

SECOND EDITION

# Infection and Autoimmunity



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Second Edition

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## CHAPTER 10

# Infection, Autoimmunity, and Vitamin D

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### 1 INTRODUCTION

For decades, researchers have noted the presence of culturable pathogens in patients with a wide range of chronic inflammatory diseases. These pathogens include Epstein–Barr virus (EBV), cytomegalovirus, hepatitis C virus, *Chlamydia pneumoniae*, *Mycobacteriaceae*, and *Borrelia* spp. Yet the prevalence of any one pathogen in a particular cohort varies widely depending on the methodology of the study, and no single pathogen has been reliably detected in all patients with a particular inflammatory diagnosis.

However, genome-based microbial detection methods that have emerged during the past decade revealed vast communities of bacteria, viruses, and fungi now known to persist in nearly every body site, called the human microbiome. This discovery has uncovered thousands of new microbial species capable of contributing to autoimmune and inflammatory disease processes. In lieu of a single pathogen causing a single disease, many of these microbes instead seem to generate dysfunction by continually interacting with other species in a shared intra-phagocytic environment. Yet to persist inside host immune cells, the microbiome must necessarily degrade the host's innate immune defences. In doing so, the pathogens also dysregulate other aspects of human metabolism by directly altering the expression of human genes. This chapter describes how the cumulative effect of these changes to the human metabolome and interactome may lead to the catastrophic failure of human metabolism so often observed in chronic idiopathic disease.

## 2 THE FIELD OF METAGENOMICS IS BORN

After completion of the Human Genome Project in 2003, researchers began to use many of the DNA-based technologies that had been developed to decode the human genome to explore the microbial genomes now being found in the human body. The results were unprecedented. Thousands of microbes that had never been identified by culture-based laboratory techniques were readily detected in human tissue and blood.

Two large-scale, multi-center collaborations spearheaded use of the genome-based technologies to better identify and characterize these previously undiscovered microbes. One was the Human Microbiome Project (HMP) (2008–2012), a US-based initiative funded by the US National Institutes of Health.<sup>1</sup> The Project, which sampled the microbial populations of 242 healthy adults across 15–18 body sites, eventually generated more than 3.5 terabases of metagenomic sequences.<sup>2</sup> The second initiative was the European-based project MetaHIT (2008–2012), which focused primarily on better characterizing the microbiome of the gut.<sup>3</sup> In concert with a number of private research teams also focused on microbiome research, these projects succeeded in identifying so many novel microbes in *Homo sapiens* that today at least 90% of cells in the human body are estimated to be bacterial, viral, fungal, or otherwise non-human in origin.

As the HMP and related projects moved forward, research shifted from the study of a dozen or so well-characterized human pathogens to the exploration of myriad microbial species that have now been identified across nearly all body sites. Metagenomic communities of microbes were shown to persist not just on the body's mucosal surfaces but also in tissue and blood. Polybacterial and chronic infections have been detected in atherosclerotic plaque.<sup>4</sup> Microbial RNA has been detected even in healthy human blood.<sup>5</sup> The lungs are now understood to harbor a microbiome as well, the composition of which differs in patients with chronic lung conditions.<sup>6</sup> Even the biofilm removed from prosthetic hip joints during revision arthroplasties has been shown to harbor dozens of distinct bacterial phylotypes.<sup>7</sup>

As of October 2013, the Genomes Online database lists 2337 completed and published bacterial genomes, with another 24,303 in progress.<sup>8</sup> Even so, each new metagenomic analysis continues to allow researchers to identify previously unknown microbes. For example, an analysis by Nasidze et al. of the human oral cavity identified 101 bacterial genera in the mouth as well as 64 genera previously unknown to science.<sup>9</sup>

Similarly, our knowledge of the chronic viruses that persist in *H. sapiens* (the virome) also has evolved rapidly. We have known for some time that several well-characterized viruses such as polyomaviruses infect and remain with most humans throughout life. Yet entirely new persistent viral populations have now been discovered. After analyzing the fecal virome of monozygotic twins and their mothers, Reyes et al. found that 81% of the reads generated from this virome did not match those of any known viruses.<sup>10</sup> In 2011, Pride et al. reported that previously uncharacterized bacteriophages dominate the oral cavity, and several phages serve as reservoirs for pathogenic gene function.<sup>11</sup>

### 3 THE HUMAN SUPERORGANISM

The approximately 20,500 genes expressed by the human genome are vastly outnumbered by the millions of genes expressed by our microbial inhabitants. The human gut microbiome alone expresses at least 9 million unique genes.<sup>12</sup> It follows that we cannot study disease by studying the human genome in isolation. The millions of proteins and metabolites expressed by the microbiome continually interact with the human genome, altering the manner in which human genes are subsequently expressed. For example, one analysis demonstrated that the expression of at least 463 human genes is changed during a single infection with *Mycobacterium tuberculosis*.<sup>13</sup>

This new understanding redefines what it means to be human – human beings are now most accurately described as superorganisms. The metabolism of the human superorganism represents a combination of microbial and human attributes. For example, the human angiotensin-converting enzyme (*ACE*) has been associated with myocardial infarction, Alzheimer's disease, diabetes mellitus, and sarcoidosis.<sup>14</sup> Yet the expression of *ACE* also has been shown to be downregulated by *Lactobacillus* and *Bifidobacteria*, microbes commonly found in dairy products.<sup>15</sup> The ability of these and other microbes to directly alter human gene expression significantly affects the pathogenesis of inflammatory conditions, albeit in ways not yet fully understood.

Proteins and metabolites generated by the microbiome permeate the body. Of the small molecules found in healthy human blood, 36% are created by the human gut microbiome.<sup>16</sup> The myriad interactions between these foreign and host proteins in the body, referred to as the interactome, affect an array of human metabolic processes. For example, the presence or absence of particular microbial metabolites in the blood of any individual cause the medication acetaminophen to be metabolized differently from person to person.<sup>17</sup>

These transgenomic interactions are complicated by the fact that the structures of many microbial proteins are identical or very similar to those expressed by the human genome. For example, some bacteria metabolize folate and glucose with a metabolism similar to that of their human hosts. Because of this overlap, the human superorganism may have great difficulty distinguishing proteins and metabolites created by the microbes from those recognized as “self”.

