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Review

Vitamin D and calcium deficits predispose for multiple chronic diseases

M. Peterlik and H. S. Cross
University of Medicine Vienna, Vienna, Austria

Abstract

There is evidence from both observational studies and clinical trials that calcium malnutrition and hypovitaminosis D are predisposing conditions for various common chronic diseases. In addition to skeletal disorders, calcium and vitamin D deficits increase the risk of malignancies, particularly of colon, breast and prostate gland, of chronic inflammatory and autoimmune diseases (e.g. insulin-dependent diabetes mellitus, inflammatory bowel disease, multiple sclerosis), as well as of metabolic disorders (metabolic syndrome, hypertension). The aim of the present review was to provide improved understanding of the molecular and cellular processes by which deficits in calcium and vitamin D cause specific changes in cell and organ functions and thereby increase the risk for chronic diseases of different aetiology. 1,25-dihydroxyvitamin D_3 and extracellular Ca^{++} are both key regulators of proliferation, differentiation and function at the cellular level. However, the efficiency of vitamin D receptor-mediated intracellular signalling is limited by the negative effects of hypovitaminosis D on extrarenal 25-hydroxyvitamin D-1α-hydroxylase activity and thus on the production of 1,25-dihydroxyvitamin D_3. Calcium malnutrition eventually causes a decrease in calcium concentration in extracellular fluid compartments, resulting in organ-specific modulation of calcium-sensing receptor activity. Hence, attenuation of signal transduction from the ligand-activated vitamin D receptor and calcium-sensing receptor seems to be the prime mechanism by which calcium and vitamin D insufficiencies cause perturbation of cellular functions in bone, kidney, intestine, mammary and prostate glands, endocrine pancreas, vascular endothelium, and, importantly, in the immune system. The wide range of diseases associated with deficits in calcium and vitamin D in combination with the high prevalence of these conditions represents a special challenge for preventive medicine.

Keywords Vitamin D insufficiency, 25-hydroxyvitamin D-1α-hydroxylase, autoimmune disease, calcium status, calcium-sensing receptor, cancer.
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Introduction

It has become common knowledge that an adequate supply of calcium and vitamin D is indispensable for optimal skeletal development as well as for maintenance of bone health in both women and men. However, it is of interest to note that inadequate intake and absorption of calcium as well as a compromised vitamin D status are not only well-known risk factors for skeletal disorders, such as osteomalacia and osteoporosis, but also contribute to the pathogenesis of frequent malignant, infectious, chronic inflammatory and autoimmune diseases. Although supported by a considerable number of epidemiological, clinical and experimental studies, this notion is not commonly accepted. This is probably owing to the difficulty in understanding how grossly different types of chronic diseases can develop out of deficits in calcium or vitamin D supply, when at the same time systemic homeostatic mechanisms maintain 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and Ca^{++} plasma levels within a normal range (cf. [1]). However, in recent years it has become clear that 1,25-$(OH)_2D_3$ and extracellular Ca^{++} are both also

Department of Pathophysiology, Center of Physiology and Pathophysiology, University of Medicine Vienna, Vienna, Austria (M. Peterlik, H. S. Cross)

Correspondence to: Meinrad Peterlik, PhD, MD, Department of Pathophysiology, Vienna General Hospital, Waehringer Guertel 18–20, A-1090 Vienna, Austria. Tel.: +43 1 40400 5120; fax: +43 1 40400 6188;
e-mail: meinrad.peterlik@meduniwien.ac.at

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involved in local control of cellular growth, differentiation and, importantly, function. In this respect, one must bear in mind that tissue concentrations of 1,25-$(OH)_2D_3$ are mainly determined by the extent of local production of 1,25-$(OH)_2D_3$ by cells that express the 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1); the enzyme mediating the conversion of 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$. Likewise, calcium concentrations in extracellular fluid compartments, such as in bone, kidney or intestine, depend primarily on the kinetics of tissue-specific calcium fluxes and not on plasma calcium levels. In the present review we will therefore address the question: What are the consequences of hypovitaminosis D and/or inadequate dietary calcium supply on local concentrations of 1,25-$(OH)_2D_3$ and calcium, and how do those changes in cell and organ functions relate to increased risks not only of skeletal disorders but also of a number of unrelated chronic diseases?

Part I: hypovitaminosis D

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is the biologically most active vitamin D compound, which not only plays a role in systemic calcium and phosphate homeostasis, but is also as a regulator of cellular proliferation, differentiation and function in a variety of organs and biological systems. The biological importance of 1,25-$(OH)_2D_3$ is highlighted by the fact that a great number of cell types are endowed with the vitamin D receptor (VDR) (e.g. [2]). which, as a member of the steroid/thyroid hormone nuclear receptor superfamily, functions as a ligand-activated transcription factor to mediate the genomic effects of the hormone.

Hypovitaminosis D: vitamin D insufficiency vs. deficiency

Vitamin D_3 or the structurally related vitamin D_2, whether synthesized in the skin or absorbed in the small intestine from the diet, are both further metabolized in the liver by hydroxylation at C25. The serum level of 25(OH)D is therefore considered a reliable indicator of the vitamin D status of a given individual. The serum concentration of 25-(OH)D varies between 25 and 125 nM in the winter months, and between 50 and 300 nM in summer time. Whereas there appears to be agreement that vitamin D deficiency is defined by 25-(OH)D concentrations less than 10 nM, vitamin D insufficiency, i.e. suboptimal vitamin D supply, is considered to be reflected by 25-(OH)D concentrations in the 25–50-nM range, because a number of studies have shown that a fall of serum 25-(OH)D levels into this range is associated with a rise of serum PTH indicative of secondary hyperparathyroidism [3,4]. More recently, in their study on an adult general urban population in France, Chapuy *et al.* [5] considered 30 nM to be 2 SD less than the average 25(OH)D-value in a normal adult population in winter time, and therefore this concentration can be used as cut-off point for diagnosis of hypovitaminosis D.

