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To cite this version:
Claire Philippe, Fabien Wauquier, Jean-François Landrier, Lauriane Bonnet, Elisabeth Miot-Noirault, et al.. GPR40 mediates potential positive effects of a saturated fatty acid enriched diet on bone. Molecular Nutrition and Food Research, Wiley-VCH Verlag, 2017, 61 (2), pp.1600219. <10.1002/mnfr.201600219>. <hal-01798936>

HAL Id: hal-01798936
https://hal-amu.archives-ouvertes.fr/hal-01798936
Submitted on 24 May 2018

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GPR40 mediates potential positive effects of a saturated fatty acid enriched diet on bone

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Received: 09/03/2016; Revised: 29/07/2016; Accepted: 02/09/2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201600219.

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Keywords: Free fatty acid receptor; GPR40; High-fat diet; Nutrition; Osteoporosis; Osteopenia

ABSTRACT

Scope: The stimulation of the free fatty acid receptor GPR40 by GW9508 prevents bone loss by inhibiting osteoclast activity, both in vitro and in vivo. Here, we questioned whether the stimulation of the GPR40 receptor by dietary fatty acids may lead to the same beneficial effect on bone.

Methods and results: We investigated (1) the impact of a fatty acid-enriched diet (HFD) on bone health in C57/Bl6 female mice depending on (2) the estrogen status (ovariectomy) and (3) the genotype (GPR40+/+ or GPR40−/−). Bone mineral density (BMD), body composition, weight, inflammation and bone remodelling parameters were monitored. HFD decreased BMD in HFD-SH-GPR40+/+ mice but OVX failed to further impact BMD in HFD-OVX-GPR40+/+ mice, while additional bone loss was observed in HFD-OVX-GPR40−/− animals. These data suggest that when stimulated by fatty acid enriched diets GPR40 contributes to counteract ovariectomy-induced bone alteration. The sparing effect is supported by the modulation of both the OPG/RANKL ratio in blood stream and the expression level of inflammatory markers in adipose tissues. Bone preservation by GPR40 stimulation is dependent on the presence of long chain saturated fatty acids.

Conclusion: GPR40 contributes to counter ovariectomy-induced bone loss in a context of saturated fatty acid enrichment.
1. **INTRODUCTION:**

A growing body of data has revealed complex relationships between dietary lipids and the skeleton [1]. Depending on the type of fatty acids, the biological activity on bone may differ. Literature has mainly evidenced that high fat diets negatively impact bone health. However, most of the studies have focused their investigations on hyper caloric diets enriched with saturated fatty acids in healthy groups without asking whether a beneficial effect could exist under certain circumstances. Regarding the unsaturated fatty acid family, the ω-6 family has been shown to enhance osteoporosis establishment while ω-3 fatty acids seem to preserve bone tissues [2-5]. These seemingly conflicting results have raised the idea that in some ways the modulation of the
lipid intakes may represent a preventive opportunity for the management of bone complications. However, mechanisms of action are poorly understood and further investigations are required to develop innovative strategies regarding fat involvement in osteoporosis management.

About a decade ago, several groups demonstrated that the G-protein coupled receptor (GPR) 40 was able to interact with medium to long-chain fatty acids and to transduce a specific signal across the cell membrane [6-11]. The role of GPR40 (also known as Free Fatty Acid Receptor-1/FFAR-1) was mainly examined in beta-cells for its involvement in insulin secretion [12-14]. Later, GPR40 roles were discovered in taste buds [15], enteroendocrine cells [16], and in the brain [17, 18].

Recently, we demonstrated *in vivo* and *in vitro* the implication of this receptor in bone homeostasis [19-22] GPR40/- mice were characterized by an osteopenic/osteoporotic phenotype when compared to wild-type littermates. In addition, phenotype onset was prevented in wild-type (WT) ovariectomized mice when a GPR40 synthetic agonist, GW9508, was given orally. We further demonstrated that prevention by GW9508 was driven by an inhibition of osteoclastogenesis in a GPR40 dependent way. This synthetic agonist was used to decipher the role of GPR40 and to avoid confounding parameters. However, since free fatty acids are natural ligands for GPR40, we further questioned the relevance of this receptor in an integrative approach investigating whether this fatty acid receptor may represent an innovative opportunity in the design of nutritional and therapeutic strategies to prevent bone loss in an ageing population.

