

MEETING REPORT

Report on recent vitamin D research (ASBMR 2012)

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Basic Biology of Vitamin D

1,25-Dihydroxyvitamin D and bone. Vitamin D is well known for its effects on promoting bone mineralization as the primary characteristic of rickets, and osteomalacia refers to decreased mineralization of the bone matrix. However, the direct effects of the vitamin D endocrine system on bone cells need further clarification. Earlier studies revealed that overexpression of VDR in osteoblasts (driven by osteocalcin promoter) increased bone mass and strength.¹ Deletion of VDR in osteocytes (using a *dmp1* cre system) did not influence bone mass or other parameters of bone metabolism.² Deletion of VDR in osteoblasts using an osteocalcin cre system, however, increased (>20%) bone mass (total and trabecular femoral bone, mainly by increase in the number of trabeculae) as reported by Anderson *et al.*³ and by S Kato (presented at the 2012 Endocrine Society Meeting as summarized in Bouillon⁴). However, the mechanisms involved are not clear, as preliminary data suggested increase bone formation (as reported by Anderson *et al.*³) and decreased bone resorption in the Japanese osteoblast-selective VDR mice. These observations imply that VDR activation can have beneficial effects (prevent rickets and osteomalacia) but can also impair bone formation or mineralization in case of exposure to very high serum concentrations of 1,25(OH)₂D.^{2,5} Such negative effects of vitamin D on mineral deposition were also observed by studying the gene expression profile after exposure to 1,25(OH)₂D (Pike's group, as reported in the previous *BoneKey* Report⁴) and now in a series of *in vitro* experiments using osteoblasts from different regions (calvaria and femur). Exposure to 1,25(OH)₂D could inhibit mineral deposition in early-stage calvaria-derived osteoblasts, whereas it enhanced mineral deposition in more mature cortical-bone-derived osteoblasts, at least in the presence of high extracellular calcium concentration.⁶ The direct effects of the vitamin D endocrine system on bone cells were also explored by analyzing mice with transgenic overexpression of *cyp27b1* in osteoblasts using an osteocalcin promoter region. At 20 weeks of age, such mice displayed a 10% increase in bone volume as well in the lumbar vertebrae as in the femoral metaphysis.⁷ This beneficial effect was due to increased bone formation with

unaltered bone resorption, indicating a direct bone anabolic effect of locally produced 1,25(OH)₂D in osteoblasts. All these new observations indicate that the vitamin D endocrine system has a yin/yang effect on bone: in most circumstances it enhances mineral deposition but in the presence of low calcium supply the vitamin D endocrine system tries to maintain serum calcium homeostasis at the expense of bone (increased resorption and decreased mineralization).

The vitamin D endocrine system started in the evolution of life on earth at the time of bony fish. This has now been studied in greater detail by exposing zebra fish to 1,25(OH)₂D and analyzing the whole transcriptome. A very large number of genes were identified in line with previous observations in mice and man (reviewed in Bouillon *et al.*⁸). Moreover, such genes were related not only to bone metabolism (such as RANKL) but also to energy and xenobiotic homeostasis,⁹ and these observations thus strengthen the idea of a wide spectrum of activities of the vitamin D endocrine system from early times onwards during the evolution of vertebrates.

24,25-Dihydroxyvitamin D. 24,25-Dihydroxyvitamin D is usually considered as a simple start of the vitamin D degradation pathway, but some older studies in chickens and growth-plate chondrocytes have suggested that it may have direct effects on bone and fracture repair.¹⁰ *cyp24a1* null mice have a high perinatal mortality, largely due to 1,25(OH)₂D-mediated hypercalcemia, as the half-life of 1,25(OH)₂D is markedly increased. Fracture repair was studied in such mice,¹¹ and endochondral bone formation, as observed in fixed long bone fractures, was markedly delayed, as shown by slower callus and collagen X formation, but fracture repair was ultimately normal. Intramembraneous bone formation was studied in such *cyp24a1* null mice by distraction osteogenesis and was also found to be delayed, but the difference did not reach significance. So it seems that 24,25-dihydroxyvitamin D has intrinsic biological effects on cartilage formation and fracture repair. The molecular mechanisms of such actions are still to be explored, although the same group reported already the existence of a specific membrane GPCR for this metabolite at