This “molecularly mimicry” is extremely common. Tens of thousands of protein–protein interactions have been documented between the genomes of *Yersinia*, *Salmonella*, *Escherichia coli*, and the human genome.<sup>18</sup> Kusalik et al. identified 19,605 pentamers from the hepatitis C virus polyprotein with a high level of similarity to the human proteome.<sup>19</sup> This high level of similarity persisted even when the team used longer peptide motifs as probes for identity scanning.

## 4 THE MICROBIOME IN HEALTH AND DISEASE

An increasing body of research demonstrates that microbiome composition often changes over time in patients with a range of chronic inflammatory diseases. This disturbance in the body’s microbial populations, or dysbiosis, is associated with a growing number of chronic conditions including types 1<sup>20</sup> and 2<sup>21</sup> diabetes, Crohn’s disease, ulcerative colitis<sup>22</sup> and psoriasis.<sup>23</sup> For example, Amar et al. recently demonstrated that 16S recombinant DNA blood serum concentrations were significantly elevated in 3280 subjects without obesity or diabetes at baseline but who later developed diabetes.<sup>24</sup>

The composition of the microbiome has even been shown to shift in patients with cancer. Kostic et al. analyzed whole genome sequences from patients with colorectal carcinomas.<sup>25</sup> The Bacteroidetes and Firmicutes phyla were depleted in tumors, whereas *Fusobacterium* sequences were enriched in carcinomas. Patients with chronic lyme disease and chronic fatigue syndrome exhibited greatly altered cerebrospinal proteomes, which also differ from those of healthy individuals.<sup>26</sup> Considering that almost all of the atypical proteins in the human body are microbial in origin, these proteomic differences directly reflect shifts in microbiome composition.

These alterations of the microbiome in health and disease involve entire microbial ecosystems. Thus, chronic disease processes driven by infection are likely due to changes in complex microbial communities rather than the acquisition of a single pathogen. It follows that Koch’s postulates, which

dictate that a single infectious disease must be caused by a single pathogen, are no longer tenable in the current era of the metagenome.

Unfortunately, studies of microbiome composition in health and disease are complicated by a host of environmental variables that can also cause significant shifts in the body's microbial populations. These include geographic location, food consumption, water intake, and the use of medications and supplements. These additional environmental factors contribute to significant variability in the microbiome across months, weeks, and even days – so much so that even the microbiomes of monozygotic twins are no more similar than those of fraternal twins.<sup>27</sup>

It follows that while the identification of the species present in patients with a given inflammatory condition can provide valuable clues about disease, we cannot focus simply on population-based studies. Instead, we must examine what the microbial genomes actually *do* to persist and to influence the body's metabolic processes.

## 5 VITAMIN D NUCLEAR RECEPTOR DYSREGULATION

Those pathogens most successful at causing disease tend to persist inside the cells of the immune system. *M. tuberculosis* and the EBV are two examples. The ability of these intracellular pathogens to persist in nucleated cells allows them to directly interfere with transcription, translation, and DNA repair processes. If the accumulation of errors resulting from this interference exceeds the capacity of cellular repair mechanisms, the interactome can become significantly dysregulated. For example, *Helicobacter pylori* infection generates significant genetic instability in gastric epithelial cells by disrupting their DNA repair mechanisms.<sup>28</sup>

Some intracellular pathogens (e.g. *M. tuberculosis* and EBV) survive by dysregulating gene expression by the vitamin D nuclear receptor (VDR). Just a decade ago, the VDR was studied almost solely in the context of calcium metabolism. Today, however, this receptor has been shown to express at least 1000 genes, with many more putative gene candidates in the pipeline.<sup>29</sup> VDR promoters are ubiquitous throughout the human genome, and genes already associated with VDR regulation are directly connected to autoimmune and inflammatory processes.<sup>29</sup> The receptor also expresses genes related to cancer, including metastasis suppressor protein 1 (*MTSS1*), which plays a key role in promoting apoptosis and repressing the cell cycle in cancerous cells.<sup>29</sup>

In addition to its key role in transcription regulation, the VDR also lies at the heart of the human innate immune response. It expresses TLR2, which allows the immune system to recognize bacterial polysaccharides. In addition, it regulates expression of the cathelicidin and  $\beta$ -antimicrobial peptides (AMPs), which play vital roles in targeting intracellular pathogens.

Thus, any microbe capable of dysregulating VDR activity would significantly disable the innate immune response, facilitating its persistence. Indeed, several of the pathogens most often linked to inflammatory disease have in fact evolved to survive in exactly this fashion. Persistent *M. tuberculosis* has evolved to slow VDR activity. When lymphoblastoid cell lines are infected with EBV, activity of the VDR is downregulated as much as 15 times.<sup>30</sup> *Mycobacterium leprae*,<sup>31</sup> cytomegalovirus,<sup>32</sup> and *Borrelia burgdorferi*<sup>33</sup> also inhibit VDR activity to varying degrees. The fungus *Aspergillus fumigatus*, common in cystic fibrosis, secretes a gliotoxin that significantly downregulates VDR expression.<sup>34</sup> In addition, bacterial species in biofilm often secrete the sulphonolipid capnine,<sup>35</sup> which we have demonstrated can inhibit VDR activation.<sup>36</sup> Indeed, disabling the innate immune response via the VDR pathway is such a logical pathogen survival mechanism that many more species capable of persisting in the same or similar fashion will likely be identified in the coming years.