2. **MATERIAL AND METHODS:**

*Ethics.* All animal procedures were approved by the institution’s animal welfare committee (Comité d’Ethique en Matière d’Expérimentation Animale Auvergne: CEMEAA) and were conducted in accordance with the European guidelines for the care and use of laboratory animals (CE 80-12).
Animals were housed in the animal facility of the INRA Research for Human Nutrition (Agreement N°: C6334514). Throughout the study, animals were housed in a controlled environment characterized by a 12:12 h light-dark cycle, 20-22°C, 50-60% relative humidity. Mice were kept one per plastic cage with free access to water. At the end of the protocol, blood was withdrawn from anesthetized animals. Then, animals were sacrificed by cervical dislocation and tissues were harvested, frozen and stored prior to investigation.

**Genotype.** GPR40-/- mice were obtained from AMGEN Inc. As described by Latour et al. (19), 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. The mice were backcrossed onto the C57BL/6 strain over 9 generations. Pups were screened by PCR performed on genomic DNA. Wild-type (WT) littermates were used as controls.

**Experimental design.** In order to accustom the mice to the texture, animals were provided with free access to a standard growth powdered diet during an acclimatization period of 3 weeks from weaning (weeks 4 to 7). On week 7, mice were randomly divided into 16 groups (n=12, individual cage), depending on the diet (6 different diets), the type of surgery (SH or OVX) and the genotype (GPR40+/+ or GPR40-/-) (Table 1 and 2). From this point, mice were provided with their specific diet. On week 9, mice were sham-operated or ovariectomized to induce bone loss. Diets were delivered ad libitum for a total of 7 weeks including 5 weeks post-surgery (Figure 1A and B). Briefly, every 48 hours, each mouse received exactly 10g of the diet in a small bowl (Figure 1) to cover their needs (about 3 to 4g per day – figure 4B). Food was changed every 48 hours to minimize fatty acid oxidation. Once a week, the food left-over was weighted to evaluate the consumption of each mouse.

**Ovariectomized mice.** Mice were either sham-operated to mimic the side effects of the surgery or ovariectomized to induce an estrogen deprivation and a subsequent bone loss.
**Diets.** Diets were purchased from our experimental facility dedicated to the conception of animal diets (INRA UE0300, UPAE Unité de Préparation des Aliments Expérimentaux, Jouy-en-Josas, France). Compositions are summarized in table 2. Diets were provided to the mice as powders and not as croquettes to avoid fatty acid heating and alteration during the croquettes’ production process. The fatty acid enrichment in high fat diets was obtained by a modulation of the corn starch content and in these cases, 42.18% of the calories were related to the fat content and 46.54% from the carbohydrate content. The total amount of calories was higher in the fatty acid enriched groups than in the control ones (464kcal/100g of diet versus 373kcal/100g of diet, respectively). The groups with fatty acid enrichment were isocaloric. This parameter was taken into account to allow group comparisons and to avoid any bias in the conclusions. The fatty acid profile of the HFD was designed to meet French dietary guidelines in terms of fatty acid ratios and set a reference point for the fatty acid content (Table 3). To test whether one fatty acid class may better activate GPR40 than another, selective enrichments of the diet were designed to produce saturated, omega-3, -6 and -9 high fat diets (Table 2).

**Bone mineral density analysis.** After removing soft tissues, left femurs were placed in PBS with 10% formaldehyde at 4°C for one week. Bone mineral density was measured using an eXplore CT 120 scanner (GE Healthcare, Canada). Acquisitions were performed with X-ray tube settings at 100 kV and 50 mA. We limited our investigation to the trabecular region of the distal metaphysis of the left femurs. ROI was set at 2000μm height. Measurements started 300μm in the proximal direction from the growth plate.

**Body mass and composition.** Mice were weighed every week throughout the experimental period and subjected to body composition analysis at the beginning and at the end of the protocol using QMR EchoMRI- 900™ system. Whole body fat and lean (excluding bone mass) mass were measured in living animals with no need of sedation.
**Tissue weight analysis.** Mice total body weight was measured weekly throughout the study. Spleen and uterus were collected from mice and weighed immediately after sacrifice.