the vitamin D workshop in Houston preceding the 2012 Endocrine Society meeting.⁴

Vitamin D and the intestine. The intestine is the primary and non-redundant target for vitamin D's action on calcium and bone homeostasis. Classical textbooks state that the vitamin D endocrine system is responsible for enhancing active intestinal calcium transport, which occurs mainly in the duodenum and jejunum. This concept is now challenged by analyzing mice that express VDR only in the cecum and colon (and low expression in the ileum) but not in the proximal gut, nor in other tissues.¹² Such mice still had the expected alopecia but serum calcium and bone homeostasis were rescued (cortical bone mass and porosity were normal). They also did not have a ricketic growth plate. This demonstrates that the whole intestine is the target for the action of the vitamin D endocrine system and this may have implications for the handling of calcium in the intestine in patients with extensive bariatric surgery.

Extra renal *cyp27b1* expression and production of 1,25-dihydroxyvitamin D. The extra renal production of 1,25(OH)₂D is very well demonstrated in a large number of tissues and is under the control of tissue-specific regulatory factors, which differ from the ones operational in the kidney. This extra renal production of the vitamin D hormone is usually considered to have para- or autocrine functions, but this has not yet been firmly confirmed by tissue- or cell-specific deletion or transgenic expression. Parathyroid-specific deletion of VDR causes mild hyperparathyroidism with increased bone turnover,¹³ and this was confirmed by the same type of experiment in San Francisco.¹⁴ Parathyroid-specific deletion of the calcium sensing receptor caused the expected increase in serum calcium and parathyroid hormone (PTH) and a right shift of the calcium/PTH dose-response curve. The surprise came with parathyroid-specific deletion of *cyp27b1*, as such mice display the expected increase in PTH, but also an unexpected decrease (–30%) of serum 1,25(OH)₂D despite a several-fold increase in renal *cyp27b1* expression. Moreover, this low serum 1,25(OH)₂D has consequences for calcium homeostasis (decreased serum calcium and increased serum phosphate) and bone (increased trabecular volume and thickness).¹⁴ These experiments indicate that the parathyroid glands would contribute to the systemic production of 1,25(OH)₂D and thus question the 'dogma' of the kidney being the exclusive origin of this metabolite for the serum compartment. Indeed many previous experiments have amply demonstrated the absence of serum 1,25(OH)₂D after total nephrectomy in humans or experimental animal models (except when inflammatory diseases such as sarcoidosis are at the origin of extra renal 1,25(OH)₂D production). The set of data, however, is still controversial, as the high serum phosphate and bone abnormalities observed in these PTH-selective *cyp27b1* null mice are not in line with low serum 1,25(OH)₂D levels. Therefore, additional experiments, such as selective renal KO of *cyp27b1*, are needed to confirm or contradict the contribution of the parathyroid glands to the systemic delivery of 1,25(OH)₂D and thus of the exclusive renal origin of serum 1,25(OH)₂D.

FGF23 and production of 1,25-dihydroxyvitamin D. The vitamin D-activating enzyme *cyp27b1* is known to be stimulated by PTH in the kidney but not in extra renal tissues. FGF23,

