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Calcium and vitamin D: roles, interactions and nutritional programming for bone development

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Abstract: The contributions of calcium and vitamin D in optimal bone development have been widely studied in the biomedical field, including in rodent models, utilizing reference diets. Additionally, studies have emphasized the interrelation between calcium, vitamin D and other hormones as it pertains to osseous tissue function, as well as the role that signaling pathways play in bone synthesis and resorption. Recently, it has been reported in literature that rodent diets consisting of lower amounts of either calcium or vitamin D than the reference diets may lead to favorable bone growth. This review will describe the properties of calcium and vitamin D, their roles on bone formation, their interactions with parathyroid hormone, osteocalcin and the function of bone signaling pathways. Additionally, this review will outline the components of rodent reference diets and suggest possible dietary modifications that ensure healthy bone development.

Keywords: calcium; vitamin D; parathyroid hormone; osteocalcin; signaling pathways; AIN-93 diet

1. Introduction

Calcium and vitamin D are essential for optimal bone function, being involved in bone mineralization, bone mass development and prevention of osteoporosis and bone fractures [1,2]. Variations in diet levels, both of calcium and vitamin D, as studied in rodent models, can influence bone development, structure and bone mineral density [3]. This approach has the long term purpose of optimizing bone development in humans, by using dietary strategies. The AIN-93 diets are reference diets, developed in order to ensure optimal bone formation, with two formulations, AIN-93G and AIN-93M. During the development of the AIN-93 diets, feeding trials in rats and mice were conducted to determine the effects of each new formulation on weight gain, calcification of kidneys and bone mineralization [4,5]. This review presents a recent literature overview of the various roles of calcium, vitamin D and their interactions, the roles of parathyroid hormone, osteocalcin and signaling pathways in balancing bone formation and resorption at a cellular level, the effects reference animal diets have on osseous tissue. The review also aims to highlight potential improvements on dietary formulations, as well as insights into possible future research on the interconnection between diet and bone health.

2. Materials and Methods

In this review, we present an outline of the most noteworthy research regarding the role of calcium and vitamin D on bone development, factors that regulate the formation and resorption of bone tissue, as well as the effect of dietary changes and nutritional programming on bone health, as reported by in vivo studies on mice. We conducted a Medline/PubMed database search for pertinent articles by using certain keywords, namely "bone calcium", "vitamin D", "signaling pathways", "parathyroid hormone", "osteocalcin", "AIN-93G diet", either individually or in combinations. We analyzed the articles in accordance with their importance for our desired study and referenced those which met our criteria: the interactions between calcium and vitamin D, their dietary levels and effect on bone tissue development. English-language articles were exclusively included in this study; the majority of articles were published between 2000 and 2024, with some exceptions we were unable to omit in view of their significance.

3. The role of calcium and vitamin D on bone tissue and their interaction

3.1. Calcium

Calcium is an ion that plays a fundamental role in various physiological processes, both in its free form and bound to proteins [1,2]. Bound calcium is a key factor in skeletal mineralization, protection of skeletal integrity and preservation of bone mineral density. Calcium is also fundamental for processes such as coagulation, enzyme and hormone secretion, cellular differentiation, immune response, muscle contraction and release of neurotransmitters. The main site of total body calcium (98,9%) is the bone, where the mineral can take the form of calcium carbonates, calcium oxalates or calcium phosphates. The most commonly found form of calcium phosphate is hydroxyapatite, which has a predominant contribution to bone structure. In the skeleton, the function of calcium is as a stable deposit and, additionally, as a mobile resevoir. This mineral may also be found in cells (1%) and in the extracellular fluid (0,1%) as well. Serum calcium has a value range between ~8.8 and 10.4 mg/dl in healthy subjects and can be found as free ions (~51%), protein-bound compounds (~40%), and ionic compounds (~9%) [6]. Ionized calcium has a physiological value between 4.4 and 5.4 mg/dl in the serum. Albumin and globulin represent the most notable serum proteins that bind calcium, while calmodulin is a calcium-binding protein found intracellularly. Ionic compounds in serum can be found in the form of calcium phosphates, calcium carbonates, and calcium oxalates [1].

Optimal bone mass levels in adulthood are directly correlated with the accumulation of bone mass during childhood and adolescence [7]. Inadequate calcium accretion has been linked to elevated risk of bone fractures and osteoporosis [8,9].

