UNIVERSITY OF IOANNINA
SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE
CHILD HEALTH SECTOR
DEPARTMENT OF PEDIATRICS

RELATIONSHIP OF VITAMIN D WITH CARDIOVASCULAR DISEASE RISK FACTORS IN ADULTS AND ADOLESCENTS AND POSSIBLE IMPACT ON ITS METABOLISM FROM THE USE OF LIPID-LOWERING MEDICATION

STEFANIA E. MAKARIOU, MD
PEDIATRICIAN

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Επιβλέπουσα
Χάλλα Άννα, Αναπληρώτρια Καθηγήτρια Παιδιατρικής του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Μέλη
Ελισάφ Μωυσής, Καθηγητής Παθολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Λυμπερόπουλος Ευάγγελος, Λέκτορας Παθολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

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Ελισάφ Μωυσής	Καθηγητής Παθολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Τσελέπης Αλέξανδρος	Καθηγητής Βιοχημείας-Κλινικής Χημείας του Τμήματος Χημείας του Πανεπιστημίου Ιωαννίνων
Χάλλα Άννα	Καθηγήτρια Παιδιατρικής με Έμφαση στην Εργαστηριακή Διερεύνηση Νοσημάτων Μεταβολισμού των Οστών και άλλων Νοσημάτων Παιδικής Ηλικίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Βλάχος Αντώνιος	Αναπληρωτής Καθηγητής Παιδοκαρδιολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Λυμπερόπουλος Ευάγγελος	Αναπληρωτής Καθηγητής Παθολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Σιώμου Αικατερίνη	Αναπληρώτρια Καθηγήτρια Παιδιατρικής με έμφαση στην Παιδιατρική Νεφρολογία του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Τίγκας Στυλιανός	Επίκουρος Καθηγητής Ενδοκρινολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

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ΜΑΡΙΑ ΚΑΠΙΤΟΠΟΥΛΟΥ

Σε όσους «περπάτησαν μαζί μου»….

Στέφανος & Θέκλα, Βασίλης & Αντιγόνη, Βαγγέλης & Μερόπη, Αλεξάνδρα, Νόρα,

Αλέξανδρος & Χριστόφορος

«….Και πήρες του καιρού τ' αλφαβητάρι

και της αγάπης λόγια φυλαχτό

για να βρει πάλι ρίζα το χορτάρι

και πήρες την ελπίδα και τη χάρη

ψηλά να πας να χτίσεις κιβωτό

με την ελπίδα μόνο και τη χάρη….»

Στίχοι Μάνου Ελευθερίου

ΠΡΟΛΟΓΟΣ

Το μακρύ ταξίδι της συγγραφής αυτής της διδακτορικής διατριβής ολοκληρώνεται. Με συνόδεψε καθ' όλη τη διάρκεια της μεταπτυχιακής μου πορείας, του αγροτικού και της ειδικότητας. Στάθηκε αφορμή απόκτησης πολλών νέων δεξιοτήτων: της επικοινωνίας με τον ασθενή και τους συναδέλφους, της διενέργειας εργαστηριακών μετρήσεων και στατιστικών αναλύσεων, της στοχευμένης αναζήτησης στη θάλασσα της βιβλιογραφίας και τελικά της συγγραφής και της παρουσίασης των αποτελεσμάτων μας. Κυρίως όμως με δίδαξε την αξία της επιμονής και της προσήλωσης στην «Ιθάκη» και στο «ωραίο ταξίδι» παρά τους «λαιστρυγώνες και τους κύκλωπες» που μπορεί να εμφανιστούν στην πορεία.