however, is a known inhibitor of *cyp27b1* in the kidney, but also in some (heart, lung, aorta, testis) but not all (parathyroid gland?) extra renal tissues expressing this enzyme. This was revealed by studying the mRNA and protein expression of this enzyme in different tissues of FGF23 null mice treated or not with FGF23 injections.¹⁵ Moreover, the inhibitory effects of FGF23 could be blocked *in vitro* and *in vivo* by an ERK1/2 inhibitor, demonstrating that ERK1/2 is part of the second signaling pathway of FGF23. FGF23 null mice develop high 1,25(OH)₂D-mediated hypercalcemia and ectopic calcifications. The contribution of locally produced 1,25(OH)₂D by extra renal expression of *cyp27b1* was explored in such null mice,¹⁶ and ectopic calcification of the heart and the aorta was highly correlated with ectopic 1- α -hydroxylase activity. Therefore, the extra renal production of 1,25(OH)₂D may have both beneficial and deleterious effects. FGF23 is a well-known inhibitor of the renal 1- α -hydroxylase, but its role in normal physiology is less well documented. A synthetic pan-specific FGFR inhibitor (at least for PGFR 1-2-3) was used to treat the consequences of FGF23 excess-mediated rickets in *hyp* or *dmp1* null mice with remarkable good effects on calcium and bone homeostasis.¹⁷ Similarly, Brommage *et al.*¹⁸ reported that *dmp4* (the mouse ortholog of the *FAM20C* gene) null mice developed severe hypophosphatemia, reduced body length, a ricketic growth plate and reduced bone mass with excessive osteoid surface and thickness, all related to very high serum FGF23 concentrations. As *FAM20C* is a kinase for several osteoblast or osteocyte products related to mineralization (*dmp1*, *phex*, *osteopontin*, *bone sialoprotein*) and as FGFR signaling is involved, a pan FGFR inhibitor may help to combat other diseases related to Sibling proteins. When the same drug was given to control mice, a marked rise in serum 1,25(OH)₂D was observed, indicating that even normal levels of FGF23 are potent inhibitors of *cyp27b1*.

Skin synthesis of vitamin D. The skin is the major origin of vitamin D, and its production is highly dependent on UV-B, but also on the concentration of 7-dehydrocholesterol (7-DHC) and the 7-DHC-dehydrogenase enzyme. Therefore, it was not a surprise that serum 25(OH)D concentrations are influenced by polymorphisms of this enzyme.^{19,20} UV-B exposure could rapidly normalize both serum 25(OH)D and 1,25(OH)₂D in vitamin D-deficient female mice but not in male mice.²¹ A possible reason could be that vitamin D-deficient female (but not male) mice decreased their skin production of this enzyme and thereby facilitated the skin production of vitamin D. Indeed, transgenic overexpression of 7-DHC-dehydrogenase in the skin reduced the UV-B-induced vitamin D production, as is already well known to happen in some mammalian species (such as cats and the feline species) with high skin expression of this enzyme, which are therefore unable to produce vitamin D and for which vitamin D is a true vitamin (reviewed in Bouillon *et al.*⁸).

Clinical Aspects of Vitamin D

Vitamin D status worldwide. The worldwide vitamin D status is frequently overlooked, as most attention is going to the situation in North America and Europe. A large study in postmenopausal osteoporotic women living in Brazil (between 8° and 12°S) revealed, however, that more than 68% of such

women had serum 25(OH)D levels below 30 ng ml^{-1} , but with very exceptional cases of severe deficiency (0.3% below 10 ng ml^{-1}).²² By contrast, serum 25(OH)D levels in the first Nation women of Canada were much lower than in White women (27–75 years), and about 10% of Aboriginal Canadian women had serum 25(OH)D levels below 10 ng ml^{-1} .²³

Serum concentrations of 25(OH)D are under the genetic control of at least three or four genes (DBP, 7-dehydrocholesterol-dehydrogenase, *cyp2R1* and most likely also *cyp24a1*).^{19,20} Whether this also applies to serum 1,25(OH)₂D is not yet known. Extensive analysis of serum 1,25(OH)₂D in a wide variety of mouse strains on a low or normal calcium intake suggests that the heritability of serum 1,25(OH)₂D is high (0.67) on both low and normal calcium intake. Three chromosomal regions not yet known to be involved in vitamin D metabolism or action (except maybe for the upstream region of VDR) were identified. This is certainly a further focus for research in these animals, as well as in humans, and several abstracts presented at the ASBMR meeting in Minneapolis confirmed a genetic factor determining the 25(OH)D status in humans.^{24,25}

The effects of vitamin D status on the bone of patients with primary hyperparathyroidism (PHP) are controversial. Indeed low 25(OH)D status is frequent in PHP, and patients with the lowest 25(OH)D level ($< 20 \text{ ng ml}^{-1}$) had higher PTH levels and lower cortical bone density (mid radius) and worse micro architecture of trabecular bone when analyzed by high-resolution peripheral quantitative computed tomography.²⁶