**Biochemical parameters.** Blood samples from 14 week old mice were collected in tubes for serum chemistry, and centrifuged. Serum was isolated, aliquoted and stored at -80°C. OPG (osteoprotegerin), RANKL (receptor activator of nuclear factor kappa-B ligand) and MCP-1 (monocyte chemoattractant protein 1) were measured by Quantikine ELISA for mouse (R&D Systems Europe).

**Statistics.** The experimental procedure was conducted with a total of 12 mice per group. The 16 groups were processed at the same time but Ctrl and HFD groups (for a total of 8 groups; see table 1) were investigated first and were the only groups fully investigated for all parameters. Statistics were performed to either investigate the role of the genotype (GPR40+/+ vs GPR40−/−), the role of the ovariectomy (Ctrl-SH vs Ctrl-OVX), the role the diet (Ctrl-SH vs Ctrl-HFD) or the role of the diet when mice were ovariectomized (HFD-SH vs HFD-OVX). Then, the obtained data were analyzed via a multivariate ANOVA followed by a Tukey’s test. Data are presented as mean ± SD. (ExcelStat Pro software - Microsoft Office 2007). Groups with significant differences (p<0.05) are indicated with different letters or (*).

### 3. RESULTS:

#### 3.1. Validation of the bone loss model

To validate ovariectomy-induced oestrogen deficiency, mice uteri were weighed and checked for atrophy. As expected, uterus mass of the ovariectomized animals was significantly lower than those isolated from sham-operated females (Figure 2). Consistently, bone mineral density (BMD) was significantly decreased in Ctrl-OVX-GPR40+/+ mice compared to Ctrl-SH-GPR40+/+ (Figure 3A). In
addition, as previously described, BMD in \textit{GPR40}^{-/-} mice was significantly lower than in \textit{GPR40}^{+/+} wild-type littermates (Figure 3A), thus validating the consistency of both the OVX and the \textit{GPR40}^{-/-} mouse models, and allowing further investigation.

3.2. \textit{GPR40} limits bone loss induced by ovariectomy upon high fat diet

As shown in figure 3B, HFD-induced bone loss in \textit{HFD-SH-GPR40}^{+/+} mice but OVX failed to further impact BMD in \textit{HFD-OVX-GPR40}^{+/+}mice. In contrast, the absence of \textit{GPR40} revealed an additional BMD decrease in \textit{HFD-OVX-GPR40}^{-/-} mice (Figure 3C). These data suggest that, when stimulated by fatty acid enriched diets, \textit{GPR40} contributes to counteract ovariectomy-induced bone alteration.

3.3. The protective effect of \textit{GPR40} is not related to mechanical protection

To ensure that \textit{GPR40} protective effects were not related to weight gain and subsequent mechanical loading due to a high fat diet, mice weight was monitored weekly throughout the experiment. Only ovariectomy significantly increased animals’ weight, whereas diet or genotype had no, or little effect on weight gain, suggesting that the absence of further bone loss in \textit{HFD-OVX-GPR40}^{+/+} is not related to a modulation of the mechanical loading (Figure 4A).

3.4. The absence of \textit{GPR40} leads to higher fat/lean mass ratio

EchoMRI data reveal that ovariectomy exerted a significant influence on body composition pattern with a decreased lean mass and an increased fat mass (Figure 4 C, D, E). HFD had no particular effect on body composition, except in the absence of \textit{GPR40}. Indeed, \textit{HFD-SH-GPR40}^{-/-} mice showed a greater percentage of fat, while HFD failed to induce such a gain in \textit{HFD-SH-GPR40}^{+/+} mice (Figure 4D and E). This difference reveals that \textit{GPR40} may contribute to limit diet-induced fat gain and adipose tissue hypertrophy.
3.5. High fat diet causes increased systemic inflammation

To elucidate the role of HFD on bone behaviour we questioned its impact on inflammatory status. Mice fed with the high fat diet exhibited both higher spleen weight and MCP-1 level in the serum \((p<0.0264 \text{ and } p<0.0001\), respectively) independently of the oestrogen status or genotype (Figure 5A and B second panels). These results support a global negative impact of the HFD on inflammatory parameters that may contribute to explain, at least in part, the subsequent loss of bone mineral density observed in HFD-SH mice. Ovariectomy or genotype had no significant impact on systemic inflammatory status.