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ABBREVIATIONS

#	
1,25$(OH)_2$VitD	1,25dihydroxyvitamin D
7-DHC	7-dehydrocholesterol
8-epiPGF_2a	8-isoprostane
24-OHase	25-hydroxyvitamin D-24-hydroxylase
25(OH)VitD	25-hydroxyvitamin D
95% CI	95% Confidence Interval
A	
AAP	American Academy of Pediatrics
AHA	American Heart Association
ANCOVA	Analysis of covariance
Apo AI	Apolipoprotein AI
Apo ApoB	Apolipoprotein B
ARYL	Arylesterase activity
AUC	Area under the curve
B	
BCAMS study	Beijing Child and Adolescent Metabolic Syndrome Study
BMI	Body mass index
BP	Blood pressure
C	
CaDDM	Calcium and Vitamin D for Diabetes Mellitus
CDC	Centers for Disease Control
CHD	Coronary heart disease
CopD	Copenhagen vitamin D
CV	Cardiovascular

CVD	Cardiovascular disease
CYP2R1	25-hydroxylase
CYP27A1	25-hydroxylase
CYP27B1	1α,25-hydroxylase

D

DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DI	Disposition index

E

e-GFR	Estimated glomerular filtration rate
EPA	Eicosapentaenoic acid
ER-NA/LRPT	Nicotinic acid/laropiprant
ESPGHAN	European Society of Paediatric Gastroenterology, Hepatology and Nutrition
EVITA	Effect of vitamin D on all-cause mortality in heart failure

F

FGF23	Fibroblast growth factor-23
FM	Fat mass

G

GSH	Glutathione
GWASs	Genome-wide association studies

H

HbA1c	Glycated haemoglobin
HDL-C	High density lipoprotein cholesterol
HF	Heart failure
HMG-CoA	Hydroxyl-methyl coenzyme A
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
hsCRP	High sensitivity C reacting protein

I

IDL	Intermediate-density lipoprotein
(IGI	Insulinogenic index
IL-6	Interleukin -6
IM	Intra-muscularly
IOM	Institute of Medicine

K

KHANES	Korean National Health and Nutrition Examination Survey

L

LDL-C	Llow density lipoprotein cholesterol
LDL-PPD	Low density lipoprotein peak particle diameter
Lp-PLA_2	Lipoprotein-associated phospholipase A_2
LURIC	Ludwigshafen Risk and Cardiovascular Health
LVH	Left ventricular hypertrophy

M

Mas	Meta-analyses
MDA	Malondialdehyde
MDRD	Modification of Diet in Renal Disease
MI	Myocardial infarction
MMP-9	Metalloproteinase-9
MetS	Metabolic syndrome

N

NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP-ATP III	National Cholesterol Education Program-Adult Treatment Panel III
NF-κB	Nuclear factor κB
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
non-HDL-C	Non-high-density lipoprotein cholesterol

NPC1L1	Niemann-Pick C1 Like 1
Nrf2	Nuclear factor-erythroid-2-related factor 2
NS	Non-significant

O

ODIN	Food-based solutions for optimal vitamin D nutrition and health through the life cycle Project
OGTT	Oral glucose tolerance test
ox-LDL	Oxidised LDL

P

PON-1	Paraoxonase activity
PPARa	Peroxisome proliferator-activated receptor alpha
PTH	Parathyroid hormone

Q

QUICKI	Quantitative insulin sensitivity check index

R

RAAS	Renin-angiotensin-aldosterone axis
RAGE	Receptor for advanced glycation-end products
RCTs	Randomized control trials
RDA	Recommended dietary allowance
Rf	Electrophoretic mobility
RNI	Reference nutritional intake
ROS	Reactive oxygen species
RXR	X-retinoic acid receptor

S

SBP	Systolic blood pressure
sdLDL	Small dense LDL particles
Si	Insulin action
SNPs	Single nucleotide polymorphisms

T

T2DM	Type 2 diabetes mellitus
TAS	Total antioxidant status
TCHOL	Total cholesterol
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TOS	Total oxidant status

V

VDBP	Vitamin D binding protein
VDRE	Vitamin D-responsive elements
VDRs	Vitamin D receptors
VitD3	Cholecalciferol
VitD	Vitamin D
ViDA	Vitamin D Assessment
VINDICATE	Vitamin D Treating Patients with Chronic Heart Failure
VITAL	Vitamin D and Omega-3 Trial
VLDL	Very low density lipoprotein

W

WBISI	Whole body sensitivity index
WC	Waist circumference
WHI	Women's Health Initiative
WHO	World Health Organization

CHAPTER I.