## 6 FLOW-ON EFFECTS OF VDR DYSREGULATION

VDR dysregulation generates a number of imbalances that further compromise homeostasis and immunity. The activated VDR is responsible for expressing *CYP24A1*, an enzyme primarily responsible for deactivating the active vitamin D metabolite 1,25-dihydroxyvitamin D (1,25-D).<sup>37</sup> In addition, inflammation associated with persistent intracellular microbes causes excess production of the enzyme *CYP27B1*. This results in increased conversion of 25-hydroxyvitamin D (25-D) into 1,25-D. Both processes cause 1,25-D concentrations to increase. Indeed, elevated concentrations of 1,25-D leaking into the bloodstream have been demonstrated in several inflammatory conditions, including tuberculosis and rheumatoid arthritis.<sup>38</sup> Our analysis found that 85 of 100 patients with autoimmune and inflammatory conditions had elevated concentrations of 1,25-D, ranging from 110 pmol/L to a high of 350 pmol/L.<sup>39</sup>

Our data indicate that, as its concentration rises, 1,25-D may also dysregulate gene expression via nuclear receptors other than the VDR.<sup>40</sup> These include the androgen receptor, the glucocorticoid receptor, and the  $\alpha$ - and  $\beta$ -thyroid receptors.<sup>41</sup> Each of these nuclear receptors also



controls multiple families of AMPs (20, 17, and 15 families, respectively, of the 22 analyzed by Brahmachary et al.<sup>42</sup>). Thus, as 1,25-D accumulates within infected cells, it may disable the activity of these AMPs as well, leaving the host increasingly immunocompromised.

## 7 SUCCESSIVE INFECTION

As acquired pathogens slow activity of the VDR and related receptors, an individual's microbiome gradually shifts towards a state that promotes overt disease. During this process of "successive infection", the host microbiome gradually shifts away from a homeostatic state. Opportunistic pathogens are progressively incorporated into the microbiome, where they alter the expression of the human genome. As a result, the interactome becomes increasingly dysregulated. Infected human cells fail to correctly express human metabolites in the presence of the proteins, enzymes, and metabolites generated by the accumulating pathogenic genomes.

In addition, any pathogen that decreases nuclear receptor AMP expression slows the innate immune response so that the host more easily acquires even more microbes, some of which may well symbiotically suppress AMP. This creates a snowball effect, in which it becomes progressively easier for the host to acquire pathogens as the strength of the immune response wanes.

As an increasing number of pathogens become incorporated into the microbiome, a patient may eventually begin to present with clinically evident symptoms characteristic of an inflammatory diagnosis. Each individual's unique symptoms vary depending on the location, species, and virulence of the pathogens they have accumulated into their microbiome. Symptoms vary between individuals because of the semi-infinite number of ways in which the microbial proteins and metabolites can interfere with those of the host.

While successive infection leads to the acquisition of more pathogens over time, the diversity of microbes in a particular niche may not necessarily increase. Microbes are highly competitive, meaning that certain pathogens often successfully outcompete larger populations of other less aggressive species. Higher levels of dysfunction may result if keystone species are lost. Indeed, some studies show that microbiome diversity is diminished in patients who are already ill. For example, lower microbial diversity has been reported in the guts of infants with atopic eczema.<sup>43</sup> In other cases, successive infection may lead to greater species diversity. In women who suffer from bacterial vaginosis, for example, the vaginal microbiome composition often shifts to become more taxon-rich than that of healthy subjects.<sup>44</sup>

## 8 COMORBIDITY

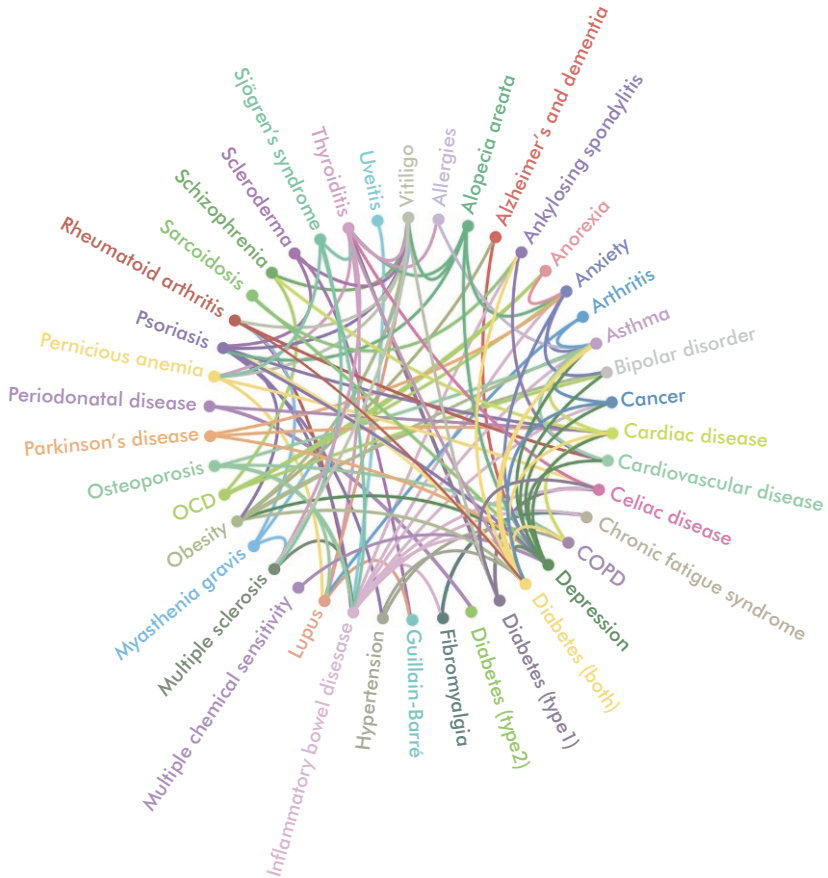
Patients with one inflammatory diagnosis are at higher risk for developing a second. For example, the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study found that baseline depressive symptoms were associated with an increased risk of coronary heart disease in the short term and stroke over the long term in otherwise healthy, European, middle-aged men.<sup>45</sup> The overlap between the symptoms and disease presentation associated with comorbid conditions directly reflects the variability inherent to the process of successive infection. No two people exposed to pathogens over time ever acquire the exact same mix of species in their microbiome. Therefore, no two individuals will ever develop an identical disease presentation, and the symptoms of patients with similar diagnoses can be expected to overlap and fluctuate with time. [Figure 1](#) shows the extent to which patients with one inflammatory disease often suffer from other comorbid conditions.