3.6. Inflammation in adipose tissue is increased by ovariectomy and enhanced in the absence of GPR40

As aforementioned, ovariectomy did not significantly impact the systemic inflammatory status. However, locally, it increased the production of the pro-inflammatory cytokine MCP-1 by the inguinal adipose tissue (Figure 5C; left and right panels). In contrast, the diet was ineffective. Regarding genotype influence, the absence of GPR40 mostly increased the production of TNFα. Interestingly, when mice were ovariectomized and fed a high fat diet, the absence of GPR40 led to a massive increase of both TNFα and MCP-1 cytokines, supporting an anti-inflammatory role for GPR40 at the adipose tissue level.

3.7. The absence of GPR40 leads to a greater production of RANKL

The analysis of OPG and RANKL expression, two major cytokines involved in bone tissue homeostasis, reveals that BMD level parallels OPG/RANKL ratio. These data suggest a key role for this molecular cross-talk between osteoblasts and osteoclasts in mediating ovariectomy, diet and genotype influences on BMD (Figure 6C; all panels). Interestingly, while no or little impact on OPG was observed in the HFD-OVX-GPR40\(^{-/-}\) group, the absence of GPR40 led to a remarkably increased
level of RANKL (Figure 6A and C; right panels). This increase correlates with the observed alteration of bone and strongly supports that HFD no longer limits a greater bone resorbing cell differentiation in the absence of GPR40.

3.8. GPR40 bone sparing effect may rely on stimulation by saturated fatty acids

Since we demonstrated the role of GPR40 in mediating HFD positive influence in OVX-induced bone loss, we questioned the specificity on the receptor. The high-fat diet combines different fatty acid groups. In order to decipher which one of these could be responsible for GPR40-related protection, fatty acid mixture composition was revised to allow diet enrichment either with saturated, ω-3, ω-6 or ω-9 fatty acids. Only the diet rich in saturated fatty acid showed similar effects to the so called HFD diet while diets rich in ω-3, ω-6 or ω-9 failed to promote GPR40-related protection of bone tissue (Figure 7). These data support a specificity for GPR40 towards saturated fatty acids that has never been reported before.

4. DISCUSSION / CONCLUSION

In this study we demonstrated for the first time that GPR40 contributes to limit ovariectomy-induced bone loss when mice were fed a high fat diet. Limitation of bone loss in this model was associated with both a restrained production of pro-inflammatory cytokines by the white adipose tissue and a controlled level of RANKL. GPR40-related protection of bone tissue was dependent on the presence of saturated fatty acids. These results are consistent with previous published data obtained with the synthetic agonist GW9508.

We also confirmed that mice lacking GPR40 exhibited osteoporotic features [19]. As expected, oestrogen deficiency following ovariectomy was confirmed by uterine horns atrophy and
led to bone loss independently of the diet or the genotype [23, 24]. Then, we provided evidence that high fat diet decreased bone mineral density in HFD-SH-GPR40+/− mice. This deleterious impact of the diet on bone parallels previous studies from Le’s and Faria’s groups showing that bone loss upon high fat diet was associated with an unbalanced osteoblast/osteoclast coupling and an increased of inflammatory status [25, 26]. In our hands, high fat diet increased MCP-1 level and spleen weight. Alteration of the OPG/RANKL ratio by the diet was also observed. Besides, bone loss observed in HFD-SH-GPR40+/− mice occurred in the absence of weight gain supporting a metabolic deleterious effect of the diet uncovered by the absence of mechanical loading [27].

Interestingly, ovariectomy failed to further impact BMD in HFD-OVX-GPR40+/− mice, while additional bone loss was observed in HFD-OVX-GPR40+/− animals. This data support a positive role for GPR40 in bone sparing effect and strongly suggest that, when stimulated by fatty acid enriched diets GPR40 contributes to counteract ovariectomy-induced bone alteration.