Intestinal calcium absorption is achieved through transcellular and paracellular mechanisms. In transcellular uptake, Ca enters enterocytes through the transient Receptor Potential Vanilloid subfamily member 6 (TRPV6), a transmembrane Ca selective channel from the TRP family of cation channels and through calbindin D9k, a calcium-binding protein located intracellularly. TRPV6 and calbindin D9k levels are positively regulated by low Ca dietary administration and by vitamin D [10]. Hormones are involved in the modulation of intestinal Ca absorption as well. Estrogens and prolactin (PRL) play a role in the augmentation of TRPV6, and in the activity of vitamin D and prolactin may facilitate calbindin D9k action as well [11]. Conversely, the paracellular mechanism, with its lumen electrochemical gradient, is strongly reliant on the existence of adequate levels of vitamin D and/or intestinal membrane integrity [10,11].

3.2. Vitamin D

Vitamin D and its metabolites are steroid hormones and hormone precursors, that are either synthesized from ultraviolet B (UVB) induced photoconversion of 7-dehydrocholesterol to the form of previtamin D3 in the skin or obtained from dietary sources or supplements. Both previtamin D2 and D3 are biologically inactive and transform to the biologically active form of vitamin D, $1,25(OH)_2D_3$, in the liver and kidney through two hydroxylation processes. Vitamin D binding protein (VDBP) transports vitamin D, as well as its metabolites in the circulation, until they arrive at their target cells, when they dissociate from the VDBP and enter the cells [12-15]. Vitamin D plays a pivotal part in maintaining bone metabolism and calcium homeostasis [14]. Calcitriol, as the active form of vitamin D, aids calcium absorption and modulates calcium reabsorption by conjugation to the vitamin D receptors (VDR) found in osteoblasts and osteoclasts [16,17]. Conversely, in case of lowered serum calcium levels, VDRs in osteoblasts can intensify bone resorption, resulting in the mobilization of calcium from the bone sites into the bloodstream, in order to ensure serum calcium homeostasis [16,17]. VDRs are involved in signalling pathways, namely those linked to the regulation of bone and calcium homeostasis, inflammation, cellular immunity, cell development, and apoptosis [18].
A diagrammatic explanation of the role of the vitamin D hormone in mineralizing the skeleton and preventing hypocalcemic tetany is presented in **Figure 1** [19]. Vitamin D plays a role in the osteoblast production of receptor activator nuclear factor-κB ligand (RANKL), which in turn stimulates osteoclastogenesis and enhances bone resorption by activating resting osteoclasts [20]. Therefore, vitamin D, along with parathyroid hormone, facilitates the mobilization of calcium from bone, in case of its dietary absence [19].

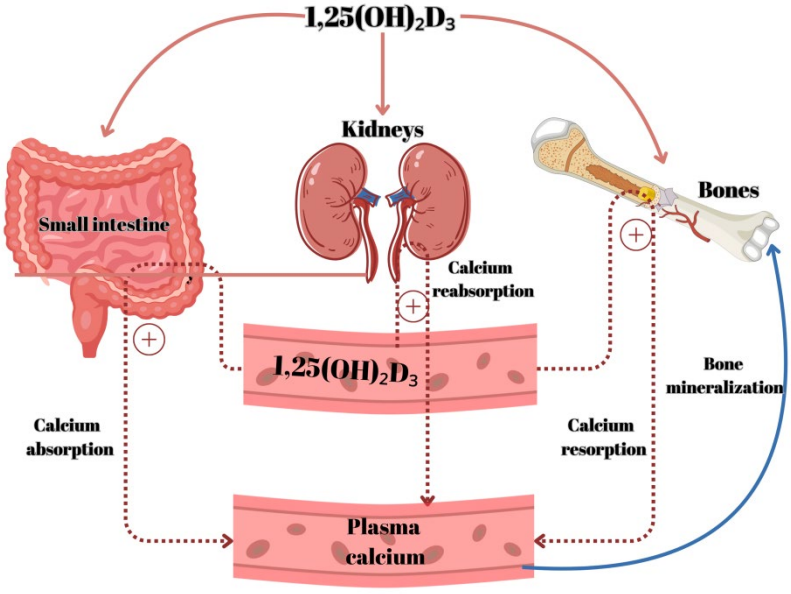

Figure 1. The role that vitamin D hormone plays in the increase of plasma calcium concentrations for skeleton mineralization and for the prevention of hypocalcemic tetany.

