(10 Suppl 2): S170-7.

Mulari, M. T., Q. Qu, et al. (2004). "Osteoblast-like cells complete osteoclastic bone resorption and form new mineralized bone matrix in vitro." Calcif Tissue Int **75**(3): 253-61.

Mundy, G. R. and L. F. Bonewald (1990). "Role of TGF beta in bone remodeling." Ann N Y Acad Sci **593**: 91-7.

Nakamura, T., Y. Imai, et al. (2007). "Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts." Cell **130**(5): 811-23.

Nakashima, T., Y. Kobayashi, et al. (2000). "Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines." Biochem Biophys Res Commun **275**(3): 768-75.

Nataf, S. (2009). "Le Tissu Osseux." Histologie, from http://histoblog.viabloga.com/texts/le-tissu-osseux--2009-.

Neer, R. M., C. D. Arnaud, et al. (2001). "Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis." N Engl J Med **344**(19): 1434-41.

Nelson, H. D., L. L. Humphrey, et al. (2002). "Postmenopausal hormone replacement therapy: scientific review." Jama **288**(7): 872-81.

New, S. A. (2003). "Intake of fruit and vegetables: implications for bone health." Proc Nutr Soc **62**(4): 889-99.

Noble, B. S., N. Peet, et al. (2003). "Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone." Am J Physiol Cell Physiol **284**(4): C934-43.

Noble, B. S., H. Stevens, et al. (1997). "Identification of apoptotic changes in osteocytes in normal and pathological human bone." Bone **20**(3): 273-82.

Noh, A. L., H. Park, et al. (2011). "L-type Ca(2+) channel agonist inhibits RANKL-induced osteoclast formation via NFATc1 down-regulation." Life Sci **89**(5-6): 159-64.

Oh, D. Y., S. Talukdar, et al. (2010). "GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects." Cell **142**(5): 687-98.

Ohtani, K., H. Shimizu, et al. (1996). "Pioglitazone hydrochloride stimulates insulin secretion in HIT-T 15 cells by inducing Ca2+ influx." J Endocrinol **150**(1): 107-11.

Okazaki, R., M. Toriumi, et al. (1999). "Thiazolidinediones inhibit osteoclast-like cell formation and bone resorption in vitro." Endocrinology **140**(11): 5060-5.

Omdahl, J. L., H. A. Morris, et al. (2002). "Hydroxylase enzymes of the vitamin D pathway: expression, function, and regulation." Annu Rev Nutr **22**: 139-66.

Orimo, H. (2010). "The mechanism of mineralization and the role of alkaline phosphatase in health and disease." J Nihon Med Sch **77**(1): 4-12.

Otto, F., A. P. Thornell, et al. (1997). "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell **89**(5): 765-71.

Oursler, M. J., C. Cortese, et al. (1991). "Modulation of transforming growth factor-beta production in normal human osteoblast-like cells by 17 beta-estradiol and parathyroid hormone." Endocrinology **129**(6): 3313-20.

Parfitt, A. M., G. R. Mundy, et al. (1996). "A new model for the regulation of bone resorption, with particular reference to the effects of bisphosphonates." J Bone Miner Res **11**(2): 150-9.

Parhami, F., Y. Tintut, et al. (2001). "Atherogenic high-fat diet reduces bone mineralization in mice." J Bone Miner Res **16**(1): 182-8.

Perez, A. V., G. Picotto, et al. (2008). "Minireview on regulation of intestinal calcium absorption. Emphasis on molecular mechanisms of transcellular pathway." Digestion **77**(1): 22-34.

Pettifor J, Prentice A, et al. (2003). The skeletal system. Nutrition and Metabolism. Oxford, Blackwell Publishing**:** C247-283.

Promislow, J. H., D. Goodman-Gruen, et al. (2002). "Protein consumption and bone mineral density in the elderly : the Rancho Bernardo Study." Am J Epidemiol **155**(7): 636-44.

Quinn, J. M. and H. Saleh (2009). "Modulation of osteoclast function in bone by the immune system." Mol Cell Endocrinol **310**(1-2): 40-51.

