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# The Effects of High Fat, Low Carbohydrate and Low Fat, High Carbohydrate Diets on Tumor Necrosis Factor Superfamily Proteins and Proinflammatory Cytokines in C57Bl/6 Mice

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## ABSTRACT

There has been considerable inconsistency regarding the potential relationship between dyslipidemia and bone metabolism. The inflammatory stimulation through the receptor activator of the nuclear factor kappa-B ligand (RANKL)/ receptor activator of the nuclear factor kappa-B (RANK)/ osteoprotegerin (OPG) pathway could be the infrastructural mechanism for hypercholesterolemia-induced bone loss.

In this study, we investigated the effect of dyslipidemia on RANKL and OPG alongside with pro-inflammatory cytokines. Thirty male C57Bl/6 mice (4 weeks old) were randomized to two purified diet groups (15 animals in each group), high fat, low carbohydrate diet (HFLCD) and its matched low fat, high carbohydrate diet (LFHCD). After 12 weeks of feeding in standard situations, the plasma concentration of lipid profile, interleukin (IL)1Beta, IL-6, tumor necrosis factor-alpha (TNF-α) and RANKL, OPG, and RANKL: OPG ratio were measured.

In the present study, although the body weight significantly increased during 12 weeks in HFLCD and LFHCD groups, there were no significant differences in food intake, food efficiency ratio and weight gain between the two groups. The LFHCD group had significantly higher median RANKL and RANKL/OPG ratio. There was no significant difference in plasma IL-1β, IL-6 and TNF-α concentration between LFHCD and HFLCD groups.

These unexpected findings from LFHCD, that seem to be as a result of its higher carbohydrate proportion in comparison to HFLCD, implicate dietary carbohydrate rather than dietary fat as a more significant nutritional factor contributing to change in RANKL level and RANKL: OPG ratio.

**Keywords:** Bone; Cytokines; Diet; Dyslipidemia; Fat; RANKL

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## INTRODUCTION

Bone is considered as a metabolically active tissue because of bone remodeling, which controls tissue



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integrity and body homeostasis.[1] The dynamic process of bone remodeling requires interaction of many cell lineages including osteoblasts, osteoclasts and secondary cell types. Osteoblasts are responsible for bone formation and are driven from bone marrow stromal cells. Osteoclasts are specialized in calcified matrix resorption and are driven from hematopoietic precursor cells. Secondary cell types such as endothelial cells and monocytes/macrophages contribute to bone turnover either by direct contact with osteogenic cells or by the secretion of cytokines or growth factors.[2] Moreover, recent studies illustrated that the immune system also plays a significant role in bone metabolism.[3] Indeed, the concept of osteoimmunology which was established more than a decade ago, was coined to focus on the reciprocal interaction between immune system and bone cells.[4,5]

The intricate bidirectional interactions between the immune and skeletal systems involve cytokines, receptors, signaling molecules and transcription factors and crosstalk among cytokines is a core of interest for osteoimmunology.[6] The receptor activator of NF-κB ligand (RANKL) which belongs to tumor necrosis factor (TNF) superfamily, is a pivotal component of this interaction.[7] This protein was first demonstrated as a T lymphocyte surface marker that provided regulatory effects on immune cell functions.[8] However, later it was shown that RANKL is expressed by osteoblasts for inducing the differentiation, activation and survival of osteoclasts.[9-11] RANKL is critical for osteoclastogenesis and has been involved in the context of bone loss with osteoporosis.[7] Osteoblasts also produce a soluble receptor; osteoprotegerin (OPG) that blocks the RANKL which binds to its receptor, receptor activator of NFκB (RANK), and prevents bone resorption.[12] Other cytokines which seem to have contribution in bone metabolism are interleukin (IL)1Beta, IL-6 and tumor necrosis factor-alpha (TNF-α) which are mainly produced by macrophages. These pro-inflammatory cytokines promote osteoclastogenesis, and are also called osteolytic cytokines on the basis of their bone resorbing effect in vivo.[13,14]