Ovariectomy-induced bone loss mainly relies on a rapid increase of osteoclast activity [28]. Besides, both osteoclast activity and differentiation are stimulated by pro-inflammatory conditions [29]. The presence or the absence of GPR40 did not significantly impact circulating level of MCP-1 or spleen weight. Therefore, while inflammation induced by high fat diet may account for the bone loss observed, the limitation of the OVX-induced bone loss by GPR40 cannot be explained by a modulation of the systemic inflammatory status. At the tissue level, ovariectomy increased TNFα and MCP-1 expression by the inguinal white adipose tissue. This increase was enhanced by the absence of GPR40 and further strengthened when combined with HFD in the HFD-OVX-GPR40−/− group. MCP-1 released by adipocytes contributes to recruit macrophages that enhance a local inflammatory vicious cycle. Interestingly, this local macrophage recruitment may involve specific populations such as Ly6C monocytes that have the ability to migrate from bone marrow to inflammation sites, get activated and then return to bone marrow where they may contribute to initiate or enhance an inflammatory context with deleterious effect on bone health [30]. This
concept is of particular interest and may explain, at least in part, the seemingly conflicting results
between the systemic and local level of MCP-1 in line with the role of GPR40 in both adipose and
bone tissues [30]. Furthermore, these results parallel the fat gain observed in the OVX mice and
strongly support that OVX and diet act in concert to increase the local production of pro-
inflammatory cytokines by the adipose tissue while the presence of GPR40 limits it.

To further decipher the mechanism of action, the modulation of the OPG/RANKL ratio was
assessed. Consistently, OPG/RANKL ratio was significantly decreased in HFD-OVX-GPR40+/−. This
unbalanced ratio was driven by an increase in RANKL production while OPG was unaffected.
According to both, the role of RANKL in osteoclast differentiation and our previous published data
[19] regarding the bone phenotype of the GPR40+/− mice, the additional decrease of bone mass
observed in HFD-OVX-GPR40+/− animals may originate from an uncovered inhibition of bone
resorption-mediated by fatty acids and GPR40 interaction. Although Gao et al. recently
demonstrated that GPR40 may promote osteogenic differentiation through Wnt/β-catenin pathway
[31], in our hands osteoblasts were only weakly affected in vivo by GPR40 [32]. This further supports
the paramount role of the osteoclast in this story [20].

To sum up the results, our data suggest that a diet enriched with saturated fatty acids leads
to decreased BMD but in a limited way due to GPR40 stimulation by fatty acids. The positive role of
GPR40 in both adipose and bone tissues contributes to counter ovariectomy-induced bone loss
(Figure 8). Regarding fatty acid specificity, GPR40 was first described as a fatty acid receptor binding
medium to long chain fatty acids independently of their degree of saturation [6, 7, 13]. Here we
demonstrate that GPR40-related bone protection seems to be preferentially mediated by saturated
fatty acids. Despite this contrast with the literature, it is worth noting that just like GPR40, GPR120
was first described as a receptor for medium to long chain fatty acids lacking specificity before Oh’s
group evidenced it as a DHA sensor [33]. Besides, our data parallel those from Cornish’s group [34].
They demonstrated that saturated fatty acids (C14:0 to C18:0) inhibited osteoclastogenesis in bone

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marrow cultures and RAW264.7 cells expressing GPR40. Still, consistent with our results regarding the influence of diets either enriched in ω-3, ω-6 or ω-9, they found that the introduction of double bonds abrogated the inhibition of osteoclastogenesis with comparable low activity between ω3 and ω6 fatty acids.

In a context of increasing prevalence of obesity in occidental countries, it appears difficult from a nutritional view point to encourage long chain saturated fat intake. Therefore, even if stimulation of the GPR40 receptor by saturated fatty acids contributes to limit excessive bone loss, this study may remain of mechanistic interest and our results rather support the relevance of pharmacologic approaches. Nevertheless, it is worth noting that GPR40 also binds medium chain saturated fatty acids such as myristic acid that have been shown to limit the prevalence of metabolic complication [35, 36]. The relevance of such a class of lipids regarding bone health prevention remains to be investigated.

