VITAMIN D AND PERIODONTAL INFECTION

Georgios Antonoglou
GEORGIOS ANTONOGLOU

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UNIVERSITY OF OULU, OULU 2015
Abstract

The aim of the present study was to examine associations between serum 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D[1,25(OH)2D]—the circulating and active forms of vitamin D—and periodontal infection.

The data were gathered from a case-control study (63 periodontitis patients and 30 periodontally healthy controls) and an intervention study among individuals with type 1 diabetes mellitus (T1DM, 80 patients at the baseline and 65 after periodontal treatment). The periodontal data and the levels of serum 25(OH)D, 1,25(OH)2D and parathyroid hormone (PTH) were available. A third data set included periodontal data and the serum level of 25(OH)D of 1262 non-smoking and non-diabetic 30–49-year-old individuals (Health 2000 Survey). Serum 25(OH)D analyses were done using enzyme-linked immunoassay and radioimmunoassay, 1,25(OH)2D analyses using enzyme-immunoassay after purification of 1,25(OH)2D by immunoeXtraction and PTH analyses using electrochemiluminescence immunoassay.

In the case-control study individuals with a low serum 1,25(OH)2D level were more likely to belong to the periodontitis group than to the periodontally healthy group and an inverse association was observed between serum 1,25(OH)2D and severity of periodontitis at the baseline of the intervention study. Serum 1,25(OH)2D increased significantly after periodontal treatment in the T1DM patients; a finding that was considered suggestive of a causal relationship between serum 1,25(OH)2D and periodontal infection. Also, serum PTH increased after periodontal treatment; this increase, which was statistically significant (p = 0.016) in patients with moderate or severe periodontitis, may partly account for the earlier observed post-treatment increase in serum 1,25(OH)2D level. Possible explanations for low serum 1,25(OH)2D in periodontal infection may be increased degradation of 1,25(OH)2D, increased use of 1,25(OH)2D, or decreased hydroxylation of 25(OH)D.

The association between serum 25(OH)D level and periodontal infection was weak, if existent. An inverse association between serum 25(OH)D and the severity of periodontal infection was observed only in the T1DM patients. Among individuals with low plaque level, those in higher 25(OH)D quintiles tended to have fewer teeth with deepened periodontal pockets than those in lower quintiles; a finding which was interpreted to mean a slight protective role of 25(OH)D against periodontal infection.

Keywords: 1,25(OH)2D, 25(OH)D, diabetes mellitus, gingival bleeding, periodontitis, serum, Vitamin D
Tiivistelmä


Tapaus-verrokki-tutkimuksessa yksilöt, joilla seerumin 1,25(OH)2D taso oli alhainen, kuului todennäköisemmin parodontiittien kuin verrokkiryhmään. Interventiotutkimuksen alkutilanteessa seerumin 1,25(OH)2D:n ja parodontaalii-infektion vaiksestaen välillä vallitsi tilastollisesti merkittävä käänteinen yhteys ja taso nousi merkittävästi infektion hoidon jälkeen. Myös seerumin PTH taso nousi parodontaalii-infektion hoidon jälkeen; nousu oli tilastollisesti merkittävä (p = 0.016) pitkälle edennyttä parodontaalii-sairastavilla. Interventiotutkimuksen tulokset viittavat kausaaliseen yhteyteen 1,25(OH)2D:n ja parodontaalii-infektion välillä. Alhainen seerumin 1,25(OH)2D pitoisuus infektion vallitessa voi selittää sen suurella käytöllä immunipuoillumukseen infektion alana tai lisääntyneellä hajoamisella. Tason nousu hoidon jälkeen tekee edellä mainittua. PTH on 25(OH)D:n hydroksylaation pääsääntöjä ja 1,25(OH)2D:n nousua hoidon jälkeen voi selittää myös seerumin PTH tason kohoaminen.


Asiain mat: 1,25(OH)2D, 25(OH)D, D-vitamiini, diabetes mellitus, ienverenvuoto, parodontiitti
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28th August, 2015

Georgios Antonoglou
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>25(OH)D</td>
<td>25- hydroxyvitamin D</td>
</tr>
<tr>
<td>1,25(OH)_{2}D</td>
<td>1,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>AL</td>
<td>Attachment Level</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CASR</td>
<td>Calsium Sensing Receptor</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
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<tr>
<td>HbA1c</td>
<td>Glycosylated Hemoglobin A1c</td>
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<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<tr>
<td>OR</td>
<td>Odds Ratio</td>
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<tr>
<td>P</td>
<td>Phosphorus</td>
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<tr>
<td>PD</td>
<td>Periodontal Pocket Depth</td>
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<tr>
<td>PRR</td>
<td>Prevalence Rate Ratio</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
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<tr>
<td>RR</td>
<td>Risk Ratio</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear factor Kappa-B Ligand</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1 (T_h1)</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2 (T_h2)</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17 (T_h17)</td>
</tr>
<tr>
<td>TREG</td>
<td>T regulatory</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
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</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals.


Contents

Abstract 7
Acknowledgments 7
Abbreviations 9
List of original publications 11
Contents 13
1 Introduction 15
2 Literature review 17
  2.1 Vitamin D – general aspects ................................................................. 17
  2.2 The storage form of Vitamin D, 25(OH)D .............................................. 18
  2.3 The active form of Vitamin D, 1,25(OH)2D ....................................... 20
    2.3.1 Functions of 1,25(OH)2D in innate immunity .................................. 21
    2.3.2 Functions of 1,25(OH)2D in adaptive immunity ............................ 23
    2.3.3 Functions of 1,25(OH)2D in bone metabolism .............................. 25
  2.4 Gingivitis and periodontitis: diagnosis and epidemiology .................. 25
  2.5 Pathogenesis of periodontitis – possible role of Vitamin D ............... 26
  2.6 Mediators of inflammation in serum linked with periodontal condition ................................................................. 27
  2.7 Serum 25(OH)D and 1,25(OH)2D in systemic immune mediated and infectious diseases ................................................................. 28
  2.8 Association between serum 25(OH)D and periodontal health status ................................................................................................. 29
    2.8.1 Clinical studies ............................................................................. 29
    2.8.2 Vitamin D supplementation studies ........................................... 30
  2.9 Association between serum 1,25(OH)2D and periodontal health status ................................................................................................. 30
  2.10 Parathyroid hormone (PTH), the main regulator of 1,25(OH)2D production ......................................................................................... 31
3 Aims of the study 33
4 Materials and methods 35
  4.1 Ethical approval .................................................................................. 35
  4.2 Study populations .............................................................................. 35
    4.2.1 Oulu Diabetes Study ..................................................................... 36
    4.2.2 Health 2000 Survey ...................................................................... 37
  4.3 Clinical oral examination and periodontal therapy ........................... 37
4.4 Laboratory analyses ................................................................. 38
4.5 Covariates and confounding factors ........................................ 39
4.5 Statistical methods ................................................................. 41

5 Results

5.1 Serum levels of Vitamin D metabolites - seasonal variation
   (Papers I-III)........................................................................... 43
5.2 Serum 1,25(OH)\textsubscript{2}D and periodontal health (Papers I, II) ............ 47
   5.2.1 Case control study (Paper I) ........................................... 47
   5.2.2 Intervention study (Paper II)............................................ 48
5.3 Serum 25(OH)D and periodontal infection (Papers I, II, IV).......... 49
5.4 Serum PTH, Ca and P concentrations and periodontal health
   (Paper IV)............................................................................. 51

6 Discussion

6.1 Association between serum 1,25(OH)\textsubscript{2}D and periodontal health
   (Papers I, II, IV).................................................................... 53
6.2 Association between serum 25(OH)D and infectious periodontal
   diseases (Papers I – III).......................................................... 54
6.3 Methodological considerations ................................................. 56
   6.3.1 Study designs and samples (Papers I-IV)............................. 56
   6.3.2 Periodontal variables ....................................................... 57

7 Summary and conclusions ......................................................... 59

References .................................................................................. 61

List of original publications .......................................................... 73
1 Introduction

Vitamin D is known as a key player in bone metabolism and calcium (Ca) homeostasis (Dusso et al. 2005). Furthermore, an increasing amount of studies support the conception that Vitamin D also has anti-inflammatory and anti-infective properties (Bruce et al. 2010). When intra-cellular Vitamin D receptors (VDR) and the expression 1,25-dihydroxy Vitamin D [1,25(OH)₂D] extrarenally by disease-associated macrophages were recognized, Vitamin D studies have focused, since the 1980s, on the immune-modulatory actions of 1,25(OH)₂D. The available evidence from the past ten years suggests that low levels of the two forms of Vitamin D—the storage form, 25-hydroxyvitamin D [25(OH)D], and its 1-α hydroxylated active form, 1,25(OH)₂D—are associated with a wide range of inflammatory/infectious conditions (Cannell et al. 2014).

The most common infectious diseases of the periodontium are gingivitis, defined as inflammation in the gingival epithelium and connective tissue, and periodontitis, in which inflammation-associated loss of periodontal connective tissue and tooth-supporting bone occurs. The etiologies of gingivitis and periodontitis are microbial (Haffajee & Socransky 1994) but this notion is being updated and expanded today (Teles et al. 2013, Lopez et al. 2015). Susceptibility to the disease varies between individuals and is affected by a number of systemic diseases and conditions. On the other hand, periodontal infection has been suggested to predispose to systemic diseases (Tonetti 2009, Borgnakke et al. 2013). To what extent the latter association is causal by nature or due to shared risk factors is so far unclear.

The host responses to microbial antigens in gingivitis and periodontitis include actions of both innate and adaptive immunity, which are known to be partly modulated by Vitamin D, especially its active form, 1,25(OH)₂D (Cannell et al. 2014). Thus, it is likely that serum Vitamin D level could be associated with the extent and severity of infectious periodontal diseases.