Hypovitaminosis D is frequently observed in housebound or hospitalized elderly people, but is a common phenomenon also in the free-living younger population: we had recently completed a large multicentre study on the calcium, vitamin D and bone status of a healthy adult Austrian population, in which we could enrol more than 1000 individuals of both sexes between 19 and 79 years of age [6,7]. We measured dietary calcium and vitamin D intake as well as parameters of calcium and bone metabolism together with bone mineral density at five skeletal sites, and were thus able to generate a fairly large database for reliable assessment of the calcium and vitamin D status and of bone health in an adult Central European population. To minimize the impact of UV radiation and cutaneous vitamin D_3 synthesis on the vitamin D status, serum 25-(OH)D levels had been determined during the winter months, i.e. between November and April. In this study, 26% of all study participants had 25-(OH)D levels less than 30 nM, and thus presented with hypovitaminosis D. In conjunction with results from similar but smaller studies (see among others [3,5,8,9]), our data lend strong support to the notion that hypovitaminosis D is a widespread phenomenon in the adult population of Central and Western Europe as well as in North America. Hypovitaminosis D must be considered an important public health problem because of numerous implications for the development of common diseases, as will be outlined in this review (cf. also [10]).

Hypovitaminosis D: differential effects on renal and extra-renal 1,25-$(OH)_2D_3$ synthesis

1,25-$(OH)_2D_3$ is synthesized mainly in the kidney from its immediate precursor 25-$(OH)D_3$, which itself is produced in the liver from vitamin D_3. Importantly, 1,25-$(OH)_2D_3$ is also produced at a number of extra-renal sites, as apart from renal tubular cells, many other cell types express the 25-(OH)D-1α-hydroxylase (CYP27B1). For example, CYP27B1 activity has been localized in osteoblasts [11], colonocytes [12,13], macrophages [14,15], synovial cells [16], keratinocytes [17], hair follicles, adrenal medulla, pancreatic islets [18], and vascular endothelial cells [19]; for details see Table 1.

It is important to note that under normal circumstances renal CYP27B1 activity is tightly regulated by serum Ca^{++} and parathyroid hormone (PTH), and also by feed-back inhibition from 1,25-$(OH)_2D_3$, as illustrated in Fig. 1 (for details see [1]). Therefore, production of 1,25-$(OH)_2D_3$ in the kidney is largely independent of 25-(OH)D serum levels. However, this is not valid for extra-renal synthesis of 1,25-$(OH)_2D_3$, as, for example, CYP27B1 expression in intestinal epithelial cells [12] is insensitive to PTH and extracellular Ca^{++} (cf. [20,21]), or, as in macrophages or endothelial cells, where it is subject to modulation primarily by pro-inflammatory cytokines such as interferon-γ (IFN-γ) [14,19,22]. In addition, 1,25-$(OH)_2D_3$ does not suppress its own synthesis in macrophages [15] as it does in renal tubular cells. Thus, at extra-renal sites, the ambient 25-$(OH)D_3$ level becomes limiting for intracellular synthesis of

Table 1 Extra-renal human tissues and cells expressing 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1)

Tissue or cells	Expression			Reference
	mRNA	Protein	Enzymatic activity	
Bone				
Osteoblasts	ND	ND	Yes	[11]
Large intestine				
Normal mucosa	Yes	Yes	ND	[23,154]
Adenoma	ND	Yes	ND	[23]
Adenocarcinoma	Yes	Yes	Yes	[12,13,23]
Breast, uterus, ovary				
(normal and neoplastic epithelial cells)	Yes	Yes	ND	[40]
Prostatic gland				
(normal and neoplastic epithelial cells)	ND	ND	Yes	[41]
Monocytes/macrophages				
Alveolar macrophages	ND	ND	Yes	[14]
Lymph nodes	ND	Yes	ND	[18]
Pancreas				
Islet cells	Yes	Yes	Yes	[18]
Acinar cells				
(normal and neoplastic)	Yes	Yes	Yes	[42]
Blood vessels				
Endothelial cells	Yes	Yes	Yes	[19]
Synovial cells	ND	ND	Yes	[16]

ND, not determined.

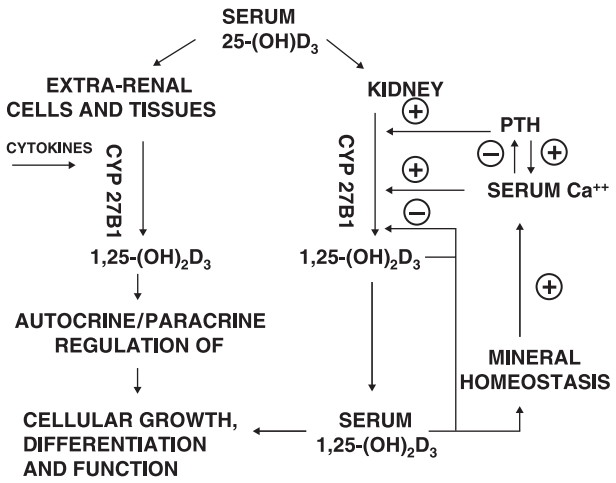

Figure 1 Differential regulation of renal and extra-renal 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) activity.

$1,25\text{-}(OH)_2D_3$. In combination with a low intrinsic production of $1,25\text{-}(OH)_2D_3$, as e.g. typically observed in normal human colonocytes [23], hypovitaminosis D could create a situation in which locally produced $1,25\text{-}(OH)_2D_3$ decreases to less than a level critical for maintenance of autocrine/paracrine regulation of cellular growth and function (cf. [24]). Therefore, even moderately low serum levels of $25\text{-}(OH)D_3$ can cause alterations of specific cell functions in the many organs and biological systems that have been identified as sites of extrarenal CYP27B1 activity (cf. Table 1). Hypovitaminosis D therefore is not only a pathogenetic factor for bone diseases such as rickets, osteomalacia and osteoporosis, but also plays an important though largely underestimated role in the development of malignant, chronic inflammatory and autoimmune diseases as well as metabolic disorders.