One of the most important factors that affect the bone metabolism is nutrition and diet.[6] During the past decades, prevalence of overweight and obesity has increased throughout the world, a phenomenon called "globesity".[15] Dyslipidemia, a common medical complication of obesity, is also a common underlying factor for osteoporosis as suggested by several epidemiological and animal studies.[16-20] However, both in human and animal studies, there are still many controversies regarding the relationship between dyslipidemia and either the bone mass or the bone loss.[21,22] Therefore, the effects of diet-induced dyslipidemia on bone mass and its mechanisms is an important subject of research.[5]

Animal models are invaluable resources for research on the effects of diet on metabolism and disease. However, diversities in diets used in animal studies make the results difficult to compare and choosing the suitable regimens to induce dyslipidemia and matching it in control groups seems challenging.[22,23] The differences in nutrients and calories between case and control groups can explain some of the conflicting results in animal research findings.[23]

Therefore, in this study, which was a sex-, age- and weight-matched interventional study, we sought to compare the effects of a purified-ingredient high fat/ high cholesterol/ low carbohydrate diet (HFLCD) on plasma concentration of RANKL, OPG and pro-inflammatory cytokines to that of a matched purified-ingredient low fat/ low cholesterol/ high carbohydrate diet (LFHCD) in male C57BlL/6 mice.

## MATERIALS AND METHODS

### Mice

Thirty male C57Bl/6 mice, 4 weeks old with mean weight of 15.40±3.39 g, were obtained from lab animal science department of Pasteur Institute of Iran. After one week for acclimatization, mice were randomly assigned to two purified diet groups (15 animals/group), with no significant differences in mean weight between the groups. The animals were housed in individual reticulated cages, under controlled conditions of temperature (21±2°C) and humidity (55%) with natural light. The animal protocol for the study was approved by the animal ethics committee of the Department of Food Science and Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Since the changes in bone structure with age in these animals are similar to the changes in human, the C57Bl/6 mice were used as an animal model in this study.[24]

**Dietary Intervention**

Mice were randomized to two dietary groups. The experimental purified diets were formulated on an energy basis to have equal total calories (4128 kcal each) and equal nutrients to kcal ratios (Table 1). The differences between the high fat and low fat diets in this study were the fat, cholesterol and carbohydrate amounts. The experimental diets formula were: 1) LFHCD group, fed with D12337 (%carbohydrate (CHO): fat: protein= 69:11:20; source of CHO=Maltodextrin, Sucrose and Corn Starch; source of fat=Soybean Oil; %cholesterol 0; Cholic Acid 0), and 2) HFLCD group, fed with D12336 (%CHO: fat: protein=45:35:20; source of CHO=Maltodextrin, Sucrose and Corn Starch; source of fat=Soybean Oil, Cocoa Butter and Coconut Oil; %cholesterol 12.5; Cholic Acid 5gm%). The protein and micronutrients in these two diets were the same (Table 1). The experimental diets were formulated and provided by Research Diet Inc. NJ, USA. All mice were allowed to have free access to diets and drinking water for 12 weeks. The food intake, spillage and residual foods were measured weekly.

**Table 1. Purified high fat low carbohydrate diet (HFLCD-D12336); Purified low fat high carbohydrate diet (LFHCD-D12337)**

| Product# | D12336 HFLCD | | D12337 LFHCD | |
|---|---|---|---|---|
| | **gm%** | **Kcal%** | **gm%** | **Kcal%** |
| Protein | 21 | 20 | 18 | 20 |
| Carbohydrate | 46 | 45 | 64 | 69 |
| Fat | 16 | 35 | 4 | 11 |
| Saturated | 53.7 | | 14.2 | |
| Monounsaturated | 25.0 | | 24.3 | |
| Polyunsaturated | 21.3 | | 61.4 | |
| Energy(Kcal/gm) | 4.13 | | 3.68 | |
| Ingredient | **gm** | **Kcal** | **Gm** | **Kcal** |
| Casein | 75 | 300 | 75 | 300 |
| Soy Protein | 130 | 520 | 130 | 520 |
| DL-Methionine | 2 | 8 | 2 | 8 |
| Corn Starch | 275 | 1100 | 522.5 | 2090 |
| Maltodextrin | 150 | 600 | 150 | 600 |
| Sucrose | 30 | 120 | 30 | 120 |
| Cellulose, BW200 | 90 | 0 | 90 | 0 |
| Soybean Oil | 50 | 450 | 50 | 450 |
| Cocoa Butter | 75 | 675 | 0 | 0 |
| Coconut Oil, 76 | 35 | 315 | 0 | 0 |
| Mineral Mix S10001 | 35 | 0 | 35 | 0 |
| Calcium carbonate | 5.5 | 0 | 5.5 | 0 |
| Sodium chloride | 8 | 0 | 8 | 0 |
| Potassium Citrate | 10 | 0 | 10 | 0 |
| Vitamin Mix V10001 | 10 | 40 | 10 | 40 |
| Choline Bitartrate | 2 | 0 | 2 | 0 |
| Cholesterol | 12.5 | 0 | 0.3 | 0 |
| Sodium Cholic Acid | 5 | 0 | 0 | 0 |
| FD&C Red Dye#40 | 0.1 | 0 | 0 | 0 |
| FD&C Blue Dye#1 | 0 | 0 | 0.1 | 0 |
| Total | 1000.1 | 4128 | 1120.4 | 4128 |
| Selenium(mg/kg) | 0.16 | | 0.14 | |