Vitamin D supplementation and vitamin D status. The dose of vitamin D₃ needed to correct vitamin D deficiency is still a matter of discussion. In a prospective randomized controlled trial (RCT) of vitamin D supplementation (400–4800 IU per day for 1 year) of postmenopausal Caucasian and AfroAmerican women with baseline 25(OH)D levels below 20 ng ml^{-1} , a daily dose of 600–800 IU of vitamin D₃ was sufficient to bring serum 25(OH)D above 20 ng ml^{-1} in 97% of cases, with no significant differences between Caucasians and AfroAmericans, or between lean and obese subjects.^{27,28} An unexpectedly high frequency ($> 10\%$) of (intermittent) hypercalcemia was observed, but was unrelated to vitamin D dose and may have been related to the combined treatment with calcium supplementation (mean intake was between 1100–1300 mg per day). This study provides clear guidelines for vitamin D supplementation but reopens the discussion about the requirement and long-term safety of combination with high calcium intake.²⁸ The dose of vitamin D needed to correct vitamin D deficiency was also evaluated by a meta-analysis of 51 RCTs on a total of > 6000 subjects using a vitamin D supplementation dose ranging from 400 to > 2000 IU per day.²⁹ A supplement of 800 IU of vitamin D₃ per day increased the mean serum 25(OH)D levels by 20 ng ml^{-1} , with the highest increase in the old elderly (> 80 years) and in subjects with the lowest baseline level. A plateau was reached after 6 months without further benefits from higher dosages. This retrospective analysis is thus fully in line with the more detailed prospective study reported by Gallagher *et al.*²⁷ In a separate analysis, the dose–response curve of 25(OH)D after vitamin D supplementation was also evaluated by a kind of meta-analysis of 60 studies mainly based on studies using between 200 and 2000 IU of vitamin D per day (or equivalent).³⁰ The observed increase in serum 25(OH)D was reasonably

predicted by the IOM formula used to calculate increase in serum 25(OH)D depending on the dose of vitamin D, whereas the formula used by the Endocrine Society task force underestimated the observed increase nearly twofold. This suggests that vitamin D replacement needs as recommended in this task force report³¹ are nearly twice higher than needed.

Intermittent high-dose vitamin D supplementation and vitamin D status. The optimal way to eliminate vitamin D deficiency in postmenopausal women is still being actively studied. A direct comparison of daily 800 IU of vitamin D₃ with a single bolus of 300 000 IU demonstrated that similar mean 25(OH)D levels were achieved (29 ng ml^{-1}), but with still a marked subgroup with 25(OH)D levels below 20 ng ml^{-1} .³² The bolus mode, however, increased transiently mean 25(OH)D levels to $> 40 \text{ ng ml}^{-1}$ 2 weeks after the bolus intake.

High intermittent doses of vitamin D (500 000 IU once a year) have been followed by transient increased risk for fractures and falls in a large RCT in Australia.³³ These authors now report³⁴ that such therapy increased not only 25(OH)D but also more than doubled the serum concentration of 1,25(OH)₂D at 1 and 3 months after the loading dose, and that the level of 1,25(OH)₂D is in fact a significant risk factor for fractures (but not for falls). Moreover, such high doses may cause excessive fluctuations in serum 25(OH)D levels over time, as shown in a comparison between intramuscular and oral administration of 600 000 IU of vitamin D₃ or vitamin D₂. Surprisingly, intramuscular vitamin D₃ only slowly increased serum 25(OH)D with a lag time of months, whereas oral D₃ caused a more rapid increase but also a more rapid decline.³⁵ As there is doubt about the safety of such high fluctuations in serum 25(OH)D, such strategy can no longer be advised for vitamin D supplementation. Another Australian study of elderly men³⁶ also found an increased fracture risk not only with low 25(OH)D levels ($< 15 \text{ ng ml}^{-1}$; hazard ratio (HR) = 4.5) but also when serum 25(OH)D levels were $> 30 \text{ ng ml}^{-1}$ (HR of 3.5). The reason for this U-shaped relationship between fractures and vitamin D status is unclear, but other large prospective studies in the US, Europe and worldwide, such as MrOS and EMAS, may soon clarify this situation.

