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MEETING REPORT

Report on recent vitamin D research: ECTS 2012 and the 15th Vitamin D workshop 2012

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Meeting Reports from the European Calcified Tissue Society 39th Annual Congress, Stockholm, Sweden, 19–23 May 2012 and the 15th Vitamin D workshop adjacent to the Endocrine Society meeting Houston, TX, USA, 20–22 June 2012.

A search on the keywords of the ECTS abstract book revealed that 92 presentations out of a total of >400 mentioned 'vitamin D', indeed a very impressive number reflecting the still rapidly growing attention for this topic. More than 220 abstracts were presented at the Vitamin D workshop.

Skeletal Effects of Vitamin D

Mice with deletion of either Klotho or FGF23 display accelerated ageing and early death but also excess production of $1,25(OH)_2D_3$ with major 'calcemic consequences'. Avoiding the excess production of $1,25(OH)_2D_3$ in these mice eliminates the calcemic effects but also avoids the accelerated ageing process.[1] Treatment of human mesenchymal stem cells from femoral heads with $1,25(OH)_2D_3$ as reported by Klotz *et al.*,[2] delayed cellular ageing without changing their multipotent capacity and thus without inducing a specific lineage commitment. This indicates that the vitamin D hormone is not responsible for replicative senescence. A proteomic approach using similar human bone marrow mesenchymal stem cells showed that exposure to 25OHD regulated a larger number of proteins than did $1,25(OH)_2D_3$ whereby 25OHD-regulated proteins were mainly involved in growth and anti-oxidant pathways, and $1,25(OH)_2D_3$ downregulated thrombospondin-1 and a protein serine-type endopeptidase inhibitor.[3] A very different pattern was, however, found in prostate progenitor cells as exposure to $1,25(OH)_2D_3$-induced interleukin 1-mediated senescence that could be further enhanced by phosphatase and tensin homologue deleted on chromosome 10 deletion. These data indicate that $1,25(OH)_2D_3$ may promote senescence in prostate stem cells and this could be a very beneficial effect in delaying their progression toward a hyperplastic, dysplastic or malignant phenotype.[4] The osteomalacia found in a large number of genetically engineered mice, with either a deletion of FGF23 or its receptor klotho or parathyroid hormone (PTH) or even in double-KO mice, is striking as most of these mice have very high serum levels of $1,25(OH)_2D_3$ and of several mineralization inhibitors such as osteopontin, dentin matrix protein 1 and matrix gla protein. The exception is the double-KO of klotho and PTH who display a normal bone phenotype, as reviewed extensively by Lanske.[5] The picture is now much better understood as Carmeliet and Lieben presented data at the Vitamin D workshop that changes the paradigm of vitamin D's action on bone mineralization. Indeed, in case of severe calcium deficiency, selective vitamin D receptor (VDR) deletion in the gut, or on high dose $1,25(OH)_2D_3$ treatment, bone will be used to maintain serum calcium homeostasis by increasing receptor activator of nuclear factor κ-B ligand-mediated bone resorption, as has been amply demonstrated *in vitro*, in animal models and in men. $1,25(OH)_2D_3$, however, also actively impairs bone mineralization by controlling the enzymes producing and degrading pyrophosphate (causing high PPi concentrations) and upregulating osteopontin, and other mineralization inhibitors, all with the aim to impair mineralization and to maintain a normal calcium homeostasis. Bone is then the victim by a combination of increased resorption and lack of mineralization, creating excess osteoid or osteomalacia. This is in fact a paradigm shift of the classical idea that vitamin D favors mineralization into a new model of a Yin/Yang situation (major beneficial effects mediated by its action on the intestine, but potentially deleterious effect by its direct action on osteoblast, osteocytes and osteoclast precursors). This certainly should draw the attention of clinicians to the clinical message that a good calcium intake is an absolute requirement for generating the beneficial vitamin D action on bone.[6] Genome-wide analysis of VDR-regulated genes confirmed these observations on mineralization inhibitory genes in mesenchymal stem cells.[7]