This suggests that various inflammatory diagnoses are best studied together. Current pragmatism often dictates that one inflammatory condition is directly causal of a second, although the mechanisms behind these assumptions remain unclear. For example, obesity is generally believed to cause diabetes. Yet, increasing evidence shows that the gut microbiome is significantly altered in patients with both conditions.<sup>46</sup> It may be more likely, then, that both obesity and diabetes gradually occur together because of successive infection or a common underlying pathogenesis.

In the same vein, patients suffering from physical inflammatory conditions are at greater risk for developing neurological dysfunction and vice versa. This suggests that neurological and autoimmune diagnoses, which are currently separated into different medical specialties, may indeed arise from similar underlying infectious processes. Indeed, dysbiosis of the microbiome has been documented in patients with a range of neurological conditions including multiple sclerosis,<sup>47</sup> autism and obsessive-compulsive disorder.<sup>48</sup>

## 9 FAMILIAL AGGREGATION

Familial aggregation must be reexamined in light of comorbid disposition. A host of studies show that the relatives of patients with inflammatory disease are more likely to be ill themselves. For example, a 2006 study by Anaya et al. of families who have a member with primary Sjögren's syndrome showed that 38% had at least one first-degree relative with an autoimmune disease versus 22% of control families.<sup>49</sup>



**Figure 1** Comorbidities among common inflammatory diseases. Each “spoke” of this wheel represents a published study appearing in MEDLINE, which shows a significant statistical relationship between one disease and another.

While this clustering of inflammatory diseases in families could be explained by the sharing of common genes, any such genes confer no known survival advantage and should subsequently have been weeded from the population. Instead, familial aggregation likely results from the sharing of common microbes. For example, infected mothers, fathers, and siblings are all major sources for *H. pylori* acquisition among young children; the infected mother serves as the main source for childhood acquisition of microbes.<sup>50</sup> Certain pathogens have been shown to cross the placental barrier and even persist in the amniotic fluid, sperm and egg.<sup>51</sup> These microbes can thus be passed easily from generation to generation.

Pathogens passed down the maternal line seem to have a particularly strong effect on the microbiome of subsequent generations. The founding microbial populations of an infant delivered by vaginal birth closely mirror those of its mother's vagina. In addition, after Cesarean delivery, differences in infants' founding microbial populations can persist for months.<sup>52</sup> Breast milk is now understood to deliver a microbiome that varies among women and depends on a host of factors. Cabrera-Rubio et al. found that the composition of the breast milk microbiome significantly changed over the course of at least the first 6 months of lactation.<sup>53</sup> The weight of the mother further affects the composition of her breast milk microbiome. Milk from obese mothers tended to contain a different and less diverse bacterial community than that obtained from healthy subjects. In addition, those mothers who underwent elective Cesarean delivery displayed different bacterial communities in their milk samples than those subjects who gave birth by vaginal delivery.

## 10 IMMUNOSUPPRESSIVE THERAPIES MAY PALLIATE SYMPTOMS BUT INCREASE DISEASE OVER THE LONG TERM

The standard of care for most autoimmune and inflammatory conditions is immunosuppression. Commonly used immunosuppressive treatments include corticosteroids, methotrexate, and tumor necrosis factor- $\alpha$  antagonists. While these therapeutic options often provide short-term symptom palliation, they have poor long-term associations with stability and relapse. Indeed, no definitive studies have identified corticosteroids capable of enhancing long-term prognosis or reducing mortality rates. For example, Gottlieb et al. reported that in sarcoidosis, steroid use leads to relapse and contributes to prolongation of disease by delaying resolution.<sup>54</sup> To date there have been nearly 150 clinical trials testing prospective agents designed to block inflammation in patients with sepsis, and all have failed.<sup>55</sup>

Most immunosuppressive medications were developed to slow what is historically believed to be an overactive immune response. However, as the inflammation and autoantibodies associated with these conditions are becoming increasingly tied to infection, the efficacy of these drugs must be reexamined. By slowing the immune response, immunosuppressive medications cause a decrease in inflammation and cytokine release. While this decrease in inflammation may allow a patient to *feel* better in the short term, the immune system may well become compromised to a point where

it can no longer correctly maintain microbiome homeostasis. This exacerbates the underlying disease state and leaves patients more vulnerable to the acquisition of new pathogens.

## 11 VITAMIN D SUPPLEMENTS ARE IMMUNOSUPPRESSIVE: 25-D PALLIATES SYMPTOMS BUT DOES NOT CURE INFLAMMATORY DISEASE

One of the most common supplements used to palliate inflammatory symptoms is cholecalciferol, a precursor for the secosteroid 25-D. While vitamin D has long been viewed solely as a nutrient, vitamin D metabolites are actually potent secosteroids. In 2008 we described some of the complexity inherent in the actions of these metabolites.<sup>37</sup> While the metabolism of a vitamin is characterized by a first-order mass-action model, metabolism of the vitamin D secosteroids is instead governed by layers of feedback and feed-forward transcriptional pathways that are tightly regulated in *H. sapiens*. This calls into question whether the word *vitamin* accurately communicates any of the primary activities of this supplement.

While proper functioning of the VDR is vital to a plethora of activities necessary for optimal human health, an increasing number of studies show that artificial supplementation of vitamin D metabolites does not result in optimal VDR activity. Under conditions of health, the VDR is activated by 1,25-D. Yet, as previously described, if 1,25-D concentrations increase in patients as they become ill, the elevated metabolite can interfere with the ability of key nuclear receptors to correctly express the AMPs under their control.<sup>40</sup>

Elevated concentrations of 25-D in the blood cause additional immunosuppression by a number of mechanisms. Dickie et al. found that 25-D slows the activity of several toll-like receptors including TLR2, TLR4, and TLR9.<sup>56</sup> A study of multiple sclerosis demonstrated that the supplement effectively slowed the immune activity of peripheral blood mononuclear cells.<sup>57</sup> Indeed, Arnson et al. argued that vitamin D has multiple immunosuppressant properties and that, on the whole, vitamin D confers an immunosuppressive effect.<sup>58</sup>

Over the past decade we performed several *in silico* experiments that suggest that blood-borne 25-D is able to directly bind into the VDR binding pocket to slow receptor activity.<sup>37</sup> Much like the microbial ligands that slow innate immunity by interfering with VDR activity, this antagonism would result in a significant decrease in the expression of AMP and TLR2. This

immunosuppressive effect progressively increases as higher doses of vitamin D are administered, resulting in the *J*- or *U*-shaped curves evident in so many of the clinical trials.<sup>59,60</sup> The resulting decrease in innate immune activity enhances pathogen survival, and homeostasis of the microbiome is more easily disrupted.