Pregnancy and second-generation effects. The consequences of vitamin D deficiency of mothers on pregnancy outcome and health of their offspring are being debated. In a RCT of Bangladeshi women, a high dose of vitamin D (35 000 IU per week from week 26 of pregnancy onwards, equivalent to 7000 IU per day) increased cord serum 25(OH)D from 16 to 41 ng ml^{-1} with mild increase in mean cord serum calcium concentration (10.8 versus 10.4 mg dl^{-1} at time of delivery) and especially a modestly lower nadir of neonatal serum calcium 3 days postpartum.³⁷ About 6% of neonates showed transient asymptomatic hypercalcemia, independent of vitamin D intake. Unfortunately, the baseline 25(OH)D level before the vitamin D supplementation was not reported. The real evaluation of potential benefit of vitamin D supplementation during pregnancy will thus require longer-term follow-up studies.

A long-term prospective study of children born from UK mothers ($n = 2840$) of the Avon Longitudinal study did not find a correlation between serum 25(OH)D during pregnancy and whole-body BMD as measured by DEXA at the age of 9.9 years, making it unlikely that vitamin D status in pregnancy, even in a

region with rather poor vitamin D status, would have permanent long-term effects on bone mass of their offspring.³⁸

Vitamin D supplementation: muscle and falls. The recommended daily dose of vitamin D for infants is now 400 IU per day in most countries. In a prospective RCT ($n = 55$; age 1 month) the effects of 1200 or 800 IU per day were compared with those of 400 IU on motor function at 6 months of age. Serum 25(OH)D levels at 6 months were 56, 40 and 32 ng ml⁻¹, respectively. Surprisingly, the motor function was advanced by 1 month in the group receiving 400 IU per day in comparison with the higher dosages.³⁹

Vitamin D supplementation (mean dose of 800 IU per day) is known to modestly decrease the risk of falls in elderly subjects. A comparison of different monthly doses of vitamin D₃ showed that a monthly dose equivalent to 800 IU per day increased mean serum 25(OH)D from 18 to 28 ng ml⁻¹, whereas a greater increase was observed by giving the equivalent of 2000 IU (> 15 ng ml⁻¹) or the equivalent of 800 IU of vitamin D₃ combined with 10 µg 25(OH)D₃ (> 25 ng ml⁻¹). Surprisingly, however, the risk of falls was higher with higher doses of vitamin D.⁴⁰ Therefore, a careful analysis of the ongoing studies with high doses of vitamin D for falls and overall health risks is needed, and generalized use of higher than the recommended supplement of 800 IU per day for the elderly should be questioned.

Vitamin D and the metabolic syndrome. A low 25(OH)D status is associated with obesity, type 2 diabetes and all aspects of the metabolic syndrome. Such an association has been now also found in non-Western countries, as 25(OH)D levels were negatively associated with fasting blood glucose levels in Thai adults.⁴¹ An intervention study in Saudi Arabian T2DM adults, however, could not demonstrate an effect on glycemic or metabolic control after 1 year of daily supplementation with 2000 IU per day that increased their 25(OH)D levels from 13 to 22 ng ml⁻¹.⁴²

Assay methodology for vitamin D metabolites. A method for measurement of serum 24,25(OH)₂D by LC/MS/MS after derivatization was presented and confirmed a nearly linear correlation ($r^2 = 0.57$) with total 25(OH)D concentrations over a very wide range (6–123 ng ml⁻¹). Such measurement⁴³ may help to better detect subtle or manifest abnormalities of the cyp24A1 gene, as such mutation may cause hypercalcemia with even modest vitamin D supplementation.^{4,44} A new family with such mutation of the 24-hydroxylase was also presented.⁴⁵

Matrix effects are well known to influence the accuracy of measurement of vitamin D metabolites, even using LC/MS/MS technology, and this could be avoided by a prior extraction method that removed phospholipids.⁴⁶

Conflict of Interest

The author declares no conflict of interest.

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