Michael McClung gave a very balanced overview on the possible beneficial effects of vitamin D in a Merck sponsored symposium. He also summarized the data indicating that alendronate with vitamin D generates better bone mineral density results than alendronate alone and this has major implications for long-term therapy of osteoporosis. Of course this is not a real surprise as all the bisphosphonates and most other anti-osteoporosis drugs were in fact a combination of the new drug plus calcium/vitamin D compared with calcium/vitamin D. Evidence-based medicine tells us that we should treat patients with a similar profile as in the randomized controlled trial (RCT) study with the same schedule as in the

experimental study. Moreover, as for all ligands of nuclear receptors there is an optimal window of activity with worse outcome when the ligand concentration is too low or too high. There is no exception for vitamin D as high concentrations of vitamin D, and certainly of $1,25(OH)_2D_3$, can have deleterious effect on falls and fractures. New data summarized by Lieben at the vitamin D workshop clearly showed that as summarized above that serum calcium homeostasis is maintained at the expense of bone and can help to explain the deleterious effects of even transient excess of vitamin D, as demonstrated by increased risk of falls and fractures early after a large bolus of vitamin D.[8] A similar U-shape relation of 25OHD levels on an extra-skeletal outcome was also observed for cardiovascular events (see below) and has been repeatedly reported, albeit based on small scale studies, on cancer prevalence and maybe even on mortality risk.[9,10]

That vitamin D has both good and bad effects on bone should not discourage industry from exploring the possible selective effects of vitamin D analogs. In fact, the contrary is true as selective estrogen-receptor modulators and selective glucocorticoids and other ligands for nuclear receptors exist or are explored because of the hope of finding analogs that retain the good and minimize the bad effects. Whether ED71 from Chugai belong to such (first generation) category for bone health was presented at the vitamin D workshop, where S Kato (Tokyo) showed that VDR+/− heterozygous mice have a slightly increased bone mass (instead of the decreased bone mass of VDR−/− mice), which was even more pronounced in mice with selective VDR deletion from osteoblasts (using a collagen 2a1 Cre model) due to decreased bone resorption.[11] ED71 increased bone mass in Japanese patients with postmenopausal osteoporosis as reviewed by Takahashi[12] at the vitamin D workshop in Houston. Daily treatment of mice with this analog confirmed that the effects are largely due to a PTH-independent decrease in receptor activator of nuclear factor κ-B ligand expression and bone resorption rather than increased bone formation. Similar results were obtained by *in vitro* studies on osteoclastogenesis. Moreover, ED71 treatment improved the locomotor activity of wild type but not of VDR KO mice, indicating that the analog may also have direct effects on muscle function.[13]

Rickets is still endemic in some regions of the world and this topic was extensively reviewed by Prentice.[14] The frequency of rickets is very low in Caucasians with a normal life style (in Denmark estimated frequency of 2/100 000) but much higher in immigrants with dark skin living in Western Europe (0.1% of such infants living in Denmark.[15] A much higher frequency was reported for Mongolia[16] with serum 25OHD levels $<8\,ng\,ml^{-1}$ in 22% of children below the age of 5 years, many of whom had clinical symptoms of rickets (soft fontanel or craniotabes in children below age 1 and other skeletal abnormalities in older children). Calcium deficiency rickets was extensively reviewed by Prentice with low calcium intake leading to high serum $1,25(OH)_2D_3$ levels followed by increased FGF23 secretion and phosphate deficiency, all leading to impaired bone mineralization, moreover this situation accelerates the 24-hydroxylase and increases vitamin D catabolism so that vitamin D deficiency is accelerated. In view of the data presented by Lieben *et al.*[6] referred to above, such high calcium deficiency-induced $1,25(OH)_2D_3$ levels may well directly impair bone mineralization and thereby cause or contribute to the clinical picture of rickets/osteomalacia.[17]