We have subsequently argued, with increasing urgency, that any subjective or objective improvements associated with vitamin D supplementation in the short term result from its ability to decrease the immunopathology associated with an effective innate immune response to elements of a patient's microbiome.<sup>61</sup> Blood-borne 25-D likely provides symptomatic relief by acting in a manner similar to the immunosuppressive medications described earlier, the use of which has been associated with high rates of relapse and instability over time.

Thus, while we are accustomed to the hypothesis that high levels of vitamin D supplementation are necessary to curb the current epidemic of chronic disease, the opposite may instead be true. Vitamin D is added to an increasing variety of food products and is more frequently used in the clinic, but the incidence of nearly every chronic condition has, in fact, increased. To minimize potential harm, we believe that blood-borne 25-D must be kept *below* the consensus immunosuppressive level of approximately 50–60 nmol/L to optimize innate immune function and overall health.

## 12 HARM FROM VITAMIN D IS INCREASINGLY SUPPORTED BY HIGH-QUALITY STUDIES

Description of vitamin D as the “sunshine vitamin” has led many to assume that the secosteroid is not likely to cause any serious harm (despite the fact that solar exposure can cause skin cancer). Yet vitamin D supplementation is increasingly being associated with a host of negative health outcomes including brain lesions,<sup>62</sup> kidney stones,<sup>63</sup> increased bone fractures<sup>64</sup> and increased incidence of allergy and atopy.<sup>65</sup>

Despite these reports, many researchers still believe that vitamin D is a panacea. The majority of studies referenced to support these assumptions, however, rely largely on surrogate outcomes and speculation. Most also fail to provide a basis in human molecular biology for the apparent benefits observed in their analyses. Some researchers assume that ancient man obtained high concentrations of vitamin D from the sun on the plains of

Africa and conclude that modern humans should do the same. However, anthropologists have no definitive data on the sun exposure of early *H. sapiens*. Other studies discuss instances in which people who live at sunnier latitudes display a lower incidence of some inflammatory diseases. Nonetheless, myriad other variables confound their analyses, and even a quick search of the literature reveals numerous, but less frequently cited, counterexamples. Others have argued for increased vitamin D supplementation on the basis that sunscreens block the production of vitamin D in the skin, but even the hypothesis that vitamin D is primarily produced by irradiation of the skin is now being questioned<sup>66</sup> and, in any case, sunscreen does not seem to block vitamin D production.<sup>67</sup>

In 2009, the US and Canadian governments commissioned the Tufts Evidence-based Practice Center to compile a report on vitamin D for the Institute of Medicine (IOM).<sup>68</sup> In an effort to address the discrepancies observed in the vitamin D literature, this report assessed all studies relating to health outcomes and vitamin D and/or calcium intake. After an evidence-based analysis, the Tufts researchers were able to support a positive association only between vitamin D intake and bone health. In the case of all other chronic or inflammatory conditions analyzed, they found no evidence to support an association between vitamin D intake and improved health. The IOM committee therefore decided not to increase the Daily Recommended Intake of vitamin D and further noted that intake of the secosteroid is associated with adverse health outcomes.

Given that the IOM report was released, the results of randomized controlled trials evaluating the use of vitamin D in a variety of inflammatory conditions continue to demonstrate little benefit, and even harm, from vitamin D supplementation. Well-designed studies recently determined that supplemental vitamin D does not significantly improve cardiovascular disease risk factors,<sup>69</sup> isolated systolic hypertension,<sup>70</sup> tuberculosis<sup>71</sup> and upper respiratory infections.<sup>72</sup> Several studies have called into question the one positive association between vitamin D intake and bone health that the IOM committee was able to support in 2010. In 2013, McAlindon et al. published the results of a randomized placebo-controlled trial showing that vitamin D3 supplementation for a period of 2 years did not reduce knee pain or loss of cartilage volume in patients with symptomatic knee osteoarthritis.<sup>73</sup> Another such study found increased risk of fracture among elderly women taking vitamin D.<sup>64</sup> A second systematic review found that vitamin D supplementation failed to improve bone health in women with breast cancer.<sup>74</sup>

### 13 THE CONCEPT OF VITAMIN D DEFICIENCY IS FLAWED: LOW BLOOD CONCENTRATIONS OF 25-D LIKELY RESULT FROM THE INFLAMMATORY DISEASE PROCESS

Despite the data discussed above, vitamin D supplementation is routinely justified based on a plethora of studies that report low concentrations of 25-D in the blood of patients with a wide variety of inflammatory conditions. Thus far, the consensus on these findings has been to assume that the low concentrations of 25-D are driving or contributing to the pathogenesis of these diseases.

However, the low concentrations of 25-D often detected in patients with inflammatory conditions may be a *result* of the inflammatory disease process rather than the cause of the inflammation. Indeed, our data suggest that under conditions of microbiome and interactome dysregulation, the body uses multiple mechanisms to naturally downregulate intracellular production of 25-D.<sup>37</sup>

Expression of the enzyme *CYP24A1* normally controls excess concentrations of 1,25-D. However, if VDR activity is slowed by the intra-phagocytic microbiome, the enzyme cannot be expressed as robustly. In addition, when 1,25-D increases, it downregulates the amount of previtamin D converted into 25-D. One of these mechanisms is antagonism of the PXR nuclear receptor and expression of the enzyme *CYP27A1*. The result is that blood concentrations of 25-D, the metabolite most commonly measured in the clinic, decrease.

The concept of vitamin D “deficiency” has been further complicated by the arbitrary ranges used to define insufficiency and deficiency. Vitamin D supplementation has become so prevalent that individuals who choose not to ingest extra amounts of the secosteroid may be deemed deficient simply because they eat unfortified foods.<sup>75</sup> Indeed, it is difficult to find unsupplemented populations to study. Nevertheless, studies of healthy individuals in populations that do not heavily supplement their food supplies with vitamin D have demonstrated that subjects’ 25-D concentrations are naturally found to be in the range we today have labeled as “deficient”.