Skeletal diseases

There certainly is no need to emphasize the important role of vitamin D in the prevention of rickets and osteomalacia. This has been attributed largely to the stimulatory effect of $1,25\text{-}(OH)_2D_3$ on transcellular absorption of calcium and phosphate in the intestine. Accrual of sufficient amounts of the two mineral ions from dietary sources is indispensable for adequate mineralization of the organic matrix. However, mineralization depends also on maturation of matrix-producing osteoblasts and expression of differentiated cell functions (production of osteocalcin, alkaline phosphatase etc.). As convincingly shown by Owen *et al.* [25], the steroid hormone co-ordinates the sequence of osteoblast development by specific VDR-mediated effects on the temporal gene expression and synthesis of several osteoblast proteins. In other words, in proliferating osteoblasts, $1,25\text{-}(OH)_2D_3$ reduces expression of type I collagen, osteopontin and osteocalcin, whereas in mature postproliferative osteoblasts, $1,25\text{-}(OH)_2D_3$ up-regulates expression of genes encoding not only for matrix proteins but also for osteocalcin. It is conceivable therefore that to the extent to which osteoblast differentiation is under autocrine/paracrine control by $1,25\text{-}(OH)_2D_3$, low CYP27B1 activity in osteoblasts [11] will become a limiting factor for mineralized matrix maturation and bone formation. A compromised vitamin D status therefore not only causes rickets and osteomalacia but is also

an important risk factor for osteoporosis, which is often caused by an insufficient rate of bone formation. In addition, hypovitaminosis D could be associated with osteoporosis because, as already mentioned, a fall in circulating 25-(OH)D levels into the 25–50-nM range induces secondary hyperparathyroidism and, consequently, increases osteoclastic bone resorption (see e.g. [3,4]).

Colorectal cancer and other malignancies

1,25-$(OH)_2D_3$, when bound to the high-affinity nuclear VDR, regulates cell growth and differentiation in multiple normal and malignant cell types. For example, 1,25-$(OH)_2D_3$ exerts antiproliferative effects in breast cancer cells which are thought to result from changes in cell-cycle regulators, such as $p21^{WAF-1/CIP-1}$, $p27^{kip1}$, cyclins, and retinoblastoma tumour suppressor protein [26]. Studies from our laboratory have shown that 1,25-$(OH)_2D_3$ inhibits growth and promotes differentiation in primary cultured human colon adenoma and carcinoma cells as well as in established human colon adenocarcinoma-derived cell lines [27–30] (for review [31]). The growth inhibitory potential of 1,25-$(OH)_2D_3$ has been traced to its ability to block up-regulation of cyclin D1 expression, which is a key element in cell-cycle control. As a number of intracellular proliferative signalling pathways, viz. the Raf-1/MEK1/ERK, and STAT-3 pathways converge at c-myc [32], and consequently engage cyclin D1 as a common downstream effector, 1,25-$(OH)_2D_3$ is potentially effective in counteracting mitogenesis independently of the nature of cellular growth-promoting factors.

Colorectal cancer

Studies from our laboratory had shown that human colon adenocarcinoma cells express CYP27B1 and are thus able to convert 25-(OH)D_3 into 1,25-$(OH)_2D_3$ [12,13,23], which we had identified as a potent antimitotic factor for human colon carcinoma cells [27–30]. We had put forward the notion that in hypovitaminosis D, the amount of 1,25-$(OH)_2D_3$ produced in the large intestinal mucosa could be too low to restrain cancer cell growth. In fact, there are several reports that a compromised vitamin D status is associated with an increased risk for colorectal cancer [33–36].

Although it had been suggested that an adequate intake of vitamin D reduces the incidence of colorectal cancer, the preventive effect of vitamin D consumption on colorectal cancer is a modest one (for review [37]). This is plausible because dietary intake of vitamin D typically supplies only a minor fraction of the daily requirement of this steroid. Therefore, the impact of low sunshine exposure on vitamin D insufficiency and consequently on colorectal cancer incidence becomes that much greater. For example, in an ecological study of cancer mortality rates in the U.S. with respect to exposure to solar radiation, Grant [38] found a highly significant ($P < 0{\cdot}001$) inverse correlation between DNA-weighted UV-B radiation (280–315 nm) and the incidence of colorectal cancer. This association persisted even after additional ecological risk factors (smoking, dietary factors, urban residence, poverty etc.) were included in the analysis [39]. Premature mortality owing to insufficient UV-B exposure among white Americans was estimated at 12% of the total number of deaths from colon cancer [38].

Other malignancies

Considering the importance of extrarenal CYP27B1-mediated production of 1,25-$(OH)_2D_3$ (cf. Table 1) for control of cell proliferation, e.g. in breast, ovary, uterus, prostate and pancreas [40–42], one would expect that vitamin D insufficiency would increase the risk of malignancies other than colorectal cancer. In fact, studies by Grant [38,39,43] as well as by Freedman *et al.* [44] on cancer mortality rates in the US and Europe, using latitude or DNA-weighted solar UV-B exposure as surrogate endpoints for photoproduction of vitamin D_3 in the skin, found a highly significant association with the incidence of breast, oesophagus, stomach, pancreas, bladder, ovary, uterus, prostate and non-melanoma skin cancer as well as non-Hodgkin lymphoma.

The long-standing assumption that low vitamin D intake is associated with increased breast cancer risk [45] has been supported by a recent study of Shin *et al.* [46], who showed in an analysis of data from the Nurses' Health Study that premenopausal women with a daily vitamin D intake of > 500 IU had a significantly lower risk (RR = 0·72) of breast cancer than those ingesting only 150 IU and less. The importance of adequate vitamin D supply for the prevention of breast cancer was particularly emphasized by Grant [38,39,43], who estimated that in the U.S. greater than 10% of premature mortality from breast cancer could be attributed to insufficient UV-B radiation.