Rachner, T. D., S. Khosla, et al. (2011). "Osteoporosis: now and the future." Lancet **377**(9773): 1276-87.

Raisz, L. G. (2005). "Pathogenesis of osteoporosis: concepts, conflicts, and prospects." J Clin Invest **115**(12): 3318-25.

Rennes, U. (2009). "Catabolisme des acides gras par les cellules périphériques (Université Rennes)."from:http://facmed.univrennes1.fr/wkf//stock/RENNES20090224050904vdavidCours_Lipides_PCEM1-1.pdf

Riggs, B. L., H. W. Wahner, et al. (1982). "Changes in bone mineral density of the proximal femur and spine with aging. Differences between the postmenopausal and senile osteoporosis syndromes." J Clin Invest **70**(4): 716-23.

Rizzoli, R. (2008). "Nutrition: its role in bone health." Best Pract Res Clin Endocrinol Metab **22**(5): 813-29.

Robinson, J. A., B. L. Riggs, et al. (1996). "Osteoclasts and transforming growth factor-beta: estrogen-mediated isoform-specific regulation of production." Endocrinology **137**(2): 615-21.

Roggia, C., Y. Gao, et al. (2001). "Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo." Proc Natl Acad Sci U S A **98**(24): 13960-5.

Ruocco, M. G., S. Maeda, et al. (2005). "I{kappa}B kinase (IKK){beta}, but not IKK{alpha}, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss." J Exp Med **201**(10): 1677-87.

Ryan, M. R., R. Shepherd, et al. (2005). "An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency." Proc Natl Acad Sci U S A **102**(46): 16735-40.

Salles, J. P. (2006). "[Growth hormone and bone]." Arch Pediatr **13**(6): 666-8.

Schmidt, J., K. Liebscher, et al. (2011). "Conjugated linoleic acids mediate insulin release through islet G protein-coupled receptor FFA1/GPR40." J Biol Chem **286**(14): 11890-4.

Schwartz, Z., C. H. Lohmann, et al. (2000). "Osteoblast proliferation and differentiation on dentin slices are modulated by pretreatment of the surface with tetracycline or osteoclasts." J Periodontol **71**(4): 586-97.

Seki, H., Y. Tani, et al. (2009). "Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1." Prostaglandins Other Lipid Mediat **89**(3-4): 126-30.

Setchell, K. D. and E. Lydeking-Olsen (2003). "Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies." Am J Clin Nutr **78**(3 Suppl): 593S-609S.

Shapiro, H., S. Shachar, et al. (2005). "Role of GPR40 in fatty acid action on the beta cell line INS-1E." Biochem Biophys Res Commun **335**(1): 97-104.

Smith, N. J., L. A. Stoddart, et al. (2009). "The action and mode of binding of thiazolidinedione ligands at free fatty acid receptor 1." J Biol Chem **284**(26): 17527-39.

Snyder, P. J., H. Peachey, et al. (1999). "Effect of testosterone treatment on bone mineral density in men over 65 years of age." J Clin Endocrinol Metab **84**(6): 1966-72.

Sun, D., A. Krishnan, et al. (2003). "Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice." J Bone Miner Res **18**(7): 1206-16.

Sun, L., Y. Peng, et al. (2006). "FSH directly regulates bone mass." Cell **125**(2): 247-60.

Sutton, A. L. and P. N. MacDonald (2003). "Vitamin D: more than a "bone-a-fide" hormone." Mol Endocrinol **17**(5): 777-91.

Takahashi, H., B. Epker, et al. (1964). "Resorption Precedes Formative Activity." Surg Forum **15**: 437-8.

Takayanagi, H., S. Kim, et al. (2002). "Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts." Dev Cell **3**(6): 889-901.

Tanaka, N., K. Sano, et al. (2008). "Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis." J Clin Gastroenterol **42**(4): 413-8.

Tatsumi, S., K. Ishii, et al. (2007). "Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction." Cell Metab **5**(6): 464-75.

Toppets, V., V. Pastoret, et al. (2004). "Morphologie, croissance et remaniement du tissu osseux." Annales de Médecine Vétérinaire **148**(1): C1-13.