Research Diets, Inc, NJ, USA

**Blood Sampling**

At the end of the study, mice were deprived of food for 12 hours through the night. They were weighed and after slightly anesthetized with CO2 gas, blood samples were collected from the heart by syringe into heparinized glass tubes and the amount of blood withdrawn was no more than 1% to 2% of the animal's body weight (e.g., 0.25-0.5 ml from a 25 g mouse). Mice were sacrificed by cervical subluxation. Plasma was isolated from blood samples after centrifugation (1500 × g for 10 min at 4°C) then frozen and stored at -80°C until further analyses.

**Plasma Analytic Procedures**

Our primary outcomes were:

Lipid Profile: Plasma levels of Triglyceride (TG), total cholesterol (TC) and HDL-C were determined using enzymatic colorimetry by TG, TC and HDL-C assay kits (Pars Azmoon Co., Tehran, Iran). LDL-C was determined using Friedwald's formula.[25]

TNF-superfamily proteins: Mouse RANKL and OPG were determined by ELISA method (sRANKL ELISA kit, KOMABIOTECH Inc., Gangseo-gu Seoul, Korea and Mouse OPG ELISA kit, Aviscera Bioscience Inc, CA, USA).

Pro-inflammatory cytokines: Plasma levels of the mouse cytokines were determined by Mouse TNF-α, IL-6 and IL-1ß ELISA kits (Koma Biotech Inc, Gangseo, and Seoul, Korea).

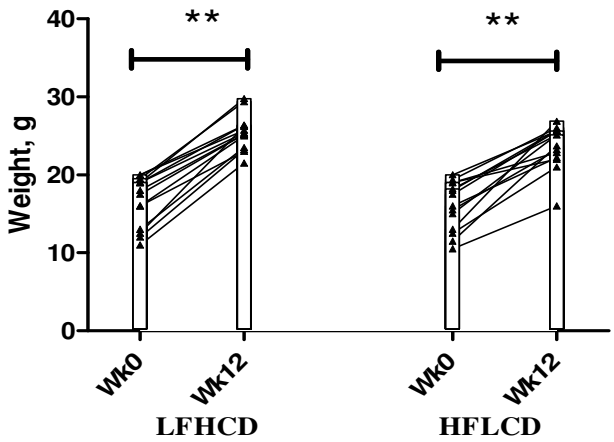
**Statistical Analysis**

As the distribution of primary outcome variables were not normal, we used the Non-parametric methods. Comparisons between the two groups were performed by Mann-Whitney U test. All statistical analyses were performed at 5% level of significance. Analyses were done using SPSS 20 (SPSS, Inc., Chicago, IL, USA) and figures were drawn using Graph-Pad Prism 5.

## RESULTS

**Food Intake, Weight Gain and Food Efficiency Ratio**

In the present study, there were no significant differences in food intake ($p$=0.35) and food efficiency ratio ($p$=0.96) between the two groups. The body weight increased significantly during the experimental period in both groups ($p$<0.001), but there were no significant differences in mean of weights after the intervention period between the two groups ($p$=0.74) (Table 2, Figure 1).