Tuohimaa *et al.* [24] conducted a longitudinal, nested case–control study on vitamin D status and prostate cancer incidence in Scandinavian countries. Finnish men with serum 25-(OH)D levels in the lowest quintile had a 90% increased risk than those in the highest quintile. This was explained by the assumption that at low serum 25-(OH)D, insufficient 1,25-$(OH)_2D_3$ is produced by local CYP27B1 activity for maintaining normal growth of prostate cells (cf. [41]).

Infectious, inflammatory and autoimmune diseases

1,25$(OH)_2D_3$ has also been identified as a potent modulator of macrophage functions as well as of B- and T-lymphocyte-mediated immune responses [15]. In particular, 1,25$(OH)_2D_3$ has a positive effect on chemotaxis of monocytes [47], promotes differentiation of monocytes into macrophages [48,49], and activates phagocytic and antimicrobial activities. The latter effects are the result of up-regulation of expression of Fc receptors [50], of an increased respiratory burst [51], as well as of enhanced production of nitric oxide [52].

Of paramount importance for the activating effect of 1,25-$(OH)_2D_3$ on various monocyte/macrophage functions is the fact that macrophages not only express the VDR but

are also endowed with CYP27B1 activity [14]. Therefore, under conditions of low serum 25-$(OH)D_3$ levels, only limited 1,25-$(OH)_2D_3$ can be produced by macrophages from the precursor. The ensuing impairment of macrophage activation and function [53] explains the well-known fact that the prevalence of infectious diseases is high in children with rickets. Likewise, low intrinsic CYP27B1 activity in macrophages and other antigen-presenting cells could be responsible for, particularly in hypovitaminosis D, tissue concentrations of 1,25-$(OH)_2D_3$ being too low to prevent pathological activation of Th-1 responses of the immune system.

This assumption is consistent with findings from epidemiological studies that a compromised vitamin D status in humans increases the risk for Th-1 cytokine-mediated autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematodes, multiple sclerosis as well as type I diabetes mellitus [54–60]. In animal models of these diseases, administration of 1,25-$(OH)_2D_3$ has been shown to prevent the development, or at least to ameliorate the symptoms of a chronic inflammatory autoimmune reaction [61–64]. Under normal conditions 1,25-$(OH)_2D_3$ inhibits $CD4^+$ T-lymphocyte proliferation and suppresses the release of typical Th-1-type cytokines, i.e. interleukin (IL)-2, IFN-γ or tumour necrosis factor-alpha (TNF-α) [61,64–67]. This effect of 1,25-$(OH)_2D_3$ has been traced to the inhibitory action of the steroid on IL-12 production by monocytes, which in turn would block differentiation of Th-0 into Th-1 lymphocytes. Extensive evaluation of the direct effects of 1,25-$(OH)_2D_3$ on human $CD4^+$ and $CD8^+$ T lymphocytes in our laboratory [68,69] showed that 1,25-$(OH)_2D_3$ had a limited effect on the constitutive expression of Th-1 and of Th-2 cytokines. Importantly, however, the steroid significantly inhibited IL-12-induced production of IFN-γ and IL-2, and, when Th-2 differentiation was induced by IL-4, it significantly expanded the percentages of cells producing IL-4 and IL-13. It must be emphasized that the predominant effect of 1,25-$(OH)_2D_3$ on T lymphocytes, particularly in the presence of IL-4, was the induction of separate $CD4^+$ and $CD8^+$ subpopulations with almost exclusive expression of IL-6. This is an important facet of the immune-modulating action of 1,25-$(OH)_2D_3$, because IL-6 may act in parallel with the steroid in modulation of T-helper effector-cell functions. We conclude from this that, *in vivo*, the composite of specific actions of 1,25-$(OH)_2D_3$ on cytokine-stimulated T-cell functions could lead to predominance of Th-2 over Th-1 responses. Consequently, vitamin D must be considered a useful agent for prevention and therapy of Th-1-mediated autoimmune diseases.

Tuberculosis

As mentioned already, hypovitaminosis D has been linked to the prevalence of infectious diseases, particularly of tuberculosis in animal species as well as in humans [70–72]. As 1α,25$(OH)_2D_3$ promotes killing specifically of mycobacteria by enhancement of nitric oxide production in macrophages, it is clear why in cases of *M. bovis* infection of vitamin D-deficient mice, lung colonization and the lesion area exceed that of vitamin D-replete mice [70].

Inflammatory bowel disease

Although Crohn's disease [73] and ulcerative colitis (UC) are two clinically distinct forms of inflammatory bowel disease (IBD), they have some basic pathogenetic features in common, such as an aberrant local immune reaction, i.e. an excessive Th/Tc1 response to luminal, mainly bacterial antigens in genetically predisposed individuals [74,75]. In addition, evidence is accumulating that links a number of environmental factors to the pathogenesis of IBD, particularly of UC [75]. As outlined earlier, vitamin D deficiency might be one of those factors [55,56]. One must assume that, as a consequence of hypovitaminosis D, local production of 1,25-$(OH)_2D_3$ by mucosal epithelial cells as well as by macrophages [14,22] within inflammatory lesions falls below a level that is critical for suppression of enhanced Th-1-cell responses, which are typically associated with chronic enterocolitis [74]. In addition, a defect in up-regulation of CYP27B1 in macrophages by immune stimuli, as observed in autoimmune diabetes mellitus [22], may contribute to ineffective suppression of Th-1 responses by endogenous 1,25-$(OH)_2D_3$.

The importance of the vitamin D endocrine system for maintenance of normal immune responses in the gut is highlighted by the recent observation that a lack of expression of the VDR aggravates symptoms in murine experimental colitis models [73]. Strong though indirect support for the assumption that vitamin D deficiency plays a role in the pathogenesis of IBD comes also from a study by Cantorna *et al.* [63] who showed that the active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$ prevents and ameliorates symptoms of IBD in an experimental mouse model, i.e. the IL-10 ko. mouse.