Previous studies of the relation between the storage form of Vitamin D, 25(OH)D, and infectious periodontal diseases do not conclusively show markedly positive effects of 25(OH)D on periodontal condition (Dietrich et al. 2004, Dietrich et al. 2005, Miley et al. 2009, Boggess et al. 2011, Jabbar et al. 2011, Millen et al. 2014). Moreover, no clear benefits of Vitamin D supplementation have been shown (Krall et al. 2001, Krall 2001, Garcia et al. 2011). So far there are no clinical studies of the association between serum 1,25(OH)₂D and periodontal infection. Well-
designed studies examining the association of both $25(OH)D$ and $1,25(OH)_{2}D$ may shed light on the role of Vitamin D in periodontal health.
2 Literature review

2.1 Vitamin D – general aspects

It is theorized that Vitamin D has existed on the earth for over 900 million years and that it was first produced by phytoplankton and zooplankton to protect them from the sun’s UV radiation (Keegan et al. 2013). Evidence of its existence was reported for the first time in 1924 (Steenbock 1924), but related research had already started some 10 years earlier (McCollum & Davis 1913). The Nobel Prize in Chemistry in 1928 was awarded to Adolf Otto Reinhold Windaus for his major contribution to research on sterols and their connection with Vitamin D.

There has been an ongoing debate on the nomenclature around Vitamin D to this day, and valid arguments have been raised on both sides; one side claims that Vitamin D can be referred to as a pro-hormone or hormone (DeLuca 2004) and the opposition suggests that Vitamin D should be regarded as a nutrient only, not a hormone or anything else (Vieth 2004).

In the present study, it was considered that Vitamin D is a hormone, primarily because it is synthesized from cholesterol like the hormones of the steroid family. Secondly, in terms of kinetics and other characteristics of the “pre-active forms”, it does have a pre-hormonal (Precursor Vitamin D₃, i.e. irradiated 7-Dehydrocholesterol), a pro-hormonal [25(OH)D], and a hormonal [1,25(OH)₂D] form (Figure 1). Moreover, it exerts biological paracrine (cell to cell) and endocrine (liver/kidney to bone) activities (Dusso et al. 2005).

The two storage form variants of Vitamin D in serum are D₂ or ergocalciferol or ercalcidol, which is obtained from diet, and D₃ or cholecalciferol or calcidiol which is produced by skin after irradiation by sunlight (Figure 1). The only difference between the two is a structural one in the side chains of their molecules, while they appear to be metabolized into the active forms in basically the same manner (Ross et al. 2011, Biancuzzo et al. 2013). In the present work, the term 25(OH)D refers collectively to both 25-Hydroxyergocalciferol (D₂) and 25-Hydroxycholecalciferol (D₃); the same is applicable to 1,25(OH)₂D which represents active forms (1,25(OH)D₂, 1,25(OH)D₃).
2.2 The storage form of Vitamin D, 25(OH)D

Vitamin D in humans starts its “life” as 7-Dehydrocholesterol in skin, advances to a pre-vitamin (or pre-hormone) after irradiation, and is rapidly converted by heat into Vitamin D₃ (Figure 1). After hydroxylation in the liver, both D₂ and D₃ turn into the so-called “storage form” or “major circulating metabolite”, i.e. 25(OH)D (Holick 2007). The standard marker of Vitamin D level is serum 25(OH)D, and it has been positively associated with several health outcomes such as bone health and negatively with immune-mediated diseases (Bischoff-Ferrari et al. 2006, Holick 2007). Although serum 25(OH)D functions well as a biomarker of ‘exposure’, its function as a biomarker of ‘effect’ is not well documented (Del Valle et al. 2011).
Fig. 1. “Classical” properties of Vitamin D. (UVB; Ultraviolet B radiation, 1-OHase; 25-hydroxyvitamin D-1α-hydroxylase, 24-OHase; 25-hydroxyvitamin D-24-hydroxylase, FGF-23; Fibroblast Growth Factor 23, RANK; Receptor Activator of Nuclear factor-KB, RANKL; Receptor Activator of Nuclear factor-KB Ligand, CaBP; Calcium Binding Protein, VDR-RXR; Vitamin D Receptor–Retinoic Acid X-Receptor Complex, TRPV6; Transient Receptor Potential Cation Channel, Subfamily V, Member 6) Reprinted by permission from N Engl J Med 2007, 357:266-81, Copyright Massachusetts Medical Society.
A second debate on the sufficiency and deficiency levels of 25(OH)D in different health outcomes is also ongoing. Although the level of ≥ 75 nmol/L of serum 25(OH)D has been recommended by some distinguished research groups in the field, the Nordic Nutrition recommendations (Andersen 2013) and the recommendations given by the US National Institutes of Health (Ross et al. 2011) state that a level of ≥ 50 nmol/L is adequate for bone and overall health. At the same time, many experts would define serum levels of < 50 nmol/L as Vitamin D deficiency and levels between 50 and 70 nmol/L as insufficiency (Holick 2007, Hansen et al. 2008).

Based on the above definitions, 20–100% of community-dwelling individuals in France and North America, are Vitamin D deficient (Chapuy et al. 1996, Holick et al. 2005, Lips et al. 2006). In Finland, Vitamin D deficiency (≤ 50 nmol/L) is rather prevalent in healthy adults; around 31% of men and 37% of women between 45 and 74 years of age are Vitamin D deficient (Miettinen et al. 2014). Lastly, Vitamin D deficiency is influenced by seasonality, and serum 25(OH)D levels vary based on the individual’s skin type and latitude of residence (Chan et al. 2009).

The Finnish authorities base their recommendations on Vitamin D intake on those of the Nordic Nutrition Council, which advises a daily intake (by supplements, diet alone, or combined) of 10 μg for all age groups and 20 μg for those older than 75 years or those not exposed to adequate sunlight (Andersen 2013). The US National Institutes of Health recommend dietary allowances of 15 μg per day for all except those older than 75 years, who should have 20 μg per day (Ross et al. 2011).

### 2.3 The active form of Vitamin D, 1,25(OH)₂D

Serum 25(OH)D is converted into its active form, 1,25(OH)₂D, which is responsible for the biological actions of Vitamin D. The hydroxylation of 25(OH)D at the 1-α position by the family of enzymes 1,25-hydroxylase (CYP27B), occurs in the kidney, which is the main source of circulating active Vitamin D (Figure 1). Attenuation of the potency of 1,25(OH)₂D—i.e. “metabolic inactivation”—on the other hand, is controlled by the family of enzymes 24-hydroxylase (CYP24A), which is controlled in a reciprocal manner by CYP27B (Christensen et al. 2013).

Production of 1,25(OH)₂D is primarily stimulated by PTH and inhibited by increased serum calcium and phosphate. In addition, insulin-like growth factor, fibroblast growth factor, and 1,25(OH)₂D itself are regulators of the genes associated with CYP27B and CYP24A (Christensen et al. 2013). In addition to the
kidney, conversion into the active form also takes place extra-renally in other organs and cells such as the prostate, breast, colon, immune cells, epithelial cells, and skin cells (Dusso et al. 2005, Cannell et al. 2014).

Most of the studied actions of 1,25(OH)2D are genomic: the binding of 1,25(OH)2D to its nuclear receptor (VDR), recognized in human leukocytes already in 1983 (Provvedini et al. 1983), yields a repositioning of helix 12 in the COOH terminus of the Vitamin D Receptor Ligand Binding Domain (VDR-LBD) and, consequently, a change in its three-dimensional structure. This change allows it to recognize and bind to the hormone response elements of 1,25(OH)2D (Dusso et al. 2004). There is a plethora of 1,25(OH)2D-VDR complex-responsive genes or elements responsible for the expression of molecules associated with bone metabolism and immune functions (Christakos et al. 2013). In addition to genomic actions, which may take effect in hours or days, Vitamin D can also act in a rapid-response manner that affects the calcium channels of cells in the kidney, intestines, and bone (Thompson & Farach-Carson 2011).

In studying Vitamin D metabolites, one should consider that serum levels of 25(OH)D and 1,25(OH)2D are not related in a straightforward manner. In terms of the complexity of their relation, the one thousand-fold difference (nmol/L vs. pmol/L, respectively) in their concentrations adds to their biphasic relation, i.e. a positive association at normal levels of 25(OH)D and a negative association at subnormal levels of 25(OH)D (Nordin & Need 2005, Rejnmark et al. 2008). These two characteristics already show that the relationship between serum 25(OH)D and serum 1,25(OH)2D is difficult to describe accurately.

### 2.3.1 Functions of 1,25(OH)2D in innate immunity

Innate immunity is activated in the early stages of defense against pathogens. Innate immune responses are triggered in response to pathogen-associated molecular patterns (PAMPs) via surveillance proteins such as Toll-like receptors (TLRs) (Takeda et al. 2003). One of these responses is promotion of the expression of CYP27B, the enzyme responsible for hydroxylation of 25(OH)D (Figure 2). An end result of this response to PAMPs is production of anti-bacterial peptides. On the other hand, the enzyme CYP24A is responsible for the inactivation of both 25(OH)D and 1,25(OH)2D.
In 2004, Wang and co-workers demonstrated for the first time that Vitamin D directly induces production of the anti-microbial peptide cathelicidin (Wang et al. 2004). Later on, Liu and co-workers (2006), using a Mycobacterium Tuberculosis model, showed that 1,25(OH)\textsubscript{2}D mediates an anti-microbial response of monocytes, but not of dendritic cells, by regulating the production of peptides such as cathelicidin and β-defensins. Notably, it has been shown that cathelicidin levels after high-dose Vitamin D administration increase only inside peripheral
blood monocytes but not in serum (Adams et al. 2009). Nonetheless, two studies (Bruce et al. 2010, Christakos & DeLuca 2011) concluded that there is not enough evidence to claim that 1,25(OH)2D is an anti-infective agent in humans.

The paracrine immunomodulatory functions of 1,25(OH)2D, resulting from the hydroxylation of 25(OH)D by innate immunity cells, include its effect on monocyte differentiation (Figure 2).

Moreover, 1,25(OH)2D has a substantial effect on the production of inflammatory mediators, that is 1,25(OH)2D inhibits the production of pro-inflammatory cytokines [interleukin-1α (IL-1α), IL-6 and tumor necrosis factor-α (TNF-α)] in lipopolysaccharide (LPS)-stimulated monocytes (Zhang et al. 2012). Furthermore, 1,25(OH)2D affects dendritic cells by reducing their production of IL-12 and IL-17 (Daniel et al. 2008).

2.3.2 Functions of 1,25(OH)2D in adaptive immunity

Adaptive immunity is activated in the later stages of defense against pathogens and includes the responses of dendritic cells, B lymphocytes, and T lymphocytes (Figure 3). Recent studies suggest that the effect of 1,25(OH)2D on the function of human adaptive immunity seems to be rather complex and extensive compared with the effects connected to innate immune functions (Bruce et al. 2010, Cantorna & Waddell 2014).