**LFHCD:low fat high carbohydrate diet (fat 11%, CHO 69%)**
**HFLCD:high fat low carbohydrate diet (fat 35%,CHO 46%)**

** P value <0.001

**Figure 1. The initial and final body weight in two groups (LFHCD, HFLCD)**

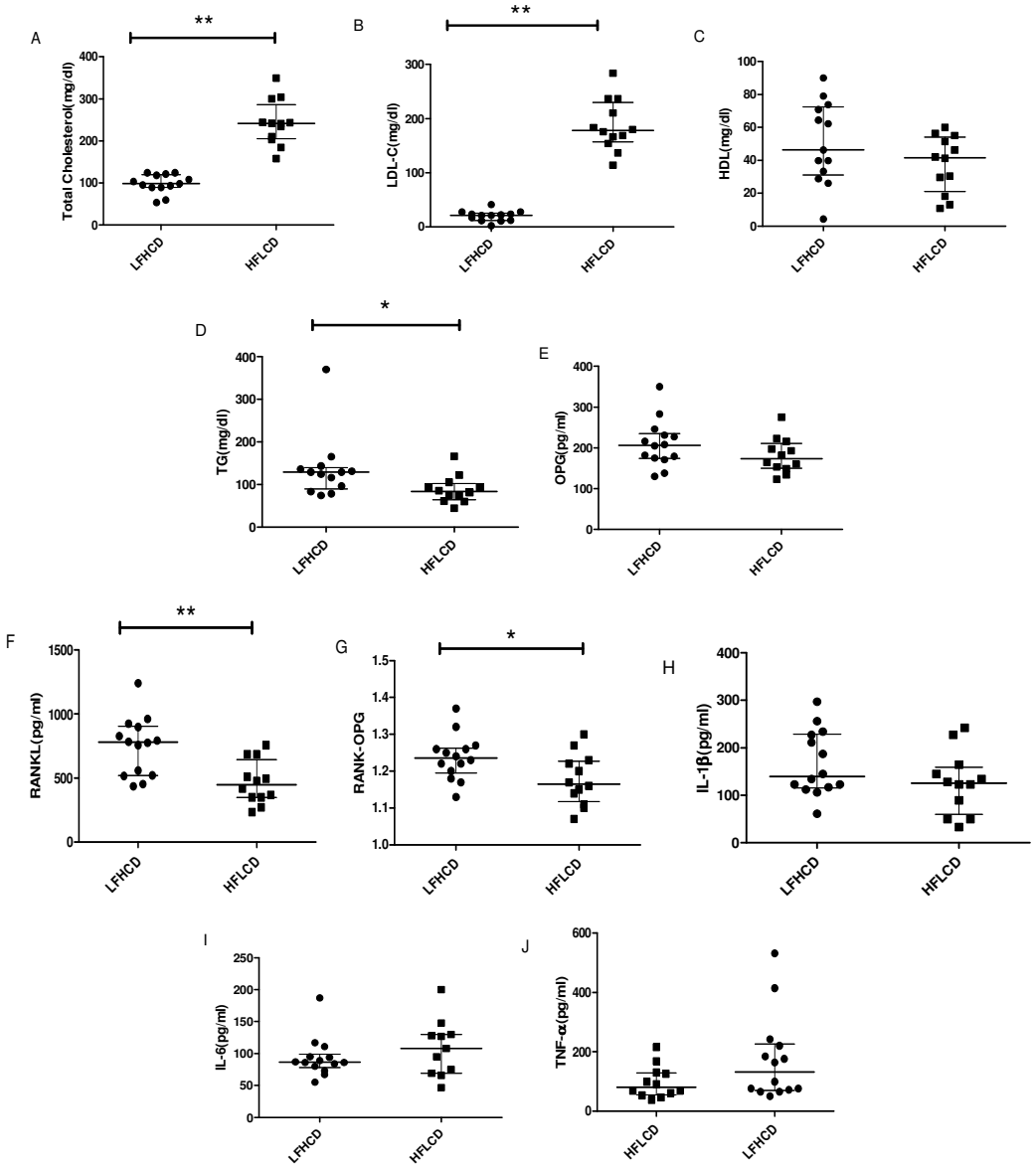



**Figure 2. Comparison of plasma lipid profile, plasma RANKL, OPG and RANKL:OPG ratio and pro-inflammatory cytokines (IL-1β, IL-6, TNFα) between the two groups. (A) Total Cholesterol, (B) LDL, (C) HDL, (D) TG, (E) OPG, (F) RANKL, (G) RANK:OPG, (H) IL-1β, (I) IL-6, (J) TNF-α.**