It is of interest to note that vitamin D deficiency, as mentioned earlier, predisposes for mycobacterial infections in animal species [70] and possibly also in man [71], which have long been speculated to be involved in the pathogenesis of CD [76].

Multiple sclerosis

Multiple sclerosis is an autoimmune disease in which overreaction of the Th-1 lymphocyte system to an as yet unidentified antigenic stimulus leads to an immune attack on the central nervous system. An association between hypovitaminosis D owing to low sunlight exposure and incidence of multiple sclerosis was first recognized by Goldberg [77,78] 30 years ago. Since then this notion has been supported by data from additional epidemiological as well as experimental animal studies (for review [60]). Interestingly, dietary supplementation with vitamin D together with calcium and magnesium decreased the relapse rate of the disease in a small group of patients with multiple sclerosis

[79]. From studies on experimental autoimmune encephalomyelitis, an animal model of the human disease, it became clear that inefficient suppression by 1,25-$(OH)_2D_3$ of Th-1-lymphocyte function is a major pathogenetic factor of the disease [80,81].

Rheumatoid arthritis

There is substantial evidence from studies with animal models of rheumatoid arthritis that the beneficial effect of vitamin D on the development of the disease results from its specific immunomodulatory effects as detailed earlier. The hypothesis that hypovitaminosis D may be involved in the pathogenesis of rheumatoid arthritis recently gained support from an 11-year prospective study of a cohort of nearly 30 000 women aged 55 years and older, which revealed an inverse association between vitamin D intake and rheumatoid arthritis [58]. In this respect it is important to note that CYP27B1 activity is present in synovial tissue [16], so that in cases of low serum 25-(OH)-D_3 local production of 1,25-$(OH)_2D_3$ is too low to sufficiently suppress the activity of Th-1 cytokines, particularly that of TNF-α, which plays a central role in the development of chronic inflammatory symptoms and bone destruction.

Diabetes mellitus type I

There is substantial evidence from studies with nonobese diabetic mice that vitamin D deficiency in early life accelerates the appearance of the symptoms of autoimmune diabetes mellitus [82] and that, conversely, 1,25-$(OH)_2D_3$ can prevent the development of the disease (cf. e.g. [83,84]). Recently, Hypponen *et al.* [57] reported the results of a large birth-cohort study highlighting the importance of vitamin D supplementation for the prevention of diabetes mellitus type I in children. Their data clearly showed that regular vitamin D intake compared with no supplementation during the first year of life was associated with an 88% risk reduction of type 1 diabetes mellitus in later life. Even children who received vitamin D irregularly had an 84% lower risk than those with no supplementation.

Metabolic syndrome

Although there are several reports that patients with diabetes mellitus type II present with low 25-(OH)D serum levels (cf. [85,86]), the mechanism through which hypovitaminosis D could contribute to the characteristic metabolic disturbances of the disease remained a matter of speculation. Recently, Chiu *et al.* [87] presented data from a study of healthy, glucose-tolerant young adults showing that serum 25(OH)D levels are positively correlated with insulin sensitivity (cf. [88]). In addition, hypovitaminosis D had a negative effect on β-cell function. Importantly, subjects with low 25-(OH)D had a threefold higher prevalence of parameters of metabolic syndrome than vitamin D-sufficient individuals.

It has been known for a long time that 1,25-$(OH)_2D_3$ is a positive regulator of insulin secretion by pancreatic β cells. Considering the fact that these cells are endowed with CYP27B1 activity [18], this explains why low 25-(OH)D levels are associated with impaired β-cell function. However, it is difficult to understand why insulin sensitivity is reduced in hypovitaminosis D, as in none of the classical target organs for insulin action, i.e. liver, adipose tissue and skeletal muscle, has there been an observed capacity to convert 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$. It remains to be seen whether a direct metabolic effect of 25-$(OH)D_3$, such as that described by Birge and Haddad [89] for skeletal muscle, could be responsible for the changes in insulin sensitivity in this and other target organs of the hormone.

Part II: calcium malnutrition and chronic diseases

Low calcium intake: a widespread phenomenon

Different levels of daily calcium intake have been recommended for infants, children and adults [90] to assure optimal whole-body calcium retention and consequently adequate development and maintenance of bone mass and mineral density (see among others e.g. [91]). A minimum of 1000 mg per day is recommended for men between 25 and 65 years of age, and also for women in the same age group, except when higher intake is necessary during pregnancy and lactation or when on oestrogen replacement therapy. Recommended for men and women alike is 1500 mg calcium per day when older than 65 years [90]. Although it is well known that particularly the elderly ingest significantly less calcium in their diet than the recommended amount [92], evidence is accumulating that calcium malnutrition is a more widespread phenomenon than previously thought: in a recent survey on the calcium, vitamin D and bone status of a healthy adult population, in which we could enrol more than 1000 individuals of both sexes [7], we found that the average daily calcium intake, independent of age and sex, was 560 mg, which is significantly less than any recommended daily allowance.

Also, a negative calcium balance certainly exists in the many individuals suffering worldwide from lactose intolerance (cf. [93]). Finally, intestinal calcium malabsorption is frequently observed in the elderly but is also associated with inflammatory diseases of the small intestine (e.g. Crohn's disease) or with all cases of vitamin D deficiency (for definition see Part I) resulting from intestinal, hepatic, renal or endocrine disorders.