As shown by Chen (2007), B cells are susceptible to various effects of 1,25(OH)2D; it inhibits proliferation of activated B cells, their apoptosis and differentiation into plasma cells, and production of IgG and IgM. However, no effect on their initial cell division is observed. In addition, differentiation of memory B cells is inhibited (Chen et al. 2007). Including a large number of effects on T cells, it’s been postulated that the overall effect is a switch from the more inflammatory T-helper 1/T-helper 17 (Th1/Th17) response to the less inflammatory Th2/TREG profile, and consequently a switch from increased production of pro-inflammatory cytokines to increased production of anti-inflammatory cytokines (Lemire et al. 1985, Boonstra et al. 2001, Daniel et al. 2008, Guillot et al. 2010, Aranow 2011, Cannell et al. 2014).
Fig. 3. 1,25(OH)_{2}D functions and adaptive immunity: interactions between vitamin D metabolites and immune cells including lymphocytes, monocytes, macrophages, and immature dendritic cells. [VDR; Vitamin D Receptor, CCL22; Chemokine (C-C motif) Ligand 22, CCR10; G Protein-Coupled Receptor-2, CP27B; 25OHD-1α hydroxylase, Ig; Immunoglobulin, T REG; T regulatory lymphocyte] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Endocrinology, Nat Clin Pract Endocrinol Metab 2008, 4:80-90, copyright 2008.

Besides the aforementioned effects on proliferation, differentiation, and apoptosis of lymphocytes, 1,25(OH)_{2}D has a broad range of effects on the production of inflammatory mediators by these cells. Human peripheral blood mononuclear cells (Lemire et al. 1984) and T lymphocytes (Rigby et al. 1987, Tsoukas et al. 1989) treated with 1,25(OH)_{2}D exhibit suppressed pro-inflammatory cytokine (IL-1, IL-2, IL-6, TNF-α, TNF-β, and interferon-γ) production (Lemire et al. 1984, Rigby et al. 1987, Tsoukas et al. 1989). On the other hand, increased production of IL-4 and IL-10 induced by CD4+ T-cells under TH2 cell culture conditions has been reported.
Other contradictory reports show that 1,25(OH)₂D inhibits the production of IL-4 by Th2 cells, but does not affect genes important in Th2 differentiation (Pichler et al. 2002).

**2.3.3 Functions of 1,25(OH)₂D in bone metabolism**

The so-called “classical” or “major actions” of 1,25(OH)₂D are related to bone metabolism; the terms refer to its high potential in elevating serum Ca and phosphate by increasing their intestinal intake and also to its direct effects on osteoblasts and osteoclasts (Bikle 2012) (Figure 1). The direct effects of 25(OH)D and primarily 1,25(OH)₂D are manifested in osteoblasts, osteoclasts, and chondrocytes, which express Vitamin D receptors (VDR) and produce 1,25(OH)₂D. Vitamin D signaling appears to be necessary for their differentiation and function and, consequently, bone formation and bone resorption (Bikle 2012). Specifically, key molecules associated with bone metabolism, for example osteopontin (Noda et al. 1990), osteocalcin (Ozono et al. 1990), RANKL (Kitazawa et al. 2003), and osteoprotegerin (OPG) (Kondo et al. 2004) are partly regulated by 1,25(OH)₂D.

**2.4 Gingivitis and periodontitis: diagnosis and epidemiology**

Gingivitis is the diagnosis for inflamed gingival tissues, in which no previous or current loss of periodontal attachment or alveolar bone is evident. It is the most common form of plaque-induced gingival disease and affects 50% to 90% of the population on a global level (Albandar & Rams 2002). Gingivitis is commonly assessed by the presence of bleeding or inflamed appearance of the marginal gingivae.

Periodontitis is distinguished from gingivitis by loss of tooth-supporting structures—periodontal ligament and alveolar bone—and damage to the root cementum. While periodontitis is always preceded by gingivitis, not all gingivitis lesions lead to periodontitis.

The diagnosis of periodontitis is based on site-by-site measurements of periodontal pocket depth (PD), attachment level (AL), and radiologically determined alveolar bone level. Chronic periodontitis, the most common form of periodontitis, has been characterized by slow continuous progression with episodes of active disease progression.

According to the Health 2000 Survey among Finnish adults aged ≥ 30 years, conducted in 2000–2001, the proportion of participants with periodontal pockets ≥
4 mm was 64%. The proportion of participants having 8 or more teeth with ≥ 4 mm pockets was 28% among men and 15% among women (Suominen-Taipale et al. 2008). Comparisons of the data from Finland with those from other European countries indicate that Finland is among the countries with a relatively high prevalence of periodontitis (König et al. 2010). In this report, the proportions of 35–44-year-olds with periodontal pockets ≥ 4 mm deep was 61% in Finland and 16% in Austria, 26% in Spain, 27% in Hungary, 35% in Denmark, and 73% in Germany. The respective figure in the UK was 59% (NHS report) and in the United States, data from the NHANES show a prevalence of pockets 4 mm or deeper in 39% of the population (35–49-year-olds) (Eke et al. 2015).

2.5 Pathogenesis of periodontitis – possible role of Vitamin D

Biofilms consist of microbial cells embedded in a matrix of extracellular polymeric substances such as polysaccharides, proteins and nucleic acids; for example microbes on the surface of teeth comprise a biofilm community. Over an extended period of dysbiosis, pathogenic microbial complexes appear on the biofilm (Berezow & Darveau 2011). A shift from the early colonizers that are termed by the research group of Socransky as the ‘orange complex’ consisting of gram-negative anaerobic species such as Prevotella intermedia and Fusobacterium nucleatum, to the more pathogenic species, ‘red complex’, such as Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola may promote the onset of periodontitis (Socransky & Haffajee 2005). In terms of the pathogenesis of periodontitis, outdated paradigms (i.e. non-specific and specific plaque theories) used to give a central, dominating role to microbes in the disease process.

A contemporary sufficient cause model for the etiology of periodontitis suggests that microbes are necessary, yet merely the initial cause of periodontal disease (Baelum & Lopez 2013), and the progression of periodontal inflammation and tissue destruction have been believed to be mainly controlled by host immune-inflammatory responses (Darveau 2010).

The molecules that effectively induce periodontal destruction include the ones associated with subgingival microflora, including so-called virulence factors (LPS, bacterial enzymes, noxious products, fimbriae, and bacterial and extracellular deoxyribonucleic acid) and molecules derived from host immune-inflammatory responses [cytokines including prostaglandins and matrix metalloproteinases (MMPs)]. These altogether act as a complex and dynamic network that challenges the defense mechanisms in periodontal tissues.
At least two of the responses of innate immunity pertinent to periodontal destruction are linked with the active form of Vitamin D, \(1,25(\text{OH})_2\text{D}\). First, genes encoding molecules expressed by the inflamed periodontal epithelium, such as human \(\beta\)-defensin 2 (hBD-2), human \(\beta\)-defensin 3 (hBD-3), and cathelicidin (LL-37), possess Vitamin D-responsive elements in their promoter region (Campbell et al. 2012). Second, it has been demonstrated that cathelicidin induced by \(1,25(\text{OH})_2\text{D}\) is normally stored in the granules of neutrophil granulocytes (De et al. 2000), which are critical to the clearance of micro-organisms invade periodontal tissues. However, the significance of these peptides against periodontal infection has been questioned (Territo et al. 1989, Dale & Fredericks 2005).

In terms of adaptive immunity in the pathogenesis of chronic periodontitis, the main cell types encountered are T-lymphocytes; e.g. \(\text{T}_{\text{H}1}, \text{T}_{\text{H}2}, \text{T}_{\text{H}17}\) and, \(\text{T}_{\text{REG}}\). It is assumed that subsets, especially, \(\text{T}_{\text{H}1}\) and \(\text{T}_{\text{H}2}\), vary between gingivitis and periodontitis as well as according to diseases severity (Yamazaki et al. 2003). The beneficial effect of \(1,25(\text{OH})_2\text{D}\) on regulatory T cell populations is connected to its capacity to down-regulate production of pro-inflammatory mediators produced by \(\text{T}_{\text{H}1}\) and \(\text{T}_{\text{H}17}\) populations (Deluca & Cantorna 2001, Bruce et al. 2010) and augment the development of \(\text{T}_{\text{H}2}\) (Boonstra et al. 2001).

On a separate note, it has been shown that many oral bacteria elicit a polyclonal B-cell response during the onset and progression of periodontitis. In spite of the reported correlation between clinical parameters of the disease and titers of some of the antibodies, their significance in the pathogenetic mechanisms of periodontitis is still debatable (Sahingur & Cohen 2004, Hwang et al. 2014). In this context, \(1,25(\text{OH})_2\text{D}\) was shown to inhibit secretion of primarily immunoglobulin IgG and to a lesser extent IgM by plasma cells (Chen et al. 2007).

### 2.6 Mediators of inflammation in serum linked with periodontal condition

Mounting evidence suggests that chronic periodontitis, most likely its severe form, is associated with low-grade systemic inflammation, i.e. with increased circulating levels of various inflammatory mediators (Loos 2005, Nibali et al. 2007).

These mediators are mainly produced in response to antigens by circulating immune cells (Preshaw & Taylor 2011), compromised (Lippitz 2013) and healthy (Preshaw & Taylor 2011) resident cells, and liver cells (Liaskou et al. 2012). In addition, leakage from severely inflamed periodontal areas may contribute to circulating mediator levels (Loos et al. 2005). C reactive protein (CRP) (D’Aiuto et
al. 2005, Salzberg et al. 2006, Paraskevas et al. 2008) and inflammatory mediators, for example, proinflammatory cytokines IL-6 (Saxlin et al. 2009, Passoja et al. 2011 Nibali et al. 2012) and TNF-α (Engebretson et al. 2007, Passoja et al. 2010, Andrukhov et al. 2011), RANKL (Nagasawa et al. 2007), and OPG (Nagasawa et al. 2007, Antonoglou et al. 2013) have been associated with periodontal inflammation and tissue destruction. Of note is that high circulating levels of IL-6 (Andriankaja et al. 2009, Passoja et al. 2011) and TNF-α (Engebretson et al. 2007, Andrukhov et al. 2011) have also been considered as susceptibility factors for periodontitis.