*$p$ value<0.05

** $p$ value <0.001

LFHCD: Low fat high carbohydrate diet (fat 11%, CHO 69%)

HFLCD: High fat low carbohydrate diet (fat 35%,CHO 46%)

TC = Total cholesterol; HDL-C = High density lipoprotein cholesterol;

LDL-C = Low density lipoprotein cholesterol; TG = Triacylglycerol

OPG = Osteoprotegerin; RANKL = Receptor activator of nuclear factor-κB ligand;

IL-1β = Interleukin 1β, IL-6 = Interleukin 6; TNF-α = Tumor necrosis factor α

**Table 2. The means of initial and final body weight, body weight gain, Food intake and food efficiency ratio.**

| Parameter | LFHCD mean(±SD) | HFLCD mean(±SD) | *P* value |
|---|---|---|---|
| Initial weight (g) | 16.70 ± 3.12 | 15.87 ± 3.14 | 0.92 |
| Final body weight (g) | 25.42 ± 2.28 | 23.64 ± 2.81 | 0.48 |
| Body weight gain (g) | 8.81 ± 2.33 | 7.56 ± 2.97 | 0.74 |
| Body weight gain (g/d) | 0.10 ± 0.03 | 0.09 ± 0.03 | 0.74 |
| Food intake (g/d) | 3.15 ± 0.20 | 3.36 ± 0.28 | 0.35 |
| Food efficiency ratio* | 3.31 ± 0.95 | 2.69 ± 1.15 | 0.96 |

LFHCD: Low fat high carbohydrate diet (fat 11%, CHO 69%)

HFLCD: High fat low carbohydrate diet (fat 35%, CHO 46%)

*Food Efficiency ratio = (body weight gain [g/d] /food intake [g/d]) ×100

**Plasma Lipid Profile and Plasma Level of RANKL, OPG, and Pro-inflammatory Cytokines**

After the intervention period, HFLCD group had significantly higher plasma levels of TC and LDL ($p$<0.001for both) and lower TG level in comparison to LFHCD group ($p$=0.01). There was no significant difference in mean of HDL-C between the two groups ($p$=0.22). Plasma RANKL concentration and RANKL:OPG ratio were significantly lower in HFLCD group compared to LFHCD group ($p$=0.001 and $p$=0.03). There were no significant differences in plasma IL-1β, IL-6 and TNF-α levels between the two groups ($p$=0.25, $p$=0.23 and $p$=0.10, respectively) (Figure 2).

## DISCUSSION

Because of the current emphasis on epidemiological analyses for showing associations between osteoporosis and dyslipidemia, the relation between lipid profile induced by high dietary fat intake and bone metabolism are of great importance. Although there were no significant differences in pro-inflammatory cytokine levels (IL-1β, IL-6 and TNF-α) between LFHCD and HFLCD groups in the present study, it was shown that mice fed with LFHCD had higher plasma RANKL concentration and RANK:OPG ratio in comparison to mice fed with HFLCD. Our findings seemed to be different from the previous lipid hypothesis of osteoporosis, which suggested that HFD increased the RANKL level in cell culture supernatants.[7]