Vitamin D Metabolism

The importance of the 24-hydroxylase for calcium and bone biology was extensively discussed in two ways. First, St Arnaud further detailed the consequences of cyp24A1 deletion on fracture repair in mice as such mice show marked delay in callus formation (probably related to decreased vascular endothelial growth factor expression and vascular invasion) and fracture healing (delay only as final healing is normal). Therefore, the question of a specific 24,25R-$(OH)_2$D receptor was also addressed and a G protein-coupled receptors was identified but also not further detailed pending patent application.[18] Tissue-specific deletion of this metabolizing enzyme will help to clarify its role. Second, several presentations dealt with cases of human 24-hydroxylase deficiency causing systemic hypercalcemia, previously known as infantile hypercalcemia. Schlingman *et al.*[19] reviewed and updated his publication. The frequency of CYP24A1 mutations in human populations is still in an exploratory phase but could well be around 1% of the Caucasian population so that deficiency of two alleles may be around 1/40 000. The affected subjects are clearly at increased risk of vitamin D toxicity when exposed to high or even fairly frequently used doses of vitamin D, especially when given as an intermittent bolus. Similar data were presented by Kaufmann *et al.*[20] who identified around 40 single nucleotide polymorphisms in the coding region of CYP24A1, some of which with clearly decreased activity when expressed *in vitro*. Whether activation mutations are also present is yet to be explored.

The metabolism of vitamin D is still unclear when it comes to precise conversion rates of vitamin D into 25OHD or from 25OHD into further metabolites. A careful study in the United Kingdom and Gambian subjects using deuterium-labeled 25OHD, given orally, demonstrated a serum half-life of about 15 days, largely in line with older studies using radio-labeled vitamin D.[21] This important information may help to clarify the nutritional vitamin D needs.[9]

Vitamin Status of the Elderly

The vitamin D status of the elderly is known to be compromised worldwide but most studies are cross-sectional and few report on the long-term 'spontaneous' evolution of their vitamin D status. Van Schoor *et al.*[22] used the Amsterdam LASA database to follow >2000 elderly subjects for 6–13 years and found that the vitamin D status of the younger population (<65 years) in fact slightly improved over time ($+5\,nmol\,l^{-1}$) whereas the age-associated decrease in 25OHD was slow ($-5\,nmol\,l^{-1}$ >13 years). This is reassuring for the large number of cohort studies that used a single measurement of 25OHD to evaluate the long-term consequences of the vitamin D status and also demonstrate that the factors determining the vitamin D status are fairly constant over time even in the elderly generally healthy population.

Supplementation with vitamin D and a good calcium intake can decrease the fracture risk of elderly subjects as shown by a meta-analysis of many meta-analyses of the primary RCTs.[23] A large longitudinal cohort study of community dwelling Swedish middle-aged and elderly subjects showed, however, that dietary intake of vitamin D was of minor importance for the occurrence of fractures or osteoporosis.[24]

A report from Saudi Arabia confirmed the amazingly low vitamin D status of this population as only 20% of Saudi women aged 60 or more had 25OHD levels $>20\,ng\,ml^{-1}$. In addition, levels of 25OHD $<20\,ng\,ml^{-1}$ and increased PTH levels were associated with decreased lower extremity muscle function,[25] in line with recent extensive meta-analysis of vitamin D/calcium intervention studies on falls.[26]

The amount of vitamin D needed to maintain a normal vitamin D status of course depends on the 25OHD level considered to be optimal. A detailed RCT using a wide range of vitamin D supplementation (from 400 to 4 800 IU per day for 6 months) in postmenopausal women with a baseline 25OHD level $<20\,ng\,ml^{-1}$ and a mean level of $15\,ng\,ml^{-1}$, demonstrated that a supplement of 600 IU per day increased serum 25OHD to $>20\,ng\,ml^{-1}$ in all subjects whereas even 4 800 IU per day could not increase serum 25OHD $>30\,ng\,ml^{-1}$ in all subjects.[27,28] The dose–response curve in Afro-Americans was not different from that in Caucasians. Obese subjects needed more vitamin D to obtain similar 25OHD levels but the curves were parallel with non-obese subjects, suggesting a dilution effect rather than a different metabolism.