A different cluster of mediators and receptors is the one which down-regulates cytokine expression or attenuates periodontal inflammation. These include for example resolvins and protectins, such as Resolvin E1, Resolvin E2 and Protectin D1 (Hasturk et al. 2006, Serhan et al. 2008), and cytokines such as IL-4 (Manhart et al. 1994, Yamazaki et al. 1994) and IL-10 (Garlet et al. 2004, Claudino et al. 2008, Passoja et al. 2010), and T-cell receptors such as Cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed by leukocytes (Aoyagi et al. 2000). Moreover, high-density lipoprotein (HDL), which is known for its capacity to bind and neutralize LPS (Murch et al. 2007), has been inversely associated with periodontal infection (Saxlin et al. 2008, Passoja et al. 2011, Haro et al. 2012). In this study it was anticipated that due to the immunemodulatory actions of Vitamin D metabolites, especially 1,25(OH)₂D, they would be associated with periodontal health in a manner similar to the above mentioned anti-inflammatory mediators.

2.7 Serum 25(OH)D and 1,25(OH)₂D in systemic immune mediated and infectious diseases

During the past ten years, increasing attention has been paid to the role of 1,25(OH)₂D—the active form of Vitamin D—in inflammatory/infectious diseases.

Serum Vitamin D level has been associated with a multitude of systemic health outcomes in humans, such as bone health, cancer, and various inflammatory/infectious diseases (Bischoff-Ferrari et al. 2006). Specifically, low levels of serum 25(OH)D and 1,25(OH)₂D have been previously linked with immune-mediated diseases, for example, inflammatory bowel disease (Crohn’s diseases and ulcerative colitis) (Ananthakrishnan et al. 2012, Nicholson et al. 2012, Augustine et al. 2014) and multiple sclerosis (Salzer et al. 2012).

Patel and co-workers (2007) demonstrated inverse associations between baseline levels of serum 25(OH)D and various measures of disease activity in
patients with inflammatory polyarthritis. In addition, serum 1,25(OH)\(_2\)D was inversely associated with the scores of the Stanford Health Assessment Questionnaire at the baseline. These observations, together with the finding that Vitamin D levels at the baseline were predictive of the above scores at one year, were interpreted to be suggestive of the potential immune-modulatory actions of Vitamin D in polyarthritis. Inverse associations between serum 25(OH)D and 1,25(OH)\(_2\)D and increased disease activity in ankylosing spondylitis (Lange et al. 2001) and rheumatoid arthritis (Colin et al. 2010, van Hamburg et al. 2012) have also been reported. Finally, the immune-modulatory role of Vitamin D has been confirmed in a treatment study among asymptomatic (free from disease) young individuals showing inverse associations between serum 25(OH)D and endothelial dysfunction and oxidative stress (Tarcin et al. 2009). In that study, Vitamin D treatment seemed to improve endothelial function, suggesting a potential beneficial effect of Vitamin D on the progression of atherosclerosis.

2.8 Association between serum 25(OH)D and periodontal health status

2.8.1 Clinical studies

So far there is only one population-based study of serum 25(OH)D and periodontal health. The authors used data from the National Health and Nutrition Examination Survey (NHANES) in the United States and showed an inverse association between serum 25(OH)D level and gingival bleeding (Dietrich et al. 2005). The same group also reported an inverse association of serum 25(OH)D with periodontal attachment loss in both men and women older than 50 years but not in younger individuals (Dietrich et al. 2004). The authors concluded that serum 25(OH)D exerts anti-inflammatory properties, but possibly at levels as high as 90-100 nmol/L. Most of the published observational studies of a variety of patient groups have shown inverse associations between serum 25(OH)D and periodontal infection. Serum 25(OH)D level and maternal periodontal disease were inversely associated during pregnancy (Boggess et al. 2011); the adjusted OR (95% confidence interval) for moderate to severe periodontal diseases among women with Vitamin D insufficiency was 2.1 (0.99 to 4.5). An inverse association between serum 25(OH)D and periodontitis, assessed with a questionnaire, was found in postmenopausal women (Jabbar et al. 2011). Zhou and co-workers (Zhou et al. 2012) investigated
the links between serum 25(OH)D, chronic obstructive pulmonary disease (COPD), and periodontal health and showed that a low serum 25(OH)D level was significantly associated with poor periodontal health and COPD.

Millen and co-workers (2013) using a cohort of 920 postmenopausal women showed that gingival bleeding and periodontal disease severity, based on PD and AL measurements, were inversely associated with serum 25(OH)D level (< 50 nmol/l vs. ≥ 50 nmol/L), whereas no association was found between serum 25(OH)D level and alveolar crest height and tooth loss.

In a longitudinal study Millen could not show any association between baseline serum 25(OH)D and subsequent five-year change in periodontal disease measures (Millen et al. 2014). To our knowledge, only one study has previously examined the effect of periodontal therapy on local and systemic levels of 25(OH)D (Liu et al. 2010); that study showed that serum 25(OH)D was systemically and locally reduced after initial periodontal therapy in aggressive periodontitis. Bashutski and co-workers (2011) demonstrated improved bone healing after periodontal surgery in conjunction with teriparatide (a recombinant form of parathyroid hormone) treatment in Vitamin D sufficient individuals compared with deficient ones.

2.8.2 Vitamin D supplementation studies

Krall and her group (2001) showed that intake of Vitamin D combined with calcium, aimed at preventing osteoporosis, had a beneficial effect on tooth retention. However, based on data from two prospective observational studies on aging and health of men, they concluded that only calcium intake had a protective role in preventing the progression of alveolar bone loss (Krall et al. 2001). Garcia (2011) found only a modest effect of calcium and Vitamin D supplementation on periodontal health and expressed a need for randomized clinical trials to confirm a possible true effect.

In summary, neither clinical nor supplementation studies unambiguously support a substantial beneficial effect of serum 25(OH)D on periodontal health.

2.9 Association between serum 1,25(OH)₂D and periodontal health status

No earlier studies examined the association between 1,25(OH)₂D and periodontal infection. Based on in vitro studies, the intracellular Vitamin D receptor-
1,25(OH)₂D complex accounts for the biologic actions of Vitamin D, which could explain a possible association between the active form of Vitamin D and periodontal inflammation and tissue destruction. One systematic review examined VDR polymorphisms, which are very closely associated with 1,25(OH)₂D functions, in relation to periodontitis (Chen et al. 2012). The authors looked into four gene loci and concluded that certain alleles (mutant allele T of TAq-I, mutant allele F of Fok-I) can be either protective or risk factors for periodontitis, although the findings were ethnic origin-specific and the need for future studies was suggested (Chen et al. 2012). Other polymorphisms in the same study, such as the ones of the loci Bsm-I and Apa-I, seemed to have no significant association with susceptibility to periodontitis (Chen et al. 2012).

2.10 Parathyroid hormone (PTH), the main regulator of 1,25(OH)₂D production

PTH is an 84 amino acid peptide secreted by the parathyroid glands and together with calcium sensing receptors (CASRs), calcitonin, and 1,25(OH)₂D regulates Ca levels in the human body (Brown 2007). In addition, it regulates formation of 1,25(OH)₂D by directly controlling expression of the enzyme 1α-hydroxylase (CYP27B), which converts serum 25(OH)D to 1,25(OH)₂D in the proximal tubular cells of the kidney (Lund & DeLuca 1966). Besides the apparent association with the pathology of parathyroid glands (e.g. tumors) and deficiencies in the direct target tissues (i.e. bone and kidney), PTH has been examined as a serum marker associated with all-cause mortality and cardiovascular disease (CVD). Recently published studies suggest that higher serum PTH levels may be associated with all-cause mortality, mostly explained by fatal CVD (van Ballegooijen et al. 2013), coronary heart disease (Grandi et al. 2011), and elevated inflammatory markers (Cheng et al. 2014). On the other hand, these findings are not unequivocally accepted in all patient groups, such as patients with chronic kidney disease (Palmer et al. 2011).

In line with the results of animal studies (Barros et al. 2003, Tokunaga et al. 2011, Vasconcelos et al. 2014) on the anabolic effect of PTH on periodontal inflammation and healing of periodontitis, an experimental study on PTH administration in the form of teriparatide showed beneficial effects on osseous regeneration (Bashutski et al. 2010). Analogous favoring effects on post-extractive socket healing were suggested by Kuroshima and his group (Kuroshima et al. 2013).
Nonetheless, there are neither observational nor experimental studies that address associations between PTH and infectious diseases in the periodontal area in humans.
3 Aims of the study

Besides its role in bone metabolism, Vitamin D is known for its immune modulatory actions, and low levels of the two Vitamin D metabolites—the storage form 25(OH)D and the active form 1,25(OH)2D—have been associated with a number infectious/inflammatory diseases. While conflicting results regarding the association between serum 25(OH)D and periodontal infection have been reported, it is not known whether a low serum 1,25(OH)2D level could be a susceptibility factor for periodontal inflammation and tissue destruction.

The specific aims of this study were

- to explore associations between serum levels of 25(OH)D and 1,25(OH)2D and the presence, extent, and severity of infectious periodontal diseases, with a hypothesis of inverse associations
- to study the effect of periodontal therapy on serum levels of 25(OH)D and 1,25(OH)2D in T1DM individuals
- to study the role of PTH in the association between serum 1,25(OH)D and periodontal health

The measures of periodontal infection used in the present studies included periodontal probing depth, bleeding associated with probing and periodontal attachment level.
4 Materials and methods

The materials of this thesis originate from two independent projects:

- The Oulu Diabetes Study, which originally explored the relationship between periodontal and general health and included two data sets
  1. case-control data from patients with moderate or severe periodontitis and age- and gender-matched periodontally healthy control subjects, and
  2. intervention data retrieved from T1DM patients with varying degrees of infectious periodontal diseases, at the baseline and after periodontal therapy.

These data were collected in clinical periodontal examinations and interviews during 2001–2007.


In this thesis only the methods that were essential for obtaining the results presented are covered. The readers are referred to the original papers (I-IV) for a more detailed description of all the methods used in the two independent projects.