Inadequate supply of calcium with the diet or intestinal calcium malabsorption must be considered important contributing factors to common diseases such as osteoporosis and hypertension (for review see [94]), but may also play a role in the development of mammary [95], prostate [96] and, particularly, colon tumours [97]. Although there is reason to believe that maintenance of adequate serum calcium levels is a prerequisite also for normal function of the innate

Table 2 Non-parathyroid human tissues and cells expressing the extracellular calcium-sensing receptor

Tissue or cells	Expression			Reference
	mRNA	Protein	Functional activity	
Bone				
Osteoblasts	Yes	Yes	Yes	[101]
Large Intestine				
Normal mucosa	Yes	Yes	ND	[134]
Adenocarcinoma	Yes	Yes	Yes	[102,105,134]
Breast				
Normal mammary gland and breast cancer cells	Yes	Yes	ND	[138]
Ovary				
Normal epithelial and cancer cells	Yes	Yes	Yes	[150]
Prostate				
Cancer cells	Yes	Yes	Yes	[151]
Monocytes/macrophages	Yes	Yes	ND	[104]
Pancreas				
Islet cells	Yes	Yes	Yes	[152]
Acinar and duct cells	Yes	Yes	Yes	[153]

ND, not determined.

immune system [98,99], a link between a negative calcium balance and a specific infectious or chronic inflammatory disease has not yet been established.

Role of the extracellular Ca^{++}-sensing receptor in local control of cellular functions

Systemic calcium homeostasis is achieved through the regulatory effects of 1,25-$(OH)_2D_3$ and of PTH on calcium handling in the intestine, kidney and bone [1]. This allows maintenance of serum Ca^{++} concentrations within a narrow range, so that even a relatively large deficit in dietary calcium induces only minute variations in extracellular fluid calcium concentrations. How these can be translated into modulation of cellular functions remained an enigma until Brown and associates identified the extracellular calcium-sensing mechanism of the parathyroid gland as mediated by a G protein-coupled type plasma membrane receptor, the Ca^{++}-sensing receptor (CaR), which transduces changes in plasma Ca concentrations along intracellular signalling pathways into modulation of PTH secretion [100]. In the past 10 years, numerous cell types have been identified as expressing the CaR, among them osteoblasts [101], renal tubular cells [100], enterocytes [102,103] and monocytes [104] etc. (for details see Table 2). As illustrated in Fig. 2, the CaR modifies intracellular signalling in a cell-type-specific manner because there are species- and cell-specific differences in the pattern of stimulatory and inhibitory G proteins that link G protein-coupled receptors to their downstream effectors (cf. [105]). As the CaR is responsive to calcium concentrations within the millimolar range found in extracellular fluids, it has an important role in local control of a wide range of cellular functions (for review [106]).

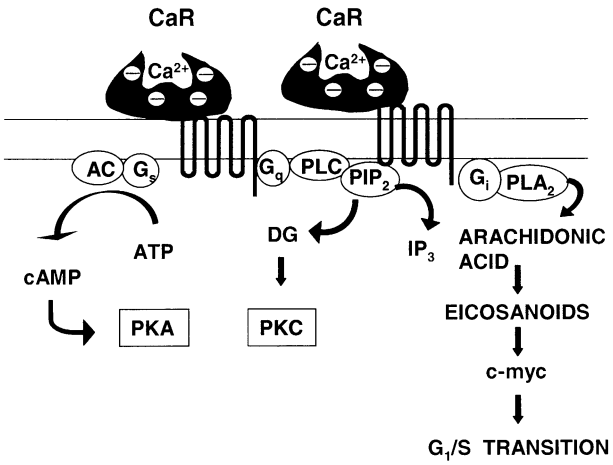

Figure 2 Coupling of the CaR to intracellular signalling pathways in human colon adenocarcinoma-derived Caco-2 cells: relevance for control of proliferation. AC, adenylate cyclase; PK, protein kinase; DG, diacylglycerol; PL, phospholipase; PIP_2, phosphoinositol diphosphate; $G_{s,\ q,\ i}$, G-coupling proteins (for details see [105]).

Osteoporosis

Recently, Riggs *et al.* [107] proposed a unitary model for involutional osteoporosis, in which, apart from hormonal imbalances, calcium malnutrition and calcium malabsorption are considered of great significance for the development of the disease.

Although calcium has a positive effect on bone mass, the mechanism by which extracellular calcium modulates bone formation has long been an enigma. Leis *et al.* [108] were the first to describe a functional Ca^{2+}-sensing mechanism

on osteoblast-like MC3T3-E1 cells. Quarles *et al.* [109] as well as Yamaguchi *et al.* [110] showed that clonal osteoblastic cell lines possess a CaR that is similar, if not identical, to the CaR of the parathyroid gland. Activation of the CaR by a rise in $[Ca^{2+}]_o$ increases the proliferative activity of osteoblastic cells, and could therefore explain the positive effects of calcium supplementation on bone mineral density.

Adequate calcium nutrition and maintenance of a normal vitamin D status must therefore be considered important measures for prophylaxis and treatment of osteoporosis [111–114]. Chapuy *et al.* [115] conducted a large randomized trial on the effect of calcium supplementation on fracture prevention in more than 3000 elderly women. They showed quite convincingly that an additional 1200 mg of calcium, when given daily with 800 IU vitamin D_3 for 18 months, reduced the incidence of hip fractures by 43% in the treatment vs. the placebo group. Most recently, Larsen *et al.* [116] presented results from a 3-year population-based intervention study showing that in approximately 5000 elderly community-dwelling residents, daily supplements of 1000 mg of calcium plus 400 IU vitamin D_3 reduced the average incidence of osteoporotic fractures at all sites of the skeleton by 16%.

Colorectal cancer and other human malignancies

There is increasing evidence from epidemiological studies that some of the most frequent human malignancies such as breast [95,117–119], prostate [96], and particularly colorectal cancer are associated with poor calcium nutrition [97,120–122]. A number of experimental and clinical studies have been undertaken to explore the possibility that calcium supplementation could reduce the risk of carcinogenesis in the mammary gland [95] as well as in the large intestine [97,123–126].