Torotra, G. and S. Grabowski (2002). Principes d'anatomie et de physiologie. Bruxelles, De Boeck Université.

Tucker, K. L., M. T. Hannan, et al. (1999). "Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women." Am J Clin Nutr **69**(4): 727-36.

Turner, A. G., F. Tjahyono, et al. (2010). "The role of the calcitonin receptor in protecting against induced hypercalcemia is mediated via its actions in osteoclasts to inhibit bone resorption." Bone **48**(2): 354-61.

Vahle, J. L., G. G. Long, et al. (2004). "Bone neoplasms in F344 rats given teriparatide [rhPTH(1-34)] are dependent on duration of treatment and dose." Toxicol Pathol **32**(4): 426-38.

Verborgt, O., G. J. Gibson, et al. (2000). "Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo." J Bone Miner Res **15**(1): 60-7.

Vettor, R., M. Granzotto, et al. (2008). "Loss-of-function mutation of the GPR40 gene associates with abnormal stimulated insulin secretion by acting on intracellular calcium mobilization." J Clin Endocrinol Metab **93**(9): 3541-50.

Wagner, E. F. (2002). "Functions of AP1 (Fos/Jun) in bone development." Ann Rheum Dis **61 Suppl 2**: ii40-2.

Wan, Y., L. W. Chong, et al. (2007). "PPAR-gamma regulates osteoclastogenesis in mice." Nat Med **13**(12): 1496-503.

Wauquier, F., L. Leotoing, et al. (2009). "Oxidative stress in bone remodelling and disease." Trends Mol Med **15**(10): 468-77.

Wei, D., J. Li, et al. (2010). "Cellular production of n-3 PUFAs and reduction of n-6-to-n-3 ratios in the pancreatic beta-cells and islets enhance insulin secretion and confer protection against cytokine-induced cell death." Diabetes **59**(2): 471-8.

Whelan, A. M., T. M. Jurgens, et al. (2006). "Natural health products in the prevention and treatment of osteoporosis: systematic review of randomized controlled trials." Ann Pharmacother **40**(5): 836-49.

Whitfield, J. F., P. Morley, et al. (2002). "Parathyroid hormone, its fragments and their analogs for the treatment of osteoporosis." Treat Endocrinol **1**(3): 175-90.

Wiktor-Jedrzejczak, W., A. Bartocci, et al. (1990). "Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse." Proc Natl Acad Sci U S A **87**(12): 4828-32.

Wiren, K. M., A. A. Semirale, et al. (2008). "Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis: a model for compartment-specific androgen action in the skeleton." Bone **43**(3): 440-51.

Wiren, K. M., X. W. Zhang, et al. (2004). "Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals." Endocrinology **145**(7): 3507-22.

Xiao, Y., J. Cui, et al. (2010). "Expression of genes associated with bone resorption is increased and bone formation is decreased in mice fed a high-fat diet." Lipids **45**(4): 345-55.

Yamamoto, A., T. Miyazaki, et al. (2002). "Possible involvement of IkappaB kinase 2 and MKK7 in osteoclastogenesis induced by receptor activator of nuclear factor kappaB ligand." J Bone Miner Res **17**(4): 612-21.

Yang, C. R. and C. C. Lai (2010). "Thiazolidinediones inhibit TNF-alpha-mediated osteoclast differentiation of RAW264.7 macrophages and mouse bone marrow cells through downregulation of NFATc1." Shock **33**(6): 662-7.

Yonezawa, T., K. Katoh, et al. (2004). "Existence of GPR40 functioning in a human breast cancer cell line, MCF-7." Biochem Biophys Res Commun **314**(3): 805-9.

Zinsou, C. (2005). "Dégradation des Lipides (Université des Antilles et de la Guyane)." from http://calamar.univ-ag.fr/deugsv/Documents/Cours/Bioch-Zinsou/Lipidegrad.pdf.

Zhao, C., N. Irie, et al. (2006). "Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis." Cell Metab **4**(2): 111-21.