Vitamin D3 participates in the maintenance of calcium homeostasis most significantly by increasing intestinal calcium absorption, as revealed by studies in VDR null mice. Conditions such as rickets, osteomalacia, hypocalcemia, and hyperparathyroidism in VDR null mice were prevented as a consequence of the administration of a high calcium rescue diet, revealing that the skeletal abnormalities of VDR ablation are caused by impaired intestinal calcium absorption and subsequent hyperparathyroidism and hypophosphatemia [21,22]. Studies in transgenic mice with VDR expression limited to the in-

testine have provided direct evidence for the crucial part that vitamin D-mediated intestinal calcium absorption plays in bone homeostasis [23]. Intestinal VDR plays an essential role in the control of bone synthesis. Transgenic expression of VDR in the intestine of VDR null mice resulted both in the restoration of calcium homeostasis and in the prevention of the rachitic phenotype in VDR null mice [23]. In situations that require increased calcium for proper function, such as pregnancy, lactation or periods of intense growth, vitamin D3 increases active intestinal calcium absorption [14]. VDR deficiency is correlated with reduced intestinal absorption of calcium, subsequently leading to a decrease in bone mineralization [21,22]. In case the absorption of intestinal calcium cannot preserve physiological serum calcium levels, vitamin D and PTH will fulfil a synergistic role in osteoclastogenesis, leading to the elimination of from bones and to increase of calcium reabsorption from the distal convoluted tubules of the kidney [2].

Several other physiological effects of vitamin D have been discovered, namely in immune function, cardiovascular health, and metabolic health [17]. Vitamin D is mainly stored in adipocytes and plays a role in the regulation of adipose tissue formation [24-27]. Patients with non-alcoholic fatty liver disease (NAFLD) were found to more likely have reduced bone mineral density and vitamin D deficiency [28]. Vitamin D supplementation can lower the risk of insulin resistance and elevated blood glucose levels [29].

3.3. Parathyroid hormone (PTH)

Parathyroid hormone (PTH) is secreted by the parathyroid glands and plays an essential role in calcium and phosphate metabolism [30]. PTH interacts with G-protein and his receptor called the PTH/PTHrP receptor type 1 (PTH1R) present primarily on the surface of osteoblasts [31,32]. PTH mobilizes bone calcium into the blood and promotes osteogenesis and osseous resorption, thus ensuring the calcium homeostasis [17,30]. The catabolic role of PTH is indirectly exerted on osteoclasts through the OPG-RANKL-RANK pathway [33], while the direct anabolic effect is evidenced by working on PTH1R in osteoblasts and osteoclasts, therefore facilitating the differentiation of precursor cells [34-37] and inhibiting mature cell apoptosis [17,37]. PTH has been shown to promote bone synthesis in mice by increasing aerobic glycolysis in osteoblasts through transduction of the insulin-like growth factor signaling pathway, thereby enhancing the synthesis function of bone in mice [38].

The principal regulator of PTH release is Ca2+. Elevated Ca levels, detected by the Ca sensing receptor in parathyroid glands, leads to inhibited PTH secretion, while lower levels of Ca2+ in extracellular fluid results in the stimulation of the parathyroid gland to secrete PTH [30]. PTH subsequently stimulates the reabsorption of Ca2+ (along with magnesium) in the distal nephron, while the reabsorption of phosphate and bicarbonate is inhibited [39].

Bone tissue mineralization through proliferation of mesenchymal stem cells is the result of binding PTH to PTH1R on osteoblastic lineage cells, leading to the synthesis of the tumor necrosis factor-related cytokine, receptor activator of nuclear factor k-B (RANK) ligand (RANKL) and osteoprotegerin (OPG) and consequent bone resorption. Additionally, PTH influences the osteocyte action in the bone matrix, inhibits sclerostin release, thus ensuring new bone synthesis [40].