Extra Skeletal Effects of Vitamin D: Cardiovascular System

The link between cardiovascular events and cardiovascular mortality has been a topic of intense research and discussion, and two good meta-analysis confirmed such an association.[29] This was confirmed by a long-term observational study on 2016 postmenopausal Danish women followed for 16 years,[30] indicating that the composed endpoint of mortality, heart failure, myocardial infarction or stroke was significantly higher when baseline 25OHD were $<20\,ng\,ml^{-1}$. A more extensive review on this topic was presented by Witham,[31] with the clear possibility that the link between vitamin D status and cardiovascular disease is either causal or due to reverse causation. There are a wide range of mechanisms whereby vitamin D could decrease the cardiovascular risk such as lowering renin production, improving vascular endothelial growth factor expression, decreasing tumor necrosis factor-mediated inflammation or decreasing PTH levels (which is a known independent risk factor for cardiovascular events). The existing intervention studies suggest that vitamin D repletion of vitamin D-deficient subjects may have a slight beneficial effect on blood pressure in hypertensive (but not in normotensive) subjects, but the overall effects are nor positive nor negative. Therefore, it is impossible to define an optimal threshold or supplementation dose for this indication. The situation is even more complex as many studies on vitamin supplementation were in fact combined vitamin D plus calcium studies, and recent extensive meta-analysis showed that high pulse-dose intake of calcium alone may have negative cardiovascular effects that offset the potential beneficial effects of vitamin D.[32] One should, however, remember that extra-skeletal and especially vascular calcifications are one of the first but most dangerous consequences of vitamin D excess and that a U-shape relationship between vitamin D status and cardiovascular events is likely. Based on the available data one cannot yet define the optimal window of 25OHD.

Extra Skeletal Effects of Vitamin D: immune System

The link between vitamin D status and immune function was reviewed by Mathieu.[33] The cellular and preclinical data are very suggestive for a role of the vitamin D endocrine system in the native as well as in the adaptive immune function. It is hard to find another natural compound with such a coherent spectrum of activities on all cells of the immune system. Intervention studies in patients with either active tuberculosis or chronic obstructive pulmonary disease, however, were negative in an intention to treatment analysis but showed beneficial effects in subgroups with either tt VDR polymorphism[34] or when the baseline 25OHD level was $<10\,ng\,ml^{-1}$.[35] A role of vitamin D in autoimmune diseases such as type 1 diabetes, multiple sclerosis or inflammatory bowel disease is plausible but needs confirmation in large RCTs. The preclinical data in these models are overwhelmingly positive both for therapy with high dose vitamin D itself as with several less calcemic analogs. In a mouse model of inflammatory bowel disease good control of the disease severity was possible by local administration of a potent less calcemic vitamin D analog.[36]

Extra Skeletal Effects of Vitamin D: the Skin

The skin has a pivotal role in the vitamin D endocrine system as it is both the origin and target of this hormone. Ultraviolet B light is essential for the photosynthesis of vitamin D but also causes DNA damage and is thus well known to cause a variety of skin cancers including melanomas. The absence of VDR increases the risk of UVB-induced skin cancers as reviewed by Bikle's and colleagues[37] at the Vitamin D workshop and by Mason.[38] Bikle's and colleagues showed data that such increased cancer risk was also present when the VDR was only deleted in the epidermis. Both groups describe compensatory mechanisms whereby $1,25(OH)_2D_3$ decreases the photodamage by increasing DNA repair mechanisms, whereby the oxidoreductase ERp57 has an essential role. Also, melanin synthesis and increasing the thickness of the epidermal layer are ways to decrease the photodamage. Local application of some (non-) genomic vitamin D analogs can reduce the risk of skin cancer.[38] The VDR-hormone ligand also directly downregulates the transcriptional activity of the β-catenin/T-cell factor signaling whereas silencing of this gene reduces skin carcinogenesis.[37] Also, activation of the hedgehog pathway increased the skin cancer risk (basal cell carcinoma) but $1,25(OH)_2D_3$ downregulates this hedgehog signaling thereby acting as a compensatory mechanism to reduce the overall cancer risk.