4.1 Ethical approval

The Ethical Committee of Oulu University Hospital, Oulu, Finland approved the Oulu Diabetes Study protocol and written informed consent was obtained from all the participants.

The Health 2000 Survey was approved by the Ethics Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland and all the participants gave written informed consent.

4.2 Study populations

The socio-demographic characteristics of the study populations are presented in Table 1. Since all data were not available for all the subjects, the numbers of subjects vary between the studies in the periodontitis and T1DM groups.
Table 1. Demographic characteristics of the subjects in the various studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>N</th>
<th>Age (mean ± SD/Range)</th>
<th>Females (%)</th>
<th>Smokers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oulu Diabetes Study</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periodontitis group</td>
<td>55</td>
<td>46.3 ± 13.5</td>
<td>61.8</td>
<td>56.4</td>
</tr>
<tr>
<td>Control group</td>
<td>30</td>
<td>41.9 ± 12.7</td>
<td>63.3</td>
<td>33.3</td>
</tr>
<tr>
<td>Paper II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1DM at the baseline</td>
<td>80</td>
<td>32.3 ± 8.6</td>
<td>57.5</td>
<td>30.0</td>
</tr>
<tr>
<td>after therapy</td>
<td>58</td>
<td>39.5 ± 12.0</td>
<td>58.6</td>
<td>22.4</td>
</tr>
<tr>
<td>Paper IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periodontitis group</td>
<td>54</td>
<td>46.3 ± 13.7</td>
<td>61.1</td>
<td>55.5</td>
</tr>
<tr>
<td>Control group</td>
<td>30</td>
<td>41.9 ± 12.7</td>
<td>63.3</td>
<td>33.3</td>
</tr>
<tr>
<td>T1DM at the baseline</td>
<td>76</td>
<td>38.4 ± 12.6</td>
<td>57.9</td>
<td>30.2</td>
</tr>
<tr>
<td>after therapy</td>
<td>53</td>
<td>40.0 ± 12.6</td>
<td>62.2</td>
<td>22.6</td>
</tr>
<tr>
<td>Health 2000 Survey</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic and non-smoker adult individuals</td>
<td>1262</td>
<td>30–49</td>
<td>60.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

4.2.1 Oulu Diabetes Study

Individuals needing prophylactic antibiotic medication in association with periodontal probing and periodontal therapy as well as those with immunosuppressive medication or antibiotics during the past four months were excluded from all the study groups.

Case-control study

A group of patients with periodontitis (n = 63) was recruited from among patients referred to specialist periodontal care at the Specialist Dental Health Care Unit, City of Oulu, Finland. They had good overall general health (individuals with rheumatoid arthritis, diabetes mellitus, and asthma were excluded) but were principally diagnosed with untreated moderate or severe periodontitis. None of the individuals had a diagnosis of cardiovascular disease.

An age- and gender-matched control group was recruited and consisted of systemically healthy individuals who had clinically healthy periodontal tissues or only minimal signs of gingival inflammation and were selected from among patients eligible for treatment in the health center (n = 20) and from among university staff and students (n = 10).
Intervention study

A group of T1DM patients (n = 80) with varying degrees of infectious periodontal disease was recruited from the Diabetes Clinic of Oulu Health Centre. A few patients were recruited from the Clinic of Internal Medicine, Oulu University Hospital, Oulu, Finland. Patient records were used to retrieve data regarding diabetic status.

4.2.2 Health 2000 Survey

The individuals included in the Health 2000 Survey belong to the main sample (n = 8028) of the population-based National Health 2000 Survey in Finland conducted by the National Institute of Health and Welfare. Oral health examinations were conducted on 6335 persons after excluding individuals with a need for prophylactic antibiotic medication in association with periodontal probing.

Restrictions by age (30−49 years) and diabetes (non-diabetics) yielded a population of 2856. After excluding those who had ever smoked, a total of 1262 remained for the present study.

4.3 Clinical oral examination and periodontal therapy

In the Oulu Diabetes Study, clinical oral examinations were performed by one calibrated examiner (a periodontal specialist), whereas in the Health 2000 Survey five calibrated dentists performed the examinations. The clinical variables recorded are shown in Table 2.
Table 2. Clinical variables used in the two studies.

<table>
<thead>
<tr>
<th>Variable</th>
<th>The Oulu Diabetes Study</th>
<th>The Health 2000 Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque</td>
<td>Visible plaque* on four sites** per tooth; the proportion (%) of sites with plaque was used in the analyses</td>
<td>Visible plaque* on one site of three indicator teeth***: the highest value of any of the indicator teeth (no visible plaque, visible plaque on gingival margins only, or visible plaque also elsewhere) was used in the analyses</td>
</tr>
<tr>
<td>Periodontal pocket depth</td>
<td>On four sites** per tooth; the proportion (%) of sites with pockets ≥ 4 mm deep was used in the analyses</td>
<td>The deepest measurement out of four measurements**** on each tooth was recorded; the number of teeth with deepened (4 mm deep or deeper) periodontal pockets was used in the analyses</td>
</tr>
<tr>
<td>Gingival bleeding</td>
<td>Bleeding on probing (yes/no) was registered 20–30 s after probing; the proportion (%) of affected sites was used in the analyses</td>
<td>Gingival bleeding measured immediately after periodontal probing was registered by sextants; the number of bleeding sextants was used in the analyses</td>
</tr>
<tr>
<td>Periodontal attachment level</td>
<td>The distance from the cemento-enamel junction to the base of the crevice/pocket on four surfaces of each tooth**. The proportion (%) of sites with AL ≥ 4 mm was used in the analyses</td>
<td>NA</td>
</tr>
</tbody>
</table>

Periodontal measurements were made using a ball pointed periodontal probe with 2-mm gradations (WHO).

*visible plaque corresponding to scores 2 and 3 of the Silness & Löe plaque index (Silness and Löe 1964)

**distobuccal, mid-buccal, mesio-buccal, mid-oral sites; third molars excluded

***the buccal surface of the most posterior tooth on the upper right side, the lingual surface of the most posterior tooth on the lower left side and the buccal surface of the lower left canine

****disto-buccal, mid-buccal, mid-oral and mesio-oral surfaces, wisdom teeth and radices excluded

With the aim to reach periodontal health, anti-infective periodontal therapy (oral hygiene education, scaling and root planning, and periodontal surgery if needed) was delivered to the T1DM patients of the Oulu Diabetes Study (Tervonen et al. 2009).

4.4 Laboratory analyses

The laboratory analyses of the biomarkers used are presented in Table 3.
Table 3. Laboratory analyses.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>The Oulu Diabetes Study*</th>
<th>The Health 2000 Survey**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated hemoglobin HbA1c (%)</td>
<td>Latex immunoturbidimetric method (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).</td>
<td>---</td>
</tr>
<tr>
<td>25(OH)D (nmol/L)</td>
<td>Enzyme-linked immunoassay (Immunodiagnostics System, IDS, Boldon, UK)</td>
<td>Radioimmunoassay (RIA, Diasorin, MN, USA)</td>
</tr>
<tr>
<td>1,25(OH)2D (pmol/L)</td>
<td>Enzyme-immunoassay procedure after purification of 1,25(OH)2D by immunoextraction, (Immunodiagnostics System, IDS, Boldon, UK)</td>
<td>---</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>Direct enzymatic methods implemented in the Advia 2400 Chemistry systems (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).</td>
<td>---</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>Electrochemiluminescence immunoassay (PTH Intact, Elecsys 2010, Roche, Espoo, Finland)</td>
<td>---</td>
</tr>
<tr>
<td>Ca (mmol/L) and P (mmol/L)</td>
<td>Abbott Architect c8000 automated clinical chemistry analyzer (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL, USA).</td>
<td>---</td>
</tr>
</tbody>
</table>

Laboratory analyses were performed in the laboratories of Oulu University Hospital, Oulu, Finland*, and the Research Unit of Seinäjoki Central Hospital and University of Tampere, Seinäjoki, Finland*, and Labquality Ltd, Helsinki, Finland**

4.5 Covariates and confounding factors

Smoking

The Oulu Diabetes Study - Smoking data were obtained by an interview and the subjects were categorized as smokers or nonsmokers.

Health 2000 Survey - Individuals who had ever smoked were excluded.
**Alcohol consumption**

The Health 2000 Survey - Information about alcohol consumption (g/week, used as a continuous variable) was obtained from a questionnaire.

**Education**

The Health 2000 Survey - Information about education was obtained from the interview and categorized into three categories: low (less than a high school education and no formal vocational qualification), intermediate (high school or vocational school) and high (university degree or graduation from a polytechnic).

**Tooth brushing frequency and dental attendance pattern**

The Health 2000 Survey - Tooth brushing frequency was obtained from a complementary questionnaire and categorized as follows: at least twice a day, once a day, and less frequently. The categories of dental attendance pattern were regular vs. irregular dental check-ups.

**Physical activity**

The Health 2000 Survey - Information about physical exercise was obtained from the basic questionnaire and was measured with the question “How often do you exercise in your leisure time so that you are at least slightly out of breath and sweating?” with the answer options being: “1. Daily; 2. 4–6 times a week; 3. 2–3 times a week; 4. Once a week; 5. 2–3 times a month; 6. Few times a year or even more rarely.”

**Lipid medication**

The Health 2000 Survey - Lipid medication was categorized into three categories: yes, no, and missing information.

**Body weight**

Body weight was assessed using a relative measure of body weight: body mass index (BMI, kg/m²).
4.6 Statistical methods

The Oulu Diabetes Study

As regards periodontal variables, the analyses were based on subject-level data using individual numbers of periodontally affected sites or categorization of the subjects into no or mild periodontitis and moderate or severe periodontitis groups, as suggested by the American Academy of Periodontology and the Centers for Disease Control and Prevention (Page & Eke 2007). Between-group comparisons were made using the independent samples T-test and adjusted means. Correlations were assessed using Spearman’s rank correlation test. The paired samples T-test and the Wilcoxon sum-rank test were used to study paired samples. To control for confounding/modifying factors, both stratification of the study populations and multivariable logistic and linear regression models were used.

Due to seasonal variation in serum 25(OH)D level, analyses in the case-control study were restricted to individuals examined in the autumn (August-October) and in the cohort study, to those examined in the winter (November-March) and spring (April-June) only. Because no clear seasonal variation could be observed in serum 1,25(OH)₂D, no restrictions with regard to season were made in the 1,25(OH)₂D analyses.