Saudi Arabia does not yet add vitamin D to their food supply. It is not surprising, then, that one study found that 100% of Saudi medical students receiving standard amounts sunlight and eating a normal diet were vitamin D insufficient or deficient according to conventional standards.<sup>75</sup> Similarly, a study of healthy Bangladeshi women not supplementing with vitamin D found that approximately 80% had 25-D concentrations less than 16 ng/mL.<sup>76</sup> A separate study of premenopausal Bangladeshi women came



to a similar conclusion.<sup>77</sup> A study of young healthy adults from the west of India, also not consuming supplemental vitamin D, found the average serum concentration of 25-D to be 17.4 ng/mL.<sup>78</sup> In 1992, before vitamin D supplementation became more common in China, a study found that healthy full-term Chinese infants had serum concentrations of 25-D ranging from an average of 5 to 14 ng/mL, a level that would be regarded as “highly deficient” by current dogma.<sup>79</sup>

It is tempting to argue that the individuals in these studies do not receive adequate exposure to sunlight. (Whether people require a certain amount of sunlight also remains a matter of debate.) Yet in each instance the authors clearly ascertain that their subjects are healthy and quite functional. Instead of assuming that these healthy subjects should be given extra vitamin D, as the current standard of care indicates, it may be prudent to consider whether the ranges we have created for vitamin D deficiency and insufficiency have a basis in human molecular science.

## 14 DISCUSSION

In just a decade, the discovery of the human microbiome has uncovered the presence of thousands of microbes capable of contributing to chronic infectious processes. Indeed, dysbiosis of the microbiome has been tied to an increasing number of inflammatory disease states. In lieu of a single pathogen causing a single chronic infectious disease, the microbiome as a whole gradually shifts away from a state of homeostasis as people become ill. Those pathogens capable of persisting inside nucleated cells are able to extensively dysregulate human metabolism by directly interfering with gene expression. A number of key pathogens associated with autoimmune disease persist by inhibiting the VDR and subsequently the innate immune response. This allows them to more easily accumulate into the microbiome over time.

Anti-inflammatory treatments that slow the immune response may provide short-term symptomatic improvement, but they do so at the expense of long-term microbiome stability. This leads to relapse and increases the likelihood of comorbid conditions over time. The secosteroid we call vitamin D is immunosuppressive. In patients with chronic inflammatory disease, low concentrations of 25-D in the bloodstream are likely a result of the disease process, rather than its cause. To allow the immune system to keep control of the microbiome, concentrations of blood-borne 25-D should be kept below the immunosuppressive threshold of 50–60 nmol/L.

## REFERENCES

1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature* 2007;**449**(7164):804–10.
2. Methé BA, Nelson KE, Pop M, Creasy HH, Giglio MG, Huttenhower C, et al. A framework for human microbiome research. *Nature* 2012;**486**(7402):215–21.
3. Ehrlich SD. *MetaHIT: The European Union Project on metagenomics of the human intestinal tract*. New York: Springer; 2011, p. 307–16.
4. Kozarov E. Bacterial invasion of vascular cell types: vascular infectology and atherogenesis. *Future Cardiol* 2012;**8**(1):123–38.
5. McLaughlin RW, Vali H, Lau PCK, Palfree RGE, De Ciccio A, Sirois M, et al. Are there naturally occurring pleomorphic bacteria in the blood of healthy humans? *J Clin Microbiol* 2002;**40**(12):4771–5.
6. Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, et al. Analysis of the lung microbiome in the “healthy” smoker and in COPD. *PLoS ONE* 2011;**6**(2):e16384–e.
7. Dempsey KE, Riggio MP, Lennon A, Hannah VE, Ramage G, Allan D, et al. Identification of bacteria on the surface of clinically infected and non-infected prosthetic hip joints removed during revision arthroplasties by 16S rRNA gene sequencing and by microbiological culture. *Arthritis Res Ther* 2007;**9**(3):R46.
8. Pagani I, Liolios K, Jansson J, Chen IM, Smirnova T, Nosrat B, et al. The Genomes OnLine Database (GOLD) v. 4: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Res* 2012;**40**(Database issue):D571–9.
9. Nasidze I, Li J, Quinque D, Tang K, Stoneking M. Global diversity in the human salivary microbiome. *Genome Res* 2009;**19**(4):636–43.
10. Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, et al. Viruses in the faecal microbiota of monozygotic twins and their mothers. *Nature* 2010;**466**(7304):334–8.
11. Pride DT, Salzman J, Haynes M, Rohwer F, Davis-Long C, White 3rd RA, et al. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. *ISME J* 2012;**6**(5):915–26.
12. Yang X, Xie L, Li Y, Wei C. More than 9,000,000 unique genes in human gut bacterial community: estimating gene numbers inside a human body. *PLoS One* 2009;**4**(6):e6074.
13. Xu Y, Xie J, Li Y, Yue J, Chen J, Chunyu L, et al. Using a cDNA microarray to study cellular gene expression altered by *Mycobacterium tuberculosis*. *Chin Med J* 2003;**116**(7):1070–3.
14. Goh KI, Cusick ME, Valle D, Childs B, Vidal M, Barabasi AL. The human disease network. *Proc Natl Acad Sci USA* 2007;**104**(21):8685–90.
15. Ramchandran L, Shah NP. Proteolytic profiles and angiotensin-I converting enzyme and alpha-glucosidase inhibitory activities of selected lactic acid bacteria. *J Food Sci* 2008;**73**(2):M75–81.
16. Hood L. Tackling the microbiome. *Science* 2012;**336**(6086):1209.
17. Clayton TA, Baker D, Lindon JC, Everett JR, Nicholson JK. Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proc Natl Acad Sci USA* 2009;**106**(34):14728–33.
18. Krishnadev O, Srinivasan N. Prediction of protein–protein interactions between human host and a pathogen and its application to three pathogenic bacteria. *Int J Biol Macromol* 2011;**48**(4):613–9.
19. Kusalik A, Bickis M, Lewis C, Li Y, Lucchese G, Marincola FM, et al. Widespread and ample peptide overlapping between HCV and *Homo sapiens* proteomes. *Peptides* 2007;**28**(6):1260–7.
20. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. *ISME J* 2010;**5**(1):82–91.

21. Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, et al. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One* 2010;**5**(2):e9085.
22. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol* 2012;**13**(9):R79.
23. Gao Z, Tseng CH, Strober BE, Pei Z, Blaser MJ. Substantial alterations of the cutaneous bacterial biota in psoriatic lesions. *PLoS One* 2008;**3**(7):e2719.
24. Amar J, Serino M, Lange C, Chabo C, Iacovoni J, Mondot S, et al. Involvement of tissue bacteria in the onset of diabetes in humans: evidence for a concept. *Diabetologia* 2011;**54**(12):3055–61.
25. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, et al. Genomic analysis identifies association of *Fusobacterium* with colorectal carcinoma. *Genome Res* 2012;**22**(2):292–8.
26. Schutzer SE, Angel TE, Liu T, Schepmoes AA, Clauss TR, Adkins JN, et al. Distinct cerebrospinal fluid proteomes differentiate post-treatment lyme disease from chronic fatigue syndrome. *PLoS One* 2011;**6**(2):e17287.
27. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, et al. Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. *Genome Res* 2012;**22**(11):2146–52.
28. Machado AM, Figueiredo C, Touati E, Maximo V, Sousa S, Michel V, et al. *Helicobacter pylori* infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells. *Clin Cancer Res* 2009;**15**(9):2995–3002.
29. Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, et al. Large-scale *in silico* and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol Endocrinol* 2005;**19**(11):2685–95.
30. Yenamandra SP, Lundin A, Arulampalam V, Yurchenko M, Pettersson S, Klein G, et al. Expression profile of nuclear receptors upon Epstein–Barr virus induced B cell transformation. *Exp Oncol* 2009;**31**(2):92–6.
31. Liu PT, Wheelwright M, Teles R, Komisopoulou E, Edfeldt K, Ferguson B, et al. MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy. *Nat Med* 2012;**18**(2):267–73.
32. Chan G, Bivins-Smith ER, Smith MS, Smith PM, Yurochko AD. Transcriptome analysis reveals human cytomegalovirus reprograms monocyte differentiation toward an M1 macrophage. *J Immunol* 2008;**181**(1):698–711.
33. Salazar JC, Duhnam-Ems S, La Vake C, Cruz AR, Moore MW, Caimano MJ, et al. Activation of human monocytes by live *Borrelia burgdorferi* generates TLR2-dependent and -independent responses which include induction of IFN- $\beta$ . *PLoS Pathog* 2009;**5**(5):e1000444.
34. Coughlan CA, Chotirmall SH, Renwick J, Hassan T, Low TB, Bergsson G, et al. The effect of *Aspergillus fumigatus* infection on vitamin D receptor expression in cystic fibrosis. *Am J Respir Crit Care Med* 2012;**186**(10):999–1007.
35. White RH. Biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid in the gliding bacterium *Cytophaga johnsonae*. *J Bacteriol* 1984;**159**(1):42–6.
36. Marshall TG. Bacterial capnine blocks transcription of human antimicrobial peptides, In: Third international conference on metagenomics, San Diego, CA, July 11–13; 2007.
37. Marshall TG. Vitamin D, discovery outpaces FDA decision making. *BioEssays* 2008;**30**(2):173–82.
38. Bell NH. Renal and nonrenal 25-hydroxyvitamin D-1 $\alpha$ -hydroxylases and their clinical significance. *J Bone Miner Res* 1998;**13**(3):350–3.
39. Blaney GP, Albert PJ, Proal AD. Vitamin D metabolites as clinical markers in autoimmune and chronic disease. *Ann NY Acad Sci* 2009;**1173**:384–90.

40. Proal AD, Albert PJ, Marshall TG. Dysregulation of the vitamin D nuclear receptor may contribute to the higher prevalence of some autoimmune diseases in women. *Ann NY Acad Sci* 2009;**1173**:252–9.
41. Marshall TG, Lee RE, Marshall FE. Common angiotensin receptor blockers may directly modulate the immune system via VDR, PPAR and CCR2b. *Theor Biol Med Model* 2006;**3**:1.
42. Brahmachary M, Schonbach C, Yang L, Huang E, Tan SL, Chowdhary R, et al. Computational promoter analysis of mouse, rat and human antimicrobial peptide-coding genes. *BMC Bioinformatics* 2006;**7**(Suppl. 5):S8.
43. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low diversity of the gut microbiota in infants with atopic eczema. *J Allergy Clin Immunol* 2012;**129**(2):434–40, 40.e1–2.
44. Oakley BB, Fiedler TL, Marrazzo JM, Fredricks DN. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Appl Environ Microbiol* 2008;**74**(15):4898–909.
45. Majed B, Arveiler D, Bingham A, Ferrieres J, Ruidavets J-B, Montaye M, et al. Depressive symptoms, a time-dependent risk factor for coronary heart disease and stroke in middle-aged men: the PRIME study. *Stroke* 2012;**43**(7):1761–7.
46. Kinross JM, Darzi AW, Nicholson JK. Gut microbiome–host interactions in health and disease. *Genome Med* 2011;**3**(3):14.
47. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. *Nature* 2011;**479**(7374):538–41.
48. Gonzalez A, Stombaugh J, Lozupone C, Turnbaugh PJ, Gordon JI, Knight R. The mind-body-microbial continuum. *Dialogues Clin Neurosci* 2011;**13**(1):55–62.
49. Anaya JM, Tobon GJ, Vega P, Castiblanco J. Autoimmune disease aggregation in families with primary Sjogren's syndrome. *J Rheumatol* 2006;**33**(11):2227–34.
50. Weyermann M, Rothenbacher D, Brenner H. Acquisition of *Helicobacter pylori* infection in early childhood: independent contributions of infected mothers, fathers, and siblings. *Am J Gastroenterol* 2009;**104**(1):182–9.
51. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008;**3**(8):e3056.
52. Grönlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr* 1999;**28**(1):19–25.
53. Cabrera-Rubio R, Collado MC, Laitinen K, Salminen S, Isolauri E, Mira A. The human milk microbiome changes over lactation and is shaped by maternal weight and mode of delivery. *Am J Clin Nutr* 2012;**96**(3):544–51.
54. Gottlieb JE, Israel HL, Steiner RM, Triolo J, Patrick H. Outcome in sarcoidosis. The relationship of relapse to corticosteroid therapy. *Chest* 1997;**111**(3):623–31.
55. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 2013;**110**(9):3507–12.
56. Dickie LJ, Church LD, Coulthard LR, Mathews RJ, Emery P, McDermott MF. Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes. *Rheumatology (Oxford)* 2010;**49**(8):1466–71.
57. Kimball S, Vieth R, Dosch HM, Bar-Or A, Cheung R, Gagne D, et al. Cholecalciferol plus calcium suppresses abnormal PBMC reactivity in patients with multiple sclerosis. *J Clin Endocrinol Metab* 2011;**96**(9):2826–34.