Extra skeletal Effects of Vitamin D: Effects on Pregnancy and Reproduction

Perinatal vitamin D status received a lot of attention over the last years as it came as a big surprise that so many young pregnant women have rather low 25OHD levels and that perinatal vitamin D status may have long-term consequences for later child health and diseases. A Danish cohort study showed that maternal vitamin D status was a good predictor of the vitamin D status of their children even at 9 months of age and that cord serum 25OHD levels (especially when $<25\,nmol\,l^{-1}$) were a predictor of body weight and length at 9 months.[39]

A large RCT with vitamin D_3 ranging from 400 to 4 000 IU per day, given to women with baseline levels of 25OHD $<40\,ng\,ml^{-1}$, during the second half of pregnancy[40,41] showed that as expected serum 25OHD increased (but not more than

mean levels of 111 nmol l^{-1} on the highest dose) but also significantly increased serum $1,25(OH)_2D_3$ (with extremely wide biological variation between 50 and 800 pg ml^{-1}) without consequences for serum or urinary calcium. When single outcomes were evaluated no effects were seen except for a marginal significance (around $P = 0.05$) for the frequency of premature delivery, preeclampsia or comorbidity.[42] These data are extremely important to define possible effect of vitamin D status on pregnancy outcome. Further details of this study were discussed by Wagner *et al*.,[41] who confirmed the lack of visible side effects for mother and newborn. An additional study funded by the Thrasher foundation allowed a combined analysis as to evaluate a large number of pregnancy outcomes without a clear overall benefit but a marginal benefit on preterm deliveries of mothers without preeclampsia ($P = 0.057$) and a real decrease in preeclampsia risk, but the analysis was not yet fully controlled for other potential confounders. A higher maternal 25OHD level was, however, significantly associated with a lower rate of co-morbidities of pregnancy. In addition, the study population may not represent the typical US population as $>60\%$ of the study population was uninsured and it is unclear whether the outcome data were fully corrected for baseline variables. Fortunately, there is a follow-up planned with a Thrasher fund grant to study the health of these children during the next 3 (and hopefully longer) years.

The extra-skeletal effects of vitamin D also include the reproductive system and reduced aromatase activity (direct negative regulation by $1,25(OH)_2D_3$) decreased female reproduction. New data presented at the vitamin D workshop[43] indicate that vitamin D-deficient or -resistant mice have reduced sperm count, motility and fertility.[44] Moreover, human VDR and 24-hydroxylase are co-expressed in human spermatozoa and low expression of this CYP24A1 was associated with subfertility making it a good marker for semen quality.[43] $1,25(OH)_2D_3$ as well as a cis-locked non-genomic agonist enhance intracellular calcium concentration and stimulate sperm motility. Finally, serum 25OHD levels were positively associated with sperm motility and morphology in normal men.

Assay Methodology

The assay methodology was extensively discussed at the Endocrine Society meeting following the Vitamin D workshop in Houston, June 2012 and M Drezner described the assay as 'Houston, we have a problem'. Indeed, when accuracy is the ultimate arbiter as this is the treatment aim, then direct assays used in $>60\%$ of all clinical assays in the United States are deviating from the values obtained by either the gold standard liquid chromatography–mass spectrometry or by assays using radioimmunoassay after extraction. So there is a strict need for further optimization of the 25OHD assays and simpler methodology is not a price to pay for accuracy or quality.

Conflict of Interest

The authors declare no conflict of interest.

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