Zhao, S., Y. K. Zhang, et al. (2002). "MLO-Y4 osteocyte-like cells support osteoclast formation and activation." J Bone Miner Res **17**(11): 2068-79.

Résumé

Rôle des acides gras dans le métabolisme osseux

Dans le contexte actuel d'allongement de l'espérance de vie, la prévalence des maladies liées à l'âge telles que l'ostéoporose est de plus en plus importante. Le coût de la prise en charge de ces pathologies constitue un problème de santé public majeur et la mise en place de stratégies de prévention nutritionnelle adaptées apparaît comme une excellente solution alternative aux traitements habituels. Pourtant, l'étude des activités biologiques des nutriments reste trop marginale pour certains tissus et certaines catégories de molécules, c'est notamment le cas du tissu osseux et des lipides, en particulier les acides gras. Ces derniers sont pourtant capables de moduler le devenir du tissu osseux que ce soit indirectement par des mécanismes systémiques ou directement au niveau de la cellule osseuse.

La perte osseuse liée au vieillissement est souvent associée à l'établissement progressif d'une inflammation chronique à bas bruit à l'échelle de l'organisme. Dans ce contexte, les niveaux de cytokines pro-inflammatoires telles que l'interleukine 6 ou le Tumor Necrosis Factor α sont augmentés et ces composés, qui sont connus pour induire la résorption osseuse, pourraient contribuer à la dégradation du squelette.

Notre hypothèse de travail était que certains Acides Gras Poly-Insaturés (AGPI) à propriétés anti-inflammatoires pouvaient éventuellement limiter l'inflammation chronique associée au vieillissement et ainsi contribuer à préserver le capital osseux. Dans cette optique, nous avons utilisé la souris SamP8 qui est un modèle de *progeria* présentant, à douze mois, un phénotype ostéoporotique. Dans ce modèle, l'administration d'un régime délétère à base d'huile de tournesol (ratio acide gras ω6 /ω3 très important) aggrave la perte osseuse en association avec une augmentation des marqueurs inflammatoires systémiques et osseux ainsi qu'une augmentation des marqueurs de la résorption osseuse. La supplémentation de ce régime tournesol avec de l'huile de bourrache ou de l'huile de poisson permet d'apporter des quantités importantes d'AGPI anti-inflammatoires (acide γ-linoléique pour l'huile de bourrache et ω3 pour l'huile de poisson). De manière intéressante, ces deux régimes supplémentés permettent, en plus de réduire les paramètres inflammatoires, de s'opposer à l'augmentation des marqueurs de résorption osseuse et de limiter la diminution de la densité minérale de l'os chez ces souris. Cette étude met donc en évidence le potentiel santé de certains AGPI au regard de la préservation du capital osseux et suggère un rôle déterminant de leurs propriétés anti-inflammatoires systémiques.

En parallèle, la description des effets directs des acides gras au niveau cellulaire par l'activation de récepteurs spécifiques occupent une place croissante dans la littérature. Récemment, le récepteur membranaire GPR40 (G Protein coupled Receptor 40) a été mis en évidence pour ses interactions avec les acides gras libres à longues chaînes et nous avons pu montrer son expression dans les précurseurs ostéoclastiques. Nous avons donc émis l'hypothèse que ce récepteur pourrait jouer un rôle dans la médiation des effets des acides gras sur les paramètres du remodelage osseux. Ce travail a permis de mettre en évidence l'existence d'un phénotype ostéoporotique chez la souris invalidée pour le GPR40. L'effet protecteur de ce récepteur semble lié principalement à un effet inhibiteur de son activation sur la différenciation des ostéoclastes. En effet, l'utilisation de l'agoniste spécifique du GPR40 empêche *in vitro* la différenciation ostéoclastique par RANKL de deux modèles cellulaires de manière GPR40-dépendante. De surcroit, cet agoniste est également capable *in vivo* de s'opposer à une perte osseuse induite chez la souris. Ces résultats révèlent pour la première fois l'implication du récepteur GPR40 dans la physiologie osseuse et apportent la connaissance d'une nouvelle possibilité de modulation directe des cellules osseuses par les acides gras.