Colorectal cancer

Already in 1985, Garland *et al.* [127] had reported the results of a 19-year prospective trial, which allowed the identification of low dietary calcium as an independent risk factor for colon cancer. Additional findings by Garland *et al.* [121] on an inverse association between nutritional calcium levels and colon cancer risk seem to indicate that the incidence of colorectal cancer can be reduced by approximately 70% if daily calcium intake is raised from 800 to 1400 mg. Although a link between low dietary calcium intake and a high incidence of colon cancer has thus been suspected for a long time [121,127], other studies on regional differences in nutritional habits and cancer incidence have been inconclusive. However, several large prospective cohort studies have consistently reported an inverse association between dietary calcium and risk of colorectal carcinoma (for review see [128]). For example, Wu *et al.* [126] analyzed data from two large prospective trials, involving more than 80 000 women and 40 000 men, and reported a significant risk reduction for distal colon cancer for subjects whose dietary calcium intake was ≥ 1250 mg compared with those who ingested 500 mg or less.

In addition, a number of experimental and clinical studies have shown that calcium supplementation reduces cellular hyperproliferation in the large intestine of humans and prevents development and recurrence of colon adenomas [129–131].

Cross *et al.* [27,132] showed that growth of human colon adenocarcinoma-derived cells could be slowed down by raising the extracellular calcium concentration in the culture medium. Cell-cycle analysis by flow cytometry indicated that transition from the G1 into the S phase seems to be a key step in regulation of colon cancer cell proliferation by $[Ca^{++}]_o$ [103]. At the same time, expression of the proto-oncogen c-myc is suppressed [29], which is also known as a cell-cycle regulatory gene acting on G1/S transition.

The ability of human colon cancer cells to react to signals from $[Ca^{++}]_o$ by changes in their proliferative activity [102] suggested the involvement of a specific extracellular calcium-sensing molecule in $[Ca^{++}]_o$–mediated cell-cycle control. In fact, human colon carcinoma cells express the parathyroid-type CaR [100] at the mRNA as well as protein level [102,133]. Immunohistochemical analysis of human colon carcinomas of different grading revealed that a majority of CaR-positive cells were confined to differentiated areas within the cancerous lesion. Poorly or undifferentiated regions were either devoid of specific immunoreactivity or contained only isolated cells expressing the CaR protein [103,134].

Molecular analysis by Southern blotting and reverse transcriptase polymerase chain reaction did not reveal any abnormalities of the CaR gene in DNA derived from normal or cancerous human colon mucosa [105]. The absence of even a single-point mutation indicates that also in colon carcinoma cells, the CaR gene encodes a functional CaR molecule. The events downstream of CaR activation that actually link the CaR to cell-cycle control probably involve inhibition of phospholipase A_2 activity [105] which, in turn, would reduce the amount of arachidonic acid available for synthesis of proliferation-stimulating prostaglandins (cf. Figure 2).

The development of tumours in the large intestine from hyperproliferative foci via adenomas into cancerous lesions could serve as a paradigm for a possible role of the CaR in modulating growth of $[Ca^{++}]_o$-sensitive proliferating normal and neoplastic cells. Proliferation, differentiation and apoptosis of colonocytes occur along the crypt axis all at the same time, requiring precise coordination between cell division at the base and cell death at the mouth of the crypt. A calcium gradient may help this synchronization if one assumes that there exists a 'calcium switch' in colonic crypt cells which triggers proliferation at low $[Ca^{++}]_o$ and induces differentiation and eventually apoptosis with $[Ca^{++}]_o$ progressively increasing in the base-to-mouth direction [135]. The CaR may well be an essential part of this switch mechanism. Owing to its preferential location at the base of the crypt [134,136] the CaR would determine the rate of

proliferation according to the ambient $[Ca^{++}]_o$. A n increase in $[Ca^{++}]_o$ will thus inhibit division of crypt base cells and facilitate their transit out of the proliferating into the differentiating compartment in the upper part of the crypt. As even neoplastic colonocytes up to a certain degree of dedifferentiation are endowed with a cell-type-specific functional CaR, the use of calcium supplements can be considered an effective chemopreventive measure against the development of colorectal cancer.

Other malignancies

Several epidemiologic and experimental studies have suggested that decreased calcium intake is associated with mammary gland carcinogenesis (for review [45]). The importance of adequate dietary intake of calcium for prevention of breast cancer is highlighted by the results of a recent study by Shin *et al.* [46], who analyzed data from a large cohort of women in the Nurses' Health Study indicating that premenopausal women, who consumed more than 800 mg calcium daily, had a 30% lower risk of breast cancer compared with women with a daily intake of ≤ 200 mg calcium.

There is long-standing evidence that normal human breast epithelial cells in culture react to an increase in ambient Ca^{++} within the physiological range by growth reduction and terminal differentiation [137]. As Cheng *et al.* [138] demonstrated the presence of CaR protein in normal as well as malignant mammary gland epithelial cells, it seems very likely that the positive effect of calcium supplementation on the incidence of breast cancer can be eventually linked to a CaR-mediated inhibitory effect of local Ca^{++} on mammary gland cell growth.

Hypertension

A number of epidemiological as well as experimental and clinical studies suggest that primary hypertension might be related to an inadequate intake of calcium (for review see McCarron *et al.* [139]). The First National Health and Nutrition Survey, 1971–74, a careful analysis of dietary habits of more than 10 000 people between the ages of 18 and 74 years, revealed a significant correlation between dietary calcium intake and arterial blood pressure [140]. Evidence that hypertension could be related to aberrant regulation of calcium homeostasis comes from experimental as well as from clinical studies [141–143]. Although addition of dietary calcium can lower diastolic blood pressure in healthy adults, it most visibly affects systolic blood pressure in patients with mild to moderate hypertension [144].

There is now cumulative evidence from more than 60 observational studies as well as randomized clinical trials that low dietary calcium is a significant risk factor for primary hypertension or, conversely, that calcium supplementation causes a consistent drop in blood pressure [94]. However, the mechanism by which minute changes in extracellular calcium could have major effects on blood pressure regulation and, consequently, on the development of primary hypertension has not yet been fully elucidated. There is preliminary evidence that activation of the renal CaR leads to enhanced prostaglandin E_2 synthesis with natriuresis as a consequence. The concomitant reduction in plasma volume would then account for the blood pressure-lowering effect of elevated extracellular calcium [145].