OPG, RANK, and RANKL are common effectors of bone, immune and vascular systems. RANKL as a key mediator of osteoclast differentiation,[4] strongly induces osteoclastogenesis and bone resorption via its binding to RANK. Meanwhile OPG blocks RANKL/RANK as a decoy receptor and inhibits osteolysis. The OPG/RANK/RANKL triad is considered as an osteoimmunomodulator complex.[12] The results of RANKL plasma concentration and RANKL:OPG ratio in our study were not in agreement with animal studies which were conducted by Graham et al.,[25] Cao et al.,[26] and Xiao et al.[27] One possible explanation might be due to different diet compositions used in these studies. Graham et al., drew conclusion about dietary effects of fat on RNAKL plasma level from comparison of high fat defined diet with natural-ingredient chow diet. Since these two different types of diet have marked differences in micro and macronutrient content, the effects of the dietary fat maybe confounded with the effects of other compositions in the diet especially phytoestrogens.[22] Phytoestrogen that structurally resemble endogenous steroidal estrogens, have been documented to affect the bone metabolism.[28] Differences in sources and amount of dietary fat and carbohydrate may account for inconsistent findings of Cao and Xiao conducted studies and present study. Dietary carbohydrates and fats have an essential role in determining fatty acid composition of circulating lipid and cell membranes. Fatty acids can affect overall inflammatory balance by several mechanisms. Dietary carbohydrate influences immune system by acting as ligands for receptors or transcription factors that regulate different signaling cascades.[29] Volek et al., showed that the diet with very low carbohydrate provided a greater reduction in plasma saturated fatty acid (SFA) compared to the LFD.[30] SFAs activate receptor signaling leading to activation of NF-κB[31] which is one of the downstream targets of RANKL/RANK pathway and is critical for osteoclastogenesis.[12] Therefore, the higher RANKL and RANKL:OPG ratio in LFHCD group compared to

HFLCD might be due to the higher carbohydrate content of LFHCD in comparison to HFLCD that requires more studies in the future.

In this study, animals were fed with HFLCD and LFHCD, which both were purified and were formulated on an energy basis to match for total calories (4,128 kcals% each) and nutrient to kcal ratios (except the fat, carbohydrate and cholesterol levels). Results of this study revealed that there was no significant difference in weight gain and food frequency ratio between the two groups after 12 weeks which could be the confirmation of using the matched test diets by energy intake. The equal means of weight gain in HFLCD and LFHCD groups in our study was in agreement with Graham et al.[25] which may relate to equal energy intake in test diets and also to the presence of cholate in HFD. The cholate in HFD could be the cause of slow rate in weight gain.[32,33] However, our findings were not in agreement with Xiao et al.[34] and Yang et al.[20] It seems that reason of this discrepancy might be due to the significant differences in energy intake between HFD and control diets and also to the lack of cholic acid in HFD in Xiao et al and Yang et al. studies.[20,34]

Plasma lipid profile of mice (TC, LDL-C, TG and HDL-C) was measured 12 weeks after the intervention in HFLCD and LFHCD groups. The HFLCD-fed mice lipid profile showed increased concentrations of plasma TC and LDL-C that was in agreement with results of Graham et al.[25] and decreased TG concentrations compared to LFHCD-fed mice. TG finding was not in agreement with several animal studies[20,27,35] and this discrepancy might be related to the lower carbohydrate content in HFLCD vs LFHCD (46% vs 69%)[32] and the presence of cholic acid in HFLCD which could depress the TG levels by preventing hepatic TG synthesis.[33] There was no significant difference in HDL-C between HFLCD and LFHCD which was not in agreement with other animal studies except for Graham et al.[25] This controversy might result from differences in diets used in these studies.[20,27] We used purified diets in comparison to Yang et al., and Xiao et al., that used chow diets. The precise composition of chow diet as a grain-based diet is difficult to predict and chow diets can have variability from batch to batch. Chows are shown to have more unrefined ingredients with multiple nutrients and non-nutrients while purified diets that were used in this study had similar main nutrients. The other plausible reason might be due to higher amounts of grain-based diet phytoestrogen content in comparison to purified diets.[23]

At the end, there was no significant difference in plasma concentration of pro-inflammatory cytokine, IL-1β, IL-1 and TNF-α between LFHCD and HFLCD fed groups in this study. A reasonable explanation might be controlling the weight gain in HFLCD group. The obesity is associated with a state of chronic inflammation[36] and elevated production of proinflammatory cytokines.[37] Another possibility could be measuring these cytokines in plasma instead of measuring them in target tissue.

This study had some limitations. The duration of experiment could have been longer and the levels of inflammatory markers were measured in plasma. The measurement in bone microenvironment or supernatant of peripheral blood mononuclear cells might have different results. We suggest further studies to be performed focusing on the effect of diets with different carbohydrate content with equal energy on pro-inflammatory cytokine levels.

These data implicate dietary carbohydrate rather than dietary fat as a more significant nutritional factor contributing to affect on bone metabolism through change in RANKL level and RANKL:OPG ratio.

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