3.4. Osteocalcin (OCN)

Osteocalcin (OCN) represents the most abundant non-collagenous bone protein in bone, composed of a chain of 46-50 aminoacid residues [41]. Adult osteoblasts have a key role in the synthesis and expression of osteocalcin [42]. OCN gains an increased affinity to Ca^{2+} through the process of carboxylation of three vitamin K-dependent glutamic acids

and the subsequent formation of bone glutamic acid protein (gla protein) or γ-carboxylated glutamic acid Protein [41,42]. The storage of carboxylated osteocalcin in the bone tissue occurs as a result, while the control of bone remodeling and resorption by osteoclasts is achieved with the release of carboxylated osteocalcin from the osseous tissue [17]. Additionally, carboxylated OCN was found to block hydroxyapatite development in bone mineralization [43,44] and to act as a chemoattractant of osteoclast precursors [45]. Nevertheless, in vivo-studies conducted on OCN-deficient mice have reported differing phenotypes, exhibiting a notable increase in both cortical and trabecular bone, as well as enhanced bone synthesis and resorption [46]. Thus, osteocalcin was considered a bone matrix protein with the role of impeding osseous formation and resorption [17,42].

4. Bone signaling pathways - RANK, RANKL, OPG

The study of signaling pathways regulating osteoclast differentiation is crucial for a thorough understanding of the skeletal system in pathological conditions [47].
RANKL is a homotrimeric transmembrane protein from the tumor necrosis factor (TNF) superfamily expressed by osteoblasts, osteocytes, bone lining cells, immune and tumor cells, leading to the differentiation of osteoclasts [30,48]. Osteoblasts expressing RANKL as a membrane-associated cytokine play a crucial role in the differentiation of osteoclasts through RANK signaling [48].
Osteoprogerin (OPG) is a soluble RANKL decoy receptor secreted by osteoblasts and osteocytes, that belongs the TNF receptor superfamily [49,50]. OPG blocks the RANKL–RANK interaction, consequently inhibiting osteoclast differentiation and activation [30,48].
Bone-forming cells play a key part both in the stimulation and inhibition of osteoclastogenesis through the RANK-RANKL-OPG axis, under differing circumstances. The functions of the axis have been verified in vivo by using genetically modified mouse models and analyzing several human mutations [30,51,52].
Both RANK and RANKL promote the survival of osteoclasts and enhance their bone resorption role. In case of inhibited RANKL–RANK signaling, osteoclastic bone resorption is impeded, which can lead to increased osseous mass. Evidence of severe osteopetrosis and a lack of osteoclast differentiation has been found in mice deficient in both RANKL and RANK [53,54]. Conversely, increased adult stage osteoclastogenesis present in OPG-deficient mice may result in severe osteoporosis [55,56]. Bone synthesis is considered to be coupled to bone resorption, though the underlying mechanisms have not been conclusively elucidated either in vivo or in vitro. It has been found that OPG-deficient mice display a high bone turnover rate [15,57]. Osteoblast function has been reported to be directly activated by osteoclastic bone resorption, with not significant correlation to serum RANKL levels [58]. The reverse signaling theory has also been suggested, which hypothesizes that the osteoclast differentiation factor RANKL signal plays a key role in osteoblastic bone synthesis [59,60].

5. AIN-93 purified diets for laboratory rodents

Rodent models have been frequently utilized to analyze the influence of early life diet on bone mineral density (BMD) and bone structure, for the purpose of developing dietary strategies that ensure optimal bone development in humans [4,61]. The American Institute of Nutrition created the rodent diets AIN-76 and AIN-76A, that have been extensively used. However, numerous nutritional and technical problems with the diets have been encountered, thus they have been revised. Consequently, two new formulations were created: AIN-93G for growth, pregnancy and lactation, and AIN-93M for maintenance at the adult stage. Significant dietary ingredients were replaced or transformed, namely the form of carbohydrate, the fat quantity and form and the sulfur amino acid

supplement. For the AIN-93G diet, the recommended carbohydrate forms and amounts are 100 g/kg diet of sucrose; 132 g/kg diet of dextrinized cornstarch, 400 g/kg diet of cornstarch. Conversely, the recommendations for the AIN-93M diet are 100 g/kg diet of sucrose, 155 g/kg diet of dextrinized starch and 470 g/kg diet of cornstarch [5].

A change in the source of fat is advised mainly to reach an optimal quantity and balance between linoleic (n-6) and linolenic (n-3) essential fatty acids, thus 7 g soybean oil/100 g diet was replaced by 5 g corn oil/100 g diet, with the purpose of increasing the amount of linolenic acid. The recommended soybean oil quantity for the AIN-93G diet is 70 g/kg diet, both for males and females during periods of rapid growth and for adult female mice during reproduction and lactation. After the accelerated growth phase is finalized or when the animals are not going through their reproductive phase, it is recommended to decrease the quantity of soybean oil to 40 g/kg diet [5].