A statistical package (STATA version 12, Stata Corp, College Station, Texas, USA) was used to perform all data analyses.

Health 2000 survey

A stratified two-stage cluster sampling design was used in the Health 2000 survey. Weighting of the sample was based on post-stratification according to sex, age, and region. Prevalence rate ratios (PRR) and 95% confidence intervals (CI) were estimated using Poisson’s regression model. Bias related to seasonal variation in serum 25(OH)D level was also assessed by adding the month of examination to the final regression models.

A statistical package (SAS Callable Sudaan release 11.0, SAS Institute, Cary, NC.) and weights were used to perform data analyses, taking into account the two-stage cluster sampling design and to correct for the effects of non-response.
5 Results

5.1 Serum levels of Vitamin D metabolites - seasonal variation (Papers I-III)

The level of serum 25(OH)D (mean ± SD) was dependent on the seasonal period of examination in both the periodontitis group and the T1DM group as follows; periodontitis group: autumn 52.2 ± 15.5 nmol/L, winter 37.0 ± 12.7 nmol/L, spring 37.7 ± 15.2 nmol/L (adjusted for disease severity p = 0.001) (Figure 4 A, Paper I) and T1DM group: autumn 56.4 ± 17.6 nmol/L, winter 41.9 ± 13.8 nmol/L nmol/L, spring 46.5 ± 12.3 nmol/L (adjusted for disease severity p = 0.004) (Figure 4 B, Paper II). The level of 25(OH)D in the control subjects (all examined in the autumn) was 56.9 ± 14.2 nmol/L (Paper I).
Fig. 4. Box plots of serum 25(OH)D levels (minimum and maximum, mean and median, upper and lower quartiles) in the periodontally healthy control subjects and periodontitis patients (A) and in the T1DM patients (B) by the period of examination.
Only slight variation was observed in serum 25(OH)D per month of examination in the Health 2000 Survey (Figure 5, Paper III).

Fig. 5. Variation in serum 25(OH)D level per month of examination in the Health 2000 Survey. Numbers of subjects are indicated above the bars.

In contrast, no similar variation was observed in regard to 1,25(OH)2D. The mean level of serum 1,25(OH)2D (± SD) in the periodontally healthy control subjects, (all examined in the autumn) was 106.8 ± 16.8 pmol/L (Figure 6 A, Paper I). In the periodontitis group the levels were 87.7 ± 35.0 pmol/L, 78.2 ± 33.2 pmol/L, and 82.5 ± 20.9 pmol/L for the subjects examined in the autumn, winter, and spring, respectively (Figure 6 A, Paper I). In the T1DM group the corresponding levels over the three seasonal periods were 68.5 ± 17.6 pmol/L, 68.8 ± 25.6 pmol/L, and 91.1 ± 33.3 pmol/L. After adjusting for periodontal disease severity, the level of serum 1,25(OH)2D was quite independent of the examination period in both the periodontitis group (p = 0.231) and the T1DM group (p = 0.133).
Fig. 6. Box plots of serum 1,25(OH)$_2$D levels (minimum and maximum, mean and median, upper and lower quartiles) in the periodontally healthy control subjects and periodontitis patients (A) and in the T1DM patients (at the baseline) (B) by the period of examination.
Serum 25(OH)D deficiency

The two commonly accepted thresholds for Vitamin D deficiency are <75 nmol/L (Ross et al. 2011) and <50 nmol/L (Nordic Council of Ministers, 2012). The proportions of individuals who had levels above these thresholds are presented in Table 4. It is evident from the data that irrespective of the season only a minority of individuals (≤10%) in various study populations had levels ≥75 nmol/L.

Table 4. Proportions of individuals with 25(OH)D levels ≥50 nmol/L and ≥75 nmol/L.

<table>
<thead>
<tr>
<th>Study</th>
<th>≥ 50 nmol/L, n (%)</th>
<th>≥ 75 nmol/L, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Oulu Diabetes Study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group (Paper I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects <em>(n=30)</em></td>
<td>21 (70.0)</td>
<td>3 (10.0)</td>
</tr>
<tr>
<td>Periodontitis group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects (n=55)</td>
<td>14 (25.5)</td>
<td>3 (5.5)</td>
</tr>
<tr>
<td>Subjects examined in the autumn (n=47)</td>
<td>16 (34.0)</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>T1DM group (Paper II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All subjects (n=77)</td>
<td>30 (40.0)</td>
<td>4 (5.0)</td>
</tr>
<tr>
<td>Subjects examined in the winter and spring (n=60)</td>
<td>20 (33.0)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>The Health 2000 Survey (n=1262) (Paper III)</td>
<td>370 (29.0)</td>
<td>35 (3.0)</td>
</tr>
</tbody>
</table>

*all examined in the autumn

5.2 Serum 1,25(OH)₂D and periodontal health (Papers I, II)

5.2.1 Case control study (Paper I)

Multivariable models (Table 5) adjusted for confounding factors such as age, plaque, smoking (in all individuals), HDL, BMI, and gender showed that individuals with a high serum 1,25(OH)₂D level were more likely to belong to the periodontally healthy control group than to the periodontitis group in both all subjects and non-smokers.
### Table 5. Parameter estimates of the the unadjusted and adjusted associations between serum \(1,25(\text{OH})_2\text{D}\) level (as a continuous variable) and periodontal condition (health vs. disease) using a logistic regression model (control group as a reference)

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>CI 95%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All individuals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>0.96</td>
<td>0.94 to 0.98</td>
<td>0.001</td>
</tr>
<tr>
<td>Model I*</td>
<td>0.97</td>
<td>0.95 to 1.00</td>
<td>0.008</td>
</tr>
<tr>
<td>Model II†</td>
<td>0.97</td>
<td>0.95 to 1.00</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Non-smokers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>0.94</td>
<td>0.90 to 0.99</td>
<td>0.002</td>
</tr>
<tr>
<td>Model I*</td>
<td>0.94</td>
<td>0.90 to 0.99</td>
<td>0.010</td>
</tr>
<tr>
<td>Model II†</td>
<td>0.94</td>
<td>0.90 to 0.99</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Model I: Adjusted for age, plaque, and smoking (in all individuals).
†Model II: adjusted for age, plaque, smoking (in all individuals), HDL, BMI, and gender.

### 5.2.2 Intervention study (Paper II)

At the baseline, the mean level (± SD) of \(1,25(\text{OH})_2\text{D}\) was 86.4 (± 28.3) pmol/L in the group with no or mild periodontitis and 62.4 (± 21.7) pmol/L in the group with moderate or severe periodontitis. Multivariable regression models showed that T1DM patients with a high serum \(1,25(\text{OH})_2\text{D}\) level were more likely to belong to the no or mild than to the moderate or severe periodontitis group (Table 6).

### Table 6. Unadjusted and adjusted associations between the serum level of \(1,25(\text{OH})_2\text{D}\) (used as a continuous variable) and periodontal disease severity (the outcome variable, moderate or severe periodontitis*** versus no or mild periodontitis***) at the baseline using logistic regression analysis

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>CI 95%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All individuals</strong> (n = 77)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}) *</td>
<td>0.96</td>
<td>0.93 to 0.98</td>
<td>0.001</td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}) **</td>
<td>0.94</td>
<td>0.90 to 0.98</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Non-smokers</strong> (n = 54)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}) *</td>
<td>0.96</td>
<td>0.93 to 0.98</td>
<td>0.009</td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}) **</td>
<td>0.95</td>
<td>0.90 to 1.00</td>
<td>0.060</td>
</tr>
</tbody>
</table>

*Unadjusted
**Adjusted for age, plaque, gender, smoking (all individuals), HbA1c, HDL, and BMI.
***Reference group
An inverse association between serum 1,25(OH)\textsubscript{2}D and the extent of chronic periodontitis was confirmed in additional regression analyses (using continuous outcome variables), in which the following estimates of the associations were obtained: the extent of AL $\geq$ 4 mm (RR = 0.99, 95% CI 0.96–0.99, p < 0.001) and the extent of PD $\geq$ 4 mm (RR = 0.99, 95% CI 0.98–1.00, p = 0.062).

The individual variation of the 1,25(OH)\textsubscript{2}D level change following periodontal therapy was considerable. After periodontal therapy we observed an increase in the average 1,25(OH)\textsubscript{2}D level from 86.4 ± 28.3 pmol/L to 114.8 ± 41.2 pmol/L (p = 0.001) in the group with no or mild periodontitis (Figure 7, left panel) and from 62.4 ± 21.7 pmol/L to a mean of 94.2 ± 28.8 pmol/L (p < 0.001) in the group with moderate or severe periodontitis (Figure 7, right panel). In the moderate or severe periodontitis group 74% of the subjects had increased 1,25(OH)\textsubscript{2}D level while only 26% had decreased or no change in these values. The smoking status of the patients did not seem to influence the observed changes.

![Fig. 7. Individual changes in serum 1,25(OH)\textsubscript{2}D after periodontal treatment in the groups with no or mild and moderate (left) or severe periodontitis (right).]

5.3 Serum 25(OH)D and periodontal infection (Papers I, II, IV)

No significant association between serum 25(OH)D level and periodontal health status (periodontal health vs. periodontitis) could be found in the case-control study among either all subjects (OR = 1.0, 95% CI 0.96–1.07) or non-smokers (OR 1.0, 95% CI 0.93–1.15) (Paper I).

At the baseline of the intervention study the T1DM patients with higher serum 25(OH)D level were more likely to belong to the group with no or mild periodontitis than to the group with moderate or severe periodontitis (OR = 0.90,
95% CI 0.83 - 0.98 among all subjects and OR = 0.88, 95% CI 0.78 - 0.99 among non-smokers). No clear response in serum 25(OH)D levels could be shown to periodontal therapy (Paper II).

After adjusting for confounding factors no obvious association between serum 25(OH)D level and numbers of teeth with ≥ 4 mm periodontal pockets or numbers of sextants with gingival bleeding could be shown in 30-49 year old non-smoking and non-diabetic individuals in the Health 2000 Survey (Table 7, Paper III). Gender did not modify the studied association effectively.

Table 7. Adjusted* associations between serum 25(OH)D level and number of teeth with periodontal pockets ≥ 4 mm and number of bleeding sextants (n=1262).