5. REFERENCES


[26] Xiao, Y., Cui, J., Li, Y. X., Shi, Y. H., Le, G. W., Expression of genes associated with bone resorption is increased and bone formation is decreased in mice fed a high-fat diet. *Lipids* 2010, 45, 345-355.


**ACKNOWLEDGEMENTS**

CP, FW, VC and YW designed the study and wrote the manuscript.

GR, JS and EMN were in charge of the bone investigation.

JF and LB were in charge of the adipose tissue investigation.

MA and FRJ were in charge of the insulin sensitivity.

AB was in charge of the microbiota investigation.

Many thanks to all the people that contributed to the ovariectomy procedures, the diet delivery, the tissue sampling.

Thanks to Vincent Poitout and AMGEN for providing us with the GPR40 mouse strain.

Thanks to Lisa Nucke for English grammar and syntax improvement.

**DISCLOSURE**

There is no conflict of interest to declare.
FIGURE LEGENDS

Figure 1. Scheme of experimental protocol design (A) and presentation of the food distribution mode (B).

Acclimatization period (weeks 4 to 7); On week 7, mice were randomized into 16 groups (n=12, individual cage) and provided with their specific diet (6 different diets; 2 different genotypes; see table 1 and 2); On week 9 mice were ovariectomized or sham-operated to induce bone loss. Diets were delivered *ad libitum* for a total of 7 weeks including 5 weeks post-surgery.

Figure 2. Ovariectomy validation. A and B : Weight analysis for uterine horns. Significantly different groups are represented by *** (p<0.0001) as analysed via a multivariate ANOVA followed by a Tukey’s test. (Ctrl : Control diet, HFD : High-fat diet, SH : Sham-operated mice, OVX : Ovariectomized mice). Data are presented as mean ± SD.

Figure 3. Bone parameters. A and B : Bone mineral density. Significantly different groups are represented by *** (p<0.0001) or ** (p<0.01) as analysed via a multivariate ANOVA followed by a Tukey’s test. (Ctrl : Control diet, HFD : High-fat diet, SH : Sham-operated mice, OVX : Ovariectomized mice). Data are presented as mean ± SD.

Figure 4. Weight, food intake and body composition parameters. Total body weight gain over the complete study of *GPR40*+/+ and *GPR40*−/− mice (A). Food intake over the complete study of *GPR40*+/+ and *GPR40*−/− mice (B). Percentage of *GPR40*+/+ and *GPR40*−/− lean gain over the complete study (C), percentage of *GPR40*+/+ and *GPR40*−/− fat gain over the complete study (D), and ratio of *GPR40*+/+ and *GPR40*−/− lean/fat over the complete study (E). Significantly different groups are represented by * (p<0.05) compared to control as analysed via a multivariate ANOVA followed by a Tukey’s test. (Ctrl : Control diet, HFD : High-fat diet, SH : Sham-operated mice, OVX : Ovariectomized mice). Data are presented as mean ± SD.
Figure 5. Inflammation parameters. A: Monocyte chemotactic protein 1 (MCP-1) level analysis in blood samples. B: Spleen weight measurement. C: MCP-1 mRNA relative expression in white adipose tissue. D: TNF-α mRNA relative expression in white adipose tissue. Significantly different groups are represented by different letters as analysed via a multivariate ANOVA followed by a Tukey’s test (p<0.05). (Ctrl: Control diet, HFD: High-fat diet, SH: Sham-operated mice, OVX: Ovariectomized mice). Data are presented as mean ± SD.

Figure 6: OPG and RANKL serum levels. A and B: osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) levels in blood samples. C: OPG/RANKL levels ratio. Significant different groups are represented by different letters as analysed via a multivariate ANOVA followed by a Tukey’s test. (p<0.05). (Ctrl: Control diet, HFD: High-fat diet, SH: Sham-operated mice, OVX: Ovariectomized mice). Data are presented as mean ± SD.

Figure 7: Bone mineral density of wild-type and knock-out GPR40 mice depending on fatty acid class-enriched diets.

Significant different groups are represented by different letters as analysed via a multivariate ANOVA followed by a Tukey’s test. Data are presented as mean ± SD.