The recommended established high protein casein quantity for AIN-93G is 200 g/kg diet, contributing to ~17% protein. L-cystine at 3 g/kg diet, rather than methionine, is considered as the optimal supplement to the AIN-93 diets, because it is present in small quantity in casein. Upon completion of the phase of accelerated growth and in non-pregnant animals, the administration of the AIN-93M diet, consisting of 140 g casein/kg diet (12% protein) and 1.8 g L-cystine/kg diet, is optimal [5].

Notable changes in the mineral mix were additionally implemented, such as the decreased amount of phosphorus, the lowered amount of manganese, the form and amount of selenium, and the addition of trace amounts of silicon, fluoride, molybdenum, boron, nickel, lithium and vanadium. The addition of trace and ultratrace elements with significant, but circumstantial, evidence for essentiality was recommended. Changes in the vitamin mix included the form and the amount of vitamin E, vitamin K, vitamin B12, choline and the amount of vitamin fill [5].

The AIN-93 diets are characterized by an optimal balance of essential nutrients, therefore having the potential to be more beneficial, in comparison with the AIN-76A diet, both in short-term and long-term in-vivo studies on laboratory rodents. AIN-93G constitutes a stable reference diet, useful for comparing results within and between laboratories, thus confirming that dietary interventions, rather than the base diet changes lead to effects detected [5]. Utilizing early life diet to influence long term health outcomes is generally known as 'nutritional programming'. In developing the AIN-93 diets, feeding trials in rats and mice were conducted with the purpose of ascertaining the role that each new formulation played on weight gain, kidney calcification and bone mineralization [4].

Novel food components, namely soy isoflavones, are capable, during early life stages, to favorably influence bone structure, bone strength and bone mass density (BMD) in female mice in early adulthood [67-70]. However, the levels of vitamin D (vit D, 1000 IU/kg) and calcium (Ca, 5 g/kg) in the AIN-93G diet may be higher than the necessary values for optimal bone development, taking into account the BMD and bone structure in mice and rats [71-73]. It has been proven that normal osseous development, measured as BMD and biomechanical bone strength, is achievable with a significantly lower level of vitamin D (25 IU/kg) in mice administered an obesogenic diet, in female mice prone to inflammation or in healthy male mice [71,72,74]. In these studies, the dietary Ca level remained constant at 5 g/kg and the animals were administered diets from weaning until 3 [71,74] or 7 months of age [72]. In growing female Sprague-Dawley rats, Ca levels were modified from 1 to 7g/kg, both lower and higher levels than in the AIN-93G reference diet, while vitamin D levels were kept at 1000 IU/kg. After the 13 week feeding trial, BMD, biomechanical bone strength and bone structure were analyzed. Bone development was concluded to be optimal if Ca intakes were 2.5 g/kg or higher [73], lower than the 5 g/kg present in the AIN-93G diet. Nevertheless, the aforementioned studies have reported the results of modifying either the level of dietary vitamin D or Ca, thus the effect of decreasing both levels on bone structure and BMD has not been comprehensively evaluated. It has been proven that decreasing the Ca level to 2.5 g/kg diet, while maintaing the vitamin

D level of the reference diet, does not significantly influence bone health [73] and that the administration of 4 g Ca/kg diet without vitamin D, leads to no variations in serum Ca and PTH levels [75]. The reduction of the levels of both vitamin D and Ca could inhibit the compensatory mechanisms that may potentially conceal the effects of one low value, when the other nutrient is in higher amount than necessary. Moreover, the benefits of a dietary modification on bone development could go unnoticed because of possibly excessive amounts of vitamin D or Ca in the diet [4]. A study using a vitamin D level of 25 IU/kg diet in a CD-1 mouse model, has demonstrated no deleterious effects on BMD or bone strength [74], while another study has reported that bone mineral content (BMC) and BMD at the femur level were decreased at 25 IU/kg diet but not at 100 IU/kg diet, in comparison to the AIN-93G reference diet level (1000 IU/kg diet) [76]. Another study evaluated whether decreased amounts of Ca and vitamin D, in comparison to the ones of the AIN-93G diet, favored optimal bone development in female mice at 2 and 4 months of age. The study utilized three level of vitamin D diet: first, 100 IU/ kg diet, a level of vitamin D that was significantly lower compared to the reference diet, still likely to favor bone development, the AIN-93G reference diet, and an intermediate one of 400 IU vitamin D/kg diet [4]. Three different calcium levels were administered for each level of vitamin D (100 IU and 400 IU/kg diet). The lowest level of dietary Ca that was analyzed was 2.5 g/kg diet, based on the study that determined that calcium levels below 2.5 g Ca/kg diet lead to harmful effects on BMD, bone strength and femur structure in rats [77]. The study utilized in vivo micro-computed tomography (μCT) to analyze longitudinal bone development, bone structure and BMD for each mouse; osseous structures at mandibular and lumbar vertebra 4 (L4) sites were assessed ex vivo at 4 months of age and bone strength at the femur neck and midpoint was tested [4]. It was concluded that, as bone structure, bone strength and BMD of female CD-1 mice in the proposed diet were predominantly similar to those of the AIN-93G diet, the reference diet may be altered, in order to include Ca and vitamin D levels that are most favourable for bone growth and development and avoid excessive administration of both nutrients [4].