58. Arnsen Y, Amital H, Shoenfeld Y. Vitamin D and autoimmunity: new aetiological and therapeutic considerations. *Ann Rheum Dis* 2007;**66**(9):1137–42.
59. Durup D, Jørgensen HL, Christensen J, Schwarz P, Heegaard AM, Lind B. A reverse J-shaped association of all-cause mortality with serum 25-hydroxyvitamin D in general practice, the CopD study. *J Clin Endocrinol Metab* 2012;**97**(8):2644–52.
60. Ahn J, Peters U, Albanes D, Purdue MP, Abnet CC, Chatterjee N, et al. Serum vitamin D concentration and prostate cancer risk: a nested case-control study. *J Natl Cancer Inst* 2008;**100**(11):796–804.
61. Albert PJ, Proal AD, Marshall TG. Vitamin D: the alternative hypothesis. *Autoimmun Rev* 2009;**8**(8):639–44.
62. Payne ME, Anderson JJ, Steffens DC. Calcium and vitamin D intakes may be positively associated with brain lesions in depressed and nondepressed elders. *Nutr Res* 2008;**28**(5):285–92.
63. Bjelakovic G, Gluud LL, Nikolova D, Whitfield K, Wetterslev J, Simonetti RG, et al. Vitamin D supplementation for prevention of mortality in adults. *Cochrane Database Syst Rev* 2011;(7):CD007470.
64. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, et al. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA* 2010;**303**(18):1815–22.
65. Hypponen E, Sovio U, Wjst M, Patel S, Pekkanen J, Hartikainen AL, et al. Infant vitamin d supplementation and allergic conditions in adulthood: northern Finland birth cohort 1966. *Ann NY Acad Sci* 2004;**1037**:84–95.
66. Becklund BR, Severson KS, Vang SV, DeLuca HF. UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production. *Proc Natl Acad Sci USA* 2010;**107**(14):6418–23.
67. Diehl JW, Chiu MW. Effects of ambient sunlight and photoprotection on vitamin D status. *Dermatol Ther* 2010;**23**(1):48–60.
68. Chung M, Balk EM, Brendel M, Ip S, Lau J, Lee J, et al. Vitamin D and calcium: a systematic review of health outcomes. *Evid Rep Technol Assess (Full Rep)* 2009;(183):1–420.
69. Wood AD, Secombes KR, Thies F, Aucott L, Black AJ, Mavroei A, et al. Vitamin D3 supplementation has no effect on conventional cardiovascular risk factors: a parallel-group, double-blind, placebo-controlled RCT. *J Clin Endocrinol Metab* 2012;**97**(10):3557–68.
70. Witham MD, Price RJ, Struthers AD, Donnan PT, Messow CM, Ford I, et al. Cholecalciferol treatment to reduce blood pressure in older patients with isolated systolic hypertension: the VitDISH randomized controlled trial. *JAMA Intern Med* 2013;**173**(18):1672–9.
71. Wejse C, Gomes VF, Rabna P, Gustafson P, Aaby P, Lisse IM, et al. Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 2009;**179**(9):843–50.
72. Murdoch DR, Slow S, Chambers ST, Jennings LC, Stewart AW, Priest PC, et al. Effect of vitamin D3 supplementation on upper respiratory tract infections in healthy adults: the VIDARIS randomized controlled trial. *JAMA* 2012;**308**(13):1333–9.
73. McAlindon T, LaValley M, Schneider E, Nuite M, Lee JY, Price LL, et al. Effect of vitamin D supplementation on progression of knee pain and cartilage volume loss in patients with symptomatic osteoarthritis: a randomized controlled trial. *JAMA* 2013;**309**(2):155–62.
74. Datta M, Schwartz GG. Calcium and vitamin D supplementation and loss of bone mineral density in women undergoing breast cancer therapy. *Crit Rev Oncol Hematol* 2013;**88**(3):613–24.
75. Al-Elq AH. The status of Vitamin D in medical students in the preclerkship years of a Saudi medical school. *J Fam Commun Med* 2012;**19**(2):100–4.

76. Islam MZ, Akhtaruzzaman M, Lamberg-Allardt C. Hypovitaminosis D is common in both veiled and nonveiled Bangladeshi women. *Asia Pac J Clin Nutr* 2006;**15**(1):81–7.
77. Islam MZ, Shamim AA, Kemi V, Nevanlinna A, Akhtaruzzaman M, Laaksonen M, et al. Vitamin D deficiency and low bone status in adult female garment factory workers in Bangladesh. *Br J Nutr* 2008;**99**(6):1322–9.
78. Shivane VK, Sarathi V, Bandgar T, Menon P, Shah NS. High prevalence of hypovitaminosis D in young healthy adults from the western part of India. *Postgrad Med J* 2011;**87**(1030):514–18.
79. Specker BL, Ho ML, Oestreich A, Yin TA, Shui QM, Chen XC, et al. Prospective study of vitamin D supplementation and rickets in China. *J Pediatr* 1992;**120**(5):733–9.