Figure 8: Conclusion

GPR40 contributes to counteract ovariectomy-induced bone alteration in the presence of fatty acid enriched diets. The sparing effect is supported by the modulation of the OPG/RANKL ratio in blood stream and the expression level of inflammatory markers in local adipose tissues. The importance of GPR40 in osteoclastogenesis have been previously described.
Figure 1: Scheme of experimental protocol design (A) and presentation of the food distribution mode (B)
Figure 2: Ovariectomy validation

A

B

Figure 3: Bone parameters

A

B

C
Figure 4: Weight, food intakes and body composition parameters

A. GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

- **Mice weight (g):**
  - GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

B. GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

- **Food intakes (g/kg/day):**
  - GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

C. GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

- **% Lean:**
  - GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

D. GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

- **% Fat:**
  - GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

E. GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>

- **Ratio Lean/Fat:**
  - GPR40<sup>+/+</sup> vs. GPR40<sup>−/−</sup>
Figure 5: Inflammation parameters

A

B

C

D

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Figure 6: OPG and RANKL serum levels
Figure 7: Bone mineral density of wild-type and knock-out GPR40 mice in function of fatty acid class-enriched diets.
Table 1: Distribution of the groups

<table>
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Table 2: Composition of high-fat diets

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<td>Sucrose</td>
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<td>Dextrin (maltodextrin)</td>
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<tr>
<td>Cellulose</td>
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<tr>
<td>Corn oil</td>
<td>7.00</td>
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<tr>
<td>Rapeseed oil</td>
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<td>6.72</td>
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<tr>
<td>Sunflower oil</td>
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<tr>
<td>Fish oil concentrated 22/60 TG (EPA/DHA)</td>
<td>-</td>
<td>0.48</td>
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<tr>
<td>Fish oil Omegavie DHA 70 TG QS ICE</td>
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<td>-</td>
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<td>Olive oil</td>
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<td>Lard</td>
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<td>16.80</td>
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<tr>
<td>L-cystine</td>
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<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
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<tr>
<td>Mineral Mix (AIN-93M) Ca normal (0.5% per kg of diet)</td>
<td>3.50</td>
<td>3.50</td>
<td>3.50</td>
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<tr>
<td>Vitamins (AIN-93M) Normal vitD (1000 UI diet)</td>
<td>1.00</td>
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<tr>
<td>t-butyl-hydroxyquinon</td>
<td>0.008</td>
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<tr>
<td>Corn starch (by difference)*</td>
<td>43.56</td>
<td>26.56</td>
<td>26.56</td>
<td>26.56</td>
<td>26.56</td>
<td>26.56</td>
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<tr>
<td>VERIFICATION</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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Table 3. Validation of fatty acid composition.

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<tr>
<th>Fatty acid</th>
<th>Control %</th>
<th>HFD %</th>
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<tr>
<td>C12:0</td>
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<td>0,18</td>
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<tr>
<td>C14:0</td>
<td>0,24</td>
<td>1,95</td>
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<tr>
<td>C16:0</td>
<td>15,29</td>
<td>25,71</td>
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<tr>
<td>C16:1 n-9</td>
<td>-</td>
<td>0,32</td>
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<tr>
<td>C16:1 n-7</td>
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<td>2,41</td>
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<tr>
<td>C17:0</td>
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<td>0,23</td>
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<tr>
<td>C17:1</td>
<td>-</td>
<td>0,2</td>
</tr>
<tr>
<td>C18:0</td>
<td>1,54</td>
<td>9,12</td>
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<tr>
<td>C18:1 n-9</td>
<td>27,07</td>
<td>36,51</td>
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<tr>
<td>C18:1n-7</td>
<td>-</td>
<td>5,98</td>
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<tr>
<td>C18:2 n-6 cis</td>
<td>55,04</td>
<td>12,91</td>
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<tr>
<td>C20:1 n-9</td>
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<td>0,34</td>
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<tr>
<td>C18:3 n-3</td>
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<td>3,07</td>
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<tr>
<td>C20:5 n-3</td>
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<td>0,6</td>
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<tr>
<td>C22:6 n-3</td>
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<td>0,48</td>
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<tr>
<td>VERIFICATION</td>
<td>100%</td>
<td>99,53%</td>
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</table>