The estimated nutrient composition of the complete diets can be found in Table 1.

Table 1. Estimated minimal nutrient composition of AIN-93G and AIN-93M rodent diets.

Estimated minimal nutrient composition of AIN-93G rodent diet	
	units/kg diet
Total energy, kcal	3766,0
Carbohydrates (%)	64,0
Proteins (%)	19,3
Fats (%)	16,7
Moisture, g	66,0
Total fat, g	70,0
Saturated, g	10,8
Monounsaturated, g	16,3
Polyunsaturated, g	49,5
Linoleic acid, g	35,7
Linolenic acid, g	4,8
Total carbohydrate, g	643,7
Complex carbohydrates, g	360,1
Simple carbohydrates, g	236,1
Cellulose, g	47,5
Total protein, g	178,6
Amino acids	
Alanine, g	4,6
Arginine, g	6,4
Aspartic acid, g	12,2
Cystine, g	3,7
Glutamic acid, g	36,3
Glycine, g	3,2
Histidine, g	4,6
Isoleucine, g	8,5
Leucine, g	15,4
Lysine, g	13,0
Methionine, g	4,6
Phenylalanine, g	8,8
Proline, g	20,5
Serine, g	9,7
Threonine, g	6,7
Thryptophan, g	2,1
Tyrosine, g	9,3
Valine, g	10,0
Total ash, g	**41,7**
Minerals	
Calcium, mg	5000,0
Phosphorus, mg	3000,0
Magnesium, mg	513,0
Sodium, mg	1039,0
Potassium, mg	3600,0
Chloride, mg	1031,0
Sulfur (inorganic), mg	300,0
Iron, mg	45,0
Zinc, mg	38,0
Manganese, mg	10,0
Copper, mg	6,0
Iodine, mg	0,2
Silicon, mg	5,0
Chromium, mg	1,0
Selenium, mg	0,18
Molybdenum, mg	0,15
Fluoride, mg	1,0
Nickel, mg	0,5
Boron, mg	0,5
Lithium, mg	0,1
Vanadium, mg	0,1
Vitamins	
Nicotinic acid, mg	30.0
Calcium panthotenate, mg	15.0
Pyridoxine, mg	6.0
Thiamin, mg	5.0
Riboflavin, mg	6.0
Folic acid, mg	2.0
Biotin, mg	0.2
Vitamin B12, µg	25
Vitamin K, µg	900.0
Vitamin A, IU	4000.0
Vitamin D, IU	1000.0
Vitamin E, IU	75.0
Other nutrients	
Choline, mg	1000.0

6. Conclusions and future perspectives

In this review, the characteristics and interactions between calcium and vitamin D have been presented, their links to different hormones and signaling pathways, as well as the role of various dietary formulations on bone formation and bone health. In summary, there is potential to re-formulate the AIN-93G diet to incorporate modified levels of calcium and vitamin D that still promote optimal bone development. In the future, the evaluation of possible benefits of dietary interventions for optimising bone health, the analysis of dietary administration of calcium and vitamin D, at lower levels than the reference diet, could create interesting research topics. As reactions may be strain specific, research groups may take into consideration the evaluation of bone development at different levels of Ca and vit D in the strain mainly utilized for the research program, before studying a dietary intervention. It is the authors' belief that the fundamental role diet plays on the physiological processes of bone growth and development present vast opportunities for further research.

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