Autoimmune diseases

In a clinical pilot study, Goldberg *et al.* [79] found that combined treatment with calcium, vitamin D and magnesium decreased the relapse rate in a small group of young, male patients with multiple sclerosis. More direct evidence for an essential role of calcium in suppression of autoimmune responses has been obtained to date from experimental animal studies: for example, Cantorna *et al.* [81] reported that the preventive effect of 1,25-$(OH)_2D_3$ on the development of chronic inflammatory symptoms in a mouse model of human multiple sclerosis depends largely on the level of dietary calcium supply. Likewise, Mathieu *et al.* [98] showed that as consequence of VDR disruption, hypocalcaemic VDR k.o. mice display important defects in macrophage function and in cellular immunity. Notably, however, immune cell function can be fully restored by normalization of calcium homeostasis. This indicates that the function of 1,25-$(OH)_2D_3$ in the immune system is redundant and that other factors such as calcium subsume the role of vitamin D. A similar situation seems to exist in human hereditary 1,25-dihydroxyvitamin D-resistant rickets [146]. Owing to loss-of-function mutations in the VDR gene patients with this disease present with hypocalcaemia and severe rickets. Maintenance treatment of the disease requires supplementation with high doses of calcium. By analogy to the VDR k.o. mouse model, one may therefore assume that restoration of serum calcium to near normal levels is responsible for those patients not exhibiting any clinically apparent immunological defects [146].

Part III: prevalence of hypovitaminosis D and calcium deficiency in a normal adult population

In a population-based cross-sectional study on calcium and vitamin D status we found that in a cohort of more than 1100 healthy adults of both sexes [6,7] the average daily intake of calcium was 568 mg. Although individual values ranged from 40 to 2170 mg of calcium per day, the great majority of study participants, i.e. 87%, ingested less than 1000 mg calcium per day and thus did not meet the recommended daily allowance [90]. In the same study, 26% of all participants had serum 25-(OH)D less than 12 ng mL^{-1} (= 30 nM), a value commonly used as the cut off-point for determination of hypovitaminosis D (cf. [5]).

When we calculated the distribution of calcium intake levels in the vitamin D-sufficient and -insufficient group, we found that in only 11% was there no evidence of a

compromised calcium and vitamin D status. Sixty-one per cent consumed less than 1000 mg calcium per day but had sufficiently high 25-(OH)D serum levels. Of the 324 subjects with serum 25-(OH)D-values indicative of hypovitaminosis D, only 58 (i.e. 5% of the entire cohort) consumed 1000 mg or more of calcium per day. Notably, 266 individuals with hypovitaminosis D had reported a daily calcium intake of less than 1000 mg. This means that 23% of the entire cohort exhibited combined vitamin D and calcium insufficiency.

Vitamin D or calcium insufficiency, respectively, contribute to the development of chronic diseases by different pathogenic mechanisms (as detailed earlier). A nutritional calcium deficit or a compromised vitamin D status has been identified as an independent risk factor, as in the case of colon cancer [127]. More often, however, the calcium and vitamin D statuses appear to act largely together, such as in the pathogenesis of osteoporosis, colorectal and breast cancer, and probably also of autoimmune diabetes type I and multiple sclerosis. With respect to osteoporosis it is commonly accepted that combined vitamin D and calcium supplementation is the basis of any therapeutic measures [115,116]. Grau *et al.* [147] in their study on the effect of vitamin D and calcium supplementation on recurrence of colorectal adenomas found that calcium supplementation was effective only in patients with normal 25-(OH)D-values. Conversely, high 25-(OH)D levels were associated with a reduced risk of adenoma recurrence only among subjects receiving calcium supplements. This may be owing to the fact that vitamin D metabolism in the colon mucosa can be manipulated by dietary calcium in favour of higher local $1,25(OH)_2D_3$ production [20,21]. Conversely, there is reason to assume that $1,25(OH)_2D_3$ augments CaR-mediated antiproliferative responses of extracellular Ca^{2+} [148,149]. Synergistic actions of calcium and vitamin D are likely to be the reason why a high intake of low-fat dairy products is associated with a reduced risk of breast cancer in premenopausal women [46]. Finally, as discussed earlier, results from studies with animal models of human autoimmune diseases indicate that calcium supplementation is necessary to optimize the therapeutic effect of vitamin D [81,98]. Because it is apparent that calcium and vitamin D in general interact mechanistically and also metabolically in homeostatic control of joint target and organ cell functions, correction of both a nutritional calcium deficit and a compromised vitamin D status is required to prevent the development of some of the most frequent chronic diseases. The fact that, as detailed earlier, almost one quarter of the healthy adult population presents with a compromised vitamin D status and, at the same time appears also to be calcium deficient, poses a special challenge for preventive medicine and public health policy alike.

Conclusion

Extensive research on the activity and function of the CYP27B1-encoded 25-(OH)D-1α-hydroxylase and of the CaR has contributed much to our understanding of how locally produced $1,25-(OH)_2D_3$ as well as extracellular calcium ions exert tissue-specific control of cellular growth, differentiation and function. Because many cell types express the 25-(OH)D-1α-hydroxylase and the CaR, different organ functions are necessarily affected by changes in the activity of either molecule. We hypothesize that this is likely to occur under conditions of hypovitaminosis D or when induced by a chronically negative calcium balance. Thereby we provide an explanation on a molecular and cellular basis for the many observations from epidemiological studies that significant associations exist between deficits in calcium and vitamin D and the pathogenesis of frequent chronic diseases. We hope that a deeper insight into the pathogenic consequences of a compromised calcium and vitamin D status will also stimulate the discussion on the importance of calcium and vitamin D substitution or even supplementation, respectively, for preventive and clinical medicine.

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