<table>
<thead>
<tr>
<th>25(OH)D</th>
<th>Teeth with periodontal pockets ≥ 4 mm</th>
<th>Sextants with gingival bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D level (nmol/L), continuous</td>
<td>0.99 (0.99 – 1.00)</td>
<td>1.00 (0.99 – 1.00)</td>
</tr>
<tr>
<td>Serum 25(OH)D level (nmol/L), categorized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Quintile (8 − 31)</td>
<td>1.0 (0.7 – 1.2)</td>
<td>1.1 (1.0 – 1.4)</td>
</tr>
<tr>
<td>II Quintile (32 − 38)</td>
<td>1.1 (0.8 – 1.4)</td>
<td>1.0 (0.8 – 1.2)</td>
</tr>
<tr>
<td>III Quintile (39 − 46)</td>
<td>1.0 (0.8 – 1.3)</td>
<td>1.0 (0.9 – 1.2)</td>
</tr>
<tr>
<td>IV Quintile (47 − 56)</td>
<td>0.8 (0.6 – 1.0)</td>
<td>0.9 (0.8 – 1.1)</td>
</tr>
<tr>
<td>V Quintile (57 − 134, reference category)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Adjusted for gender, age, education, presence of dental plaque, number of teeth (offset variable), dental attendance pattern, tooth brushing frequency, lipid medication, BMI and alcohol consumption.

The numbers of teeth with deepened (≥ 4mm) periodontal pockets in 25(OH)D quintiles according to oral hygiene level are presented in Table 8.

Table 8. Proportions of teeth* (%) with deepened periodontal pockets ≥ 4 mm (95% CI) over serum 25(OH)D quintiles stratified by oral hygiene level

<table>
<thead>
<tr>
<th>Oral hygiene level</th>
<th>I (8 − 31)</th>
<th>II (32 − 38)</th>
<th>III (39 − 46)</th>
<th>IV (47 − 56)</th>
<th>V (57 − 134)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>7.1 (4.6−10.8)</td>
<td>4.7 (2.5−8.9)</td>
<td>7.1 (4.9−10.4)</td>
<td>2.6 (2−4)</td>
<td>5.2 (3.7−7.3)</td>
</tr>
<tr>
<td>Moderate</td>
<td>8.7 (6.7−11.3)</td>
<td>11.6 (9.2−14.6)</td>
<td>9.9 (8.0−12.3)</td>
<td>9.8 (8.1−12.6)</td>
<td>10.8 (8.4−14.0)</td>
</tr>
<tr>
<td>Poor</td>
<td>23.5 (14.6−37.8)</td>
<td>25.9 (17.4−38.8)</td>
<td>22.9 (16.0−32.8)</td>
<td>18.7 (13.5−25.8)</td>
<td>22.9 (15.8−33.0)</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, education, number of teeth (offset variable), dental attendance pattern, tooth brushing frequency, lipid medication, BMI, and alcohol consumption.
A somewhat lower number of teeth with deepened periodontal pockets was found in the higher quintiles of serum 25(OH)D than in the lower in individuals with good oral hygiene.

5.4 Serum PTH, Ca and P concentrations and periodontal health (Paper IV)

We determined serum PTH, Ca and P levels in the case-control and the cohort studies.

The age-, gender-, and plaque-adjusted means (± SE) of serum PTH showed no statistically significant differences ($p = 0.878$) between periodontitis patients ($2.9 \pm 0.2$ pmol/L) and periodontally healthy control subjects ($3.0 \pm 0.2$ pmol/L). The mean PTHs after adjustments for age, gender, plaque, and HbA1c were $2.0 \pm 0.9$ pmol/L and $2.4 \pm 1.3$ pmol/L, respectively, in the T1DM patients with no or mild vs. moderate or severe periodontitis; the difference was not statistically significant ($p = 0.178$) (Paper IV).

Fig. 8. Box-plots of serum PTH levels in the periodontitis patients and in the periodontally healthy subjects (left) and in the T1DM patients with no or mild (middle) and moderate or severe periodontitis (right) at the baseline (BL) and in the follow-up examination (FU) (minimum and maximum, mean and median, upper and lower quartiles).
However, when we examined the response of serum PTH to periodontal therapy in the T1DM group, a moderate increase in PTH levels, especially in the group with moderate or severe periodontitis, was observed ($p = 0.016$, Figure 8). In 47% of the patients, more often in those with moderate or severe periodontitis, the increase in serum PTH was followed by an increase in serum $1,25(\text{OH})_2\text{D}$ level (Figure 9, Paper IV). Overall, the individual changes in serum PTH ($\Delta$ PTH) did not correlate with the changes in serum $1,25(\text{OH})_2\text{D}$ ($\Delta 1,25(\text{OH})_2\text{D}$) ($r = 0.02$, $p = 0.883$) (Figure 9 Paper IV).

Fig. 9. Absolute changes in serum $1,25(\text{OH})_2\text{D}$ level in relation to absolute changes in serum PTH level in T1DM patients with no or mild and moderate or severe periodontitis. In both studies serum Ca and P levels appeared to be within narrow/normal limits and not associated with periodontal health.
6 Discussion

6.1 Association between serum 1,25(OH)₂D and periodontal health (Papers I, II, IV)

In support of the posed hypothesis a significantly lower serum level of 1,25(OH)₂D—the active form of Vitamin D—was observed in periodontitis patients than in periodontally healthy individuals in the case-control study (Paper I). In addition, a significantly lower serum 1,25(OH)₂D level was observed in T1DM patients with moderate or severe periodontitis than in those with no or mild periodontitis at the baseline of the intervention study (Paper II).

To be able to characterize the nature of the association between periodontal infection and serum 1,25(OH)₂D level, we then used the intervention data and showed a significant increase in serum 1,25(OH)₂D level after elimination of periodontal infection (Paper II). This observation was interpreted to be suggestive of a true causal association between serum 1,25(OH)₂D level and periodontal infection.

Finally, to indirectly verify the possible causality in the association between serum 1,25(OH)₂D and periodontal infection, we studied one possible mechanism behind the increased serum 1,25(OH)₂D, namely whether more 25(OH)D was converted into the hydroxylated form after periodontal therapy. We observed in some individuals—more often in those with moderate or severe periodontitis—a moderate post-treatment increase in serum PTH, the main regulator of the enzyme 1-α hydroxylase (Fig. 9, Paper IV).

In line with the above findings, a number of studies show inverse associations between serum 1,25(OH)₂D and some inflammatory and infectious diseases (i.e. human sepsis, rheumatoid arthritis, and Crohn’s diseases) (Oelzner et al. 1998, Nguyen et al. 2013, Prosnitz et al. 2013, Augustine et al. 2014) which, like periodontitis, induce an increased systemic inflammatory burden (Loos et al. 2000, Noack et al. 2001, Paraskevas et al. 2008). In these studies three possible explanations for low serum 1,25(OH)₂D in diseased subjects were postulated by the research groups: 1) impaired 1-α-hydroxylation of 25(OH)D, 2) increased binding of 1,25(OH)₂D on immune-competent cells, and a less likely explanation, 3) increased degradation of 1,25(OH)₂D (Oelzner et al. 1998, Nguyen et al. 2013, Prosnitz et al. 2013, Augustine et al. 2014).
One explanation for the low level of serum 1,25(OH)_{2}D during periodontal infection in our subjects may be based on its actions on immunity reactions, possibly higher binding on immune-competent cells in inflammation/infection. Comparable notions have been postulated previously in other inflammatory/infectious diseases (Cannell et al. 2014). The post-treatment increase in serum PTH observed in some subjects (Figs. 8 and 9, Paper IV) may offer another partial or complementary explanation; the 1,25(OH)_{2}D increase could be due to improved 1-α hydroxylation of 25(OH)D associated with a decreased inflammatory/infectious burden in these patients. Correspondingly in Crohn’s disease, an increase in serum 1,25(OH)_{2}D level after therapies was explained by increased PTH and improved 1-α hydroxylation of 25(OH)D in the kidney (Prosnitz et al. 2013, Augustine et al. 2014). The biological mechanism suggested by in vitro or animal studies involves an inflammation-induced upregulated expression of CASRs yielding reduced PTH secretion and consequently reduced hydroxylation of serum 25(OH)D (Cannaf et al. 2005, Cannaf et al. 2008). An additional explanation for low serum 1,25(OH)_{2}D in periodontal disease may be decreased degradation.

While the present series of studies support a lower level and possibly higher use of 1,25(OH)_{2}D in periodontal infection, they cannot directly assess the actual alleviating or protective role of serum 1,25(OH)_{2}D. Nonetheless, due to the wide range of serum 1,25(OH)_{2}D in the two studies, (approximately from 20 pmol/L to 190 pmol/L) the findings may be considered clinically significant (Papers I and II). Further studies to characterize the immune-modulatory role of 1,25(OH)_{2}D in the periodontal area are needed.

6.2 Association between serum 25(OH)D and infectious periodontal diseases (Papers I – III)

The hypothesis of a lower serum 25(OH)D level in periodontitis patients vs. periodontally healthy individuals could not be confirmed in the case-control study (Table 5, Paper I). On the contrary, in the intervention study, T1DM patients with a low serum 25(OH)D level were more likely to belong to the group of moderate or severe than in the no or mild periodontitis group (Table 6, Paper II). However, unlike serum 1,25(OH)_{2}D, serum 25(OH)D did not respond to anti-infective periodontal therapy in the same patient group.

To further study the possible association between infectious periodontal diseases and serum 25(OH)D, we used data from a large sample of non-smoking
and non-diabetic 30–49-year-old individuals in the National Health 2000 Survey (Table 7, Paper III). While this study showed no significant association between serum 25(OH)D level or Vitamin D intake and periodontal pocketing and gingival bleeding after adjusting for confounding factors, a slightly lower number of teeth with deepened periodontal pockets was observed in individuals in higher than in lower quintiles of serum 25(OH)D, but only in those with good oral hygiene (Table 8, Paper III).

The overall conclusion to be drawn from the above studies (Papers I-III) is that the association between infectious periodontal diseases and serum 25(OH)D, if present, is weak and may be evident only in selected groups of individuals. In agreement with earlier studies among individuals with a compromised immune status, such as COPD patients (Zhou et al. 2012) or pregnant women (Boggess et al. 2011), an inverse association was found between serum 25(OH)D and periodontal infection in the present T1DM patients (Paper II). A plausible scenario behind this may be that in COPD and pregnancy Chary et al. 2015, Heulens et al. 2015, Persson et al. 2015) the metabolism of 25(OH)D is distorted in diabetes (Schneider et al. 1977, Hamed et al. 2011). That a slightly protective effect of high serum 25(OH)D on periodontal infection was seen only in individuals with good oral hygiene in the present study (Paper III) may be related to the possibility that the beneficial effect of 25(OH)D was overwhelmed by the effect of plaque in individuals with only moderate or poor oral hygiene.

Our finding of a lack of any significant association between periodontal pocketing and serum 25(OH)D level in non-smoking and non-diabetic 30–49-year-old Finnish adults is in line with the findings of the only existing population-based study (Dietrich et al. 2004), in which periodontal attachment loss was not associated with serum 25(OH)D level in < 50-year-old individuals in United States. In contrast to our findings on gingival bleeding, the same group (Dietrich et al. 2005) concluded that serum 25(OH)D may reduce susceptibility to gingival inflammation through its anti-inflammatory actions.

One explanation for the weakness of the association between serum 25(OH)D and periodontal condition in our studies may have largely to do with the overall low serum 25(OH)D level. Compared to the suggested threshold of 90-100 nmol/L for beneficial immune effects of 25(OH)D (Dietrich et al. 2005) the levels were clearly low; in various groups of the present series of studies ≤ 10% of the subjects had levels ≥ 75 nmol/L (Table 4).

In summary, contrary to the observed significant and consistent association of high serum 1,25(OH)2D level with periodontal health (Tables 5 and 6, Papers I and
II), our results show only a weak inverse association between serum 25(OH)D and periodontal infection (Table 8, Papers II and III), meaning that the beneficial effects of high serum 25(OH)D on periodontal condition, at least at the population level, are limited. Considering the different chemical and biological properties of the two Vitamin D metabolites and their complex mutual relationship, this is not surprising. Whether periodontal health is related to serum 25(OH)D at higher levels, for example at levels suggested for its beneficial immune effects, 90–100 nmol/L, remains open.

6.3 Methodological considerations

6.3.1 Study designs and samples (Papers I-IV)

Among the strengths of the present series of studies was the availability of various data sets [i.e the case-control data (Paper I), the intervention data (Papers II and IV) and the data of the Health 2000 Survey (Paper III)], enabling us to study the associations between the two Vitamin D metabolites and infectious periodontal diseases from different aspects. Another strength was the intervention study making it possible to more closely examine the nature of the associations between Vitamin D metabolites and periodontal infection. Compared with the study designs used, a randomized controlled trial (RCT), the gold standard for clinical trials, with a group with delayed periodontal treatment would have been a more powerful tool for studying the relationship between Vitamin D and periodontal infection. Moreover, including a non-diabetic cohort (Paper II) matched with the T1DM individuals would have allowed us to draw concrete conclusions about the effect of diabetes on the studied associations.

No a priori power analyses were performed for the present series of studies. The sample sizes in the Oulu Diabetes Study (Papers I and II)—originally planned for a study of the effect of anti-inflammatory periodontal therapy on glycemic control of T1DM—were fairly small. This naturally means there is a need to verify our results, especially the preliminary results concerning 1,25(OH)2D, in larger samples. The sample of non-smoking and non-diabetic Finnish adults aged 30–49 years from the population-based national Health 2000 Survey (Paper III) was fairly large (n = 1262), but the cross-sectional study design in this study precluded making conclusions about the temporal sequence of events, i.e. the exposure and
the outcome, and consequently limited assertions about the nature of the studied associations.

6.3.2 Periodontal variables

The Oulu Diabetes Study (Papers I, II and IV): All the clinical examinations were performed by one examiner (a specialist in periodontology) after a careful calibration procedure. Several periodontal measures including dental plaque, probing pocket depth, bleeding on probing, and periodontal attachment level (on four sites per tooth), indicative of both current and past periodontal infection and tissue destruction, were used. All the subjects in the periodontitis group had from moderate to severe periodontitis, whereas the degree of periodontitis varied from mild to severe in the T1DM group.

The Health 2000 Survey (Paper III): The three periodontal measures used were relatively crude estimators of periodontal health; plaque was measured on only three indicator teeth, and periodontal pocket depths—although measured from four surfaces per tooth—were registered only from the deepest site. Lastly, gingival bleeding was recorded only by sextants. The reason for the crude measures was the large number of individuals on whom periodontal measurements were performed (> 6000 individuals). Another reason that may have contributed to the negative result, i.e. lack of association between serum 25(OH)D and periodontal infection may be the overall low level of periodontal infection in the studied population; by restricting to non-diabetics and non-smokers, we had a study population with an admittedly low risk for periodontitis. The inter- and intra-examiner agreement in measuring periodontal infection was considered acceptable (Vehkalahti et al. 2004).

In terms of confounding factors, those associated with the outcome and the exposure based on unadjusted associations, and known established confounding factors were in focus (Papers I–III); of these, diabetes mellitus together with its glycemic control, and age are among the most important. To control for confounding, both restriction of the study samples and adjusting for confounding factors in multivariate analyses were used.

Smoking, a thoroughly discussed problem in periodontal research (Spiekerman et al. 2003), is strongly associated with general health status and is surely a factor that may confound associations between periodontal infection and systemic conditions including serum Vitamin D level (Manavi et al. 2015).

It was not possible to fully control the effect of smoking by adjusting the regression models for smoking based on self-reported dichotomized (yes/no) data.
(Hujoel et al. 2002) in the Oulu Diabetes Study (Papers I and II). Therefore, the same associations were studied also in non-smokers. For the same reason, restriction to non-smokers was done in the Health 2000 Survey (Paper III).

A second strong determinant for periodontal infection and tissue destruction is diabetes mellitus (Lalla & Papapanou 2011). Therefore, restriction to non-diabetics was done in the case-control study (Paper I) and in the Health 2000 Survey (Paper III). In addition, to consider the confounding effect of the glycemic status of the T1DM patients on the studied associations in the intervention study, adjustments were made for the 3-year level of the metabolic control of diabetes mellitus (Tervonen & Oliver 1993, Tervonen & Karjalainen 1997).

By restricting the study sample to 30–49-year-olds in the Health 2000 Survey, we reduced the biological effects of aging and the effects of age-related diseases and medications on the disease outcome and also eliminated confounding due to the high prevalence of edentulism and high rates of lost teeth in the elderly part of the Finnish population.

Of other disease determinants not included in the present study, one is genetic polymorphism of the genes encoding the hydroxylases of 25(OH)D as well as the Vitamin D receptor genes, which may affect the studied associations (McCullough et al. 2009, Chen et al. 2012).
7 Summary and conclusions

In the case-control study, the level of serum 1,25(OH)₂D was significantly lower in the periodontitis patients than in the periodontally healthy controls (Paper I). In addition, at the baseline of the intervention study among T1DM patients, a statistically significant inverse association was found between the extent/severity of periodontal infection and serum 1,25(OH)₂D level (Paper II). Based on these observations, it was hypothesized that more 1,25(OH)₂D was used, i.e. more was bound to the intracellular VDR receptors of immune-competent cells during periodontal infection. An alternative explanation could be increased degradation of 1,25(OH)₂D during infection.

A statistically significant increase in serum 1,25(OH)₂D level was observed after anti-infective periodontal treatment in the group of T1DM patients (Paper II). This finding might be suggestive of causality in the nature of the association between periodontal infection and serum 1,25(OH)₂D.

To examine other mechanisms behind the above increase in serum 1,25(OH)₂D, we then studied serum PTH, the main regulator of 1-α hydroxylase, the enzyme that hydroxylates 25(OH)D to 1,25(OH)₂D (Paper IV). Based on the observed increase in serum PTH after periodontal treatment in some patients (47%), we also hypothesized that more 25(OH) could have been hydroxylated to 1,25(OH)₂D after resolution of infection in these individuals, but this explained only partly the increase in 1,25(OH)D.

In line with earlier studies among immune-compromised individuals, a statistically significant inverse association between periodontal infection and serum 25(OH)D was found in the group of patients with T1DM (Paper II). However, no response in serum 25(OH)D level to periodontal therapy could be observed in the same patient group. Infectious periodontal diseases were not associated with serum 25(OH)D level in non-smoking and non-diabetic Finnish adults aged 30–49 years. However, a slightly lower number of teeth with deepened periodontal pockets (≥ 4 mm) was observed in higher than in lower 25(OH)D quintiles in individuals with good oral hygiene (Paper III).

To summarize, the present series of studies showed that infectious periodontal diseases were associated differently with the two Vitamin D metabolites; while the results may support a substantial association between infectious periodontal diseases and serum 1,25(OH)₂D, no consistent association was found between periodontal infection and serum 25(OH)D. Measuring both metabolites of Vitamin D and simultaneously studying their association with periodontal infection can be
considered a strength of this study. In order to fully evaluate the role of Vitamin D in periodontal infection, future studies measuring $25(OH)D$ and $1,25(OH)_{2}D$ both in serum and locally in the periodontal area are needed. Furthermore, the possible causality in the association between $1,25(OH)_{2}D$ and periodontal infection should be verified in non-diabetic individuals.
References


61


List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals.


Reprinted with permission from John Wiley and Sons (I, IV), Endocrine Society (II) and the American Academy of Periodontology (III).

Original publications are not included in the electronic version of the dissertation.
1294. Koskenkorva, Timo (2015) Outcome after tonsillectomy in adult patients with recurrent pharyngitis
1296. Timlin, Ulla (2015) Adolescent's adherence to treatment in psychiatric care
1297. Aatsinki, Sanna-Mari (2015) Regulation of hepatic glucose homeostasis and Cytochrome P450 enzymes by energy-sensing coactivator PGC-1α
1300. Pakanen, Lasse (2015) Thrombomodulin and catecholamines as post-mortem indicators of hypothermia
1304. Ijäs, Hilkka (2015) Gestational diabetes: metformin treatment, maternal overweight and long-term outcome
1308. Myllymäki, Satu-Marja (2015) Specific roles of epithelial integrins in chemical and physical sensing of the extracellular matrix to regulate cell shape and polarity

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