BACKGROUND

1.1 Intoduction

During the last two decades, the scientific interest in vitamin D (VitD) has increased exponentially. However, the importance of VitD for bone health has already been known for almost 100 years. In the early 1920s, VitD administration was found to be the cure for rickets, a bone disease that affected infants and toddlers in many industrialized countries in Europe and North America (up to 80% in some cities) during the 19th and early 20th century [1, 2]. Nowadays, the importance and safety of VitD are well understood and VitD has an established role in the regulation of calcium and phosphorus metabolism and bone homeostasis not only in infants, who are protected from rickets with a daily dose of 400 IU VitD [3], but in all age groups. However, the scientific interest has moved from infancy to older ages, since low VitD status seems to be a re-emerging public health problem even in developed countries and approximately one billion people in the world are estimated to have either deficient or insufficient 25-hydroxyvitamin D (25(OH)VitD) levels which is the measured index for vitamin D status [4]. Hypovitaminosis D has been implicated not only in the pathogenesis of many chronic skeletal but also in non-skeletal diseases which originate in adolescence and are prevalent in adulthood [4]. These include cardiovascular diseases, obesity, metabolic syndrome and its characteristics (central obesity, hypertension, atherogenic dyslipidemia and disturbed carbohydrate metabolism), diabetes, malignancies, infections, neuropsychiatric and autoimmune diseases (Table 1) [5]. The above assumptions originally relied on the findings from a large number of epidemiological studies. Later laboratory proof came from the detection of vitamin D receptors (VDRs) in many tissues and cells which can bind the active metabolite 1,25dihydroxyvitamin D ($1,25(OH)_2$VitD) but also have the capacity to convert 25(OH)vitD to $1,25(OH)_2$D locally and act in an autocrine fashion. Indeed, VitD may control about 0.5–5.0% of the total human genome, i.e. 100–1250 genes [6]. Some of the diseases where VitD insufficiency/deficiency is implicated are shown on Table 1.

Cardiovascular disease (CVD) is a leading cause of mortality worldwide, estimated to account for 30% of all deaths [7]. Although CVD events occur most frequently during or after the fifth decade of life, precursors of CVD are shown to originate in childhood and adolescence [8]. Thus, identifying modifiable risk factors and treatments as soon as possible remains a high priority for CVD prevention. Several CVD risk factors have been

recognized so far, among which is VitD deficiency, as an emerging new one, since low serum 25(OH)VitD levels have been linked to poor cardiovascular outcomes [9, 10]. Compared to adults, however, very little is known about the possible association between VitD and CVD risk factors in youth.

Table 1. Non-skeletal diseases associated with hypovitaminosis D

All cause mortality
Metabolic Syndrome
Hypertension
Impaired glucose metabolism and type 2 diabetes
Dyslipidemia
Cardiovascular diseases (myocardial infarction, coronary insufficiency, coronary calcification, increased carotid intimae median thickness)
Heart failure
Peripheral arterial disease
Stroke
Renal disease
Autoimmune diseases (lupus erythematosus, multiple sclerosis, type 1 diabetes)
Cancer (bowel, breast, prostate, non-Hodgkin lymphoma)
Respiratory diseases (wheezing illness, autoimmune interstitial lung diseases)
Liver fibrosis
Psychiatric diseases (depression, autism, schizophrenia)
Loss of gait performance and falls in the elderly
Lower androgen levels

Subsequently, VitD is an attractive potential target for interventional research, as serum 25(OH)VitD levels are modifiable, ideally during childhood and adolescence, treatment is relatively inexpensive and can have an impact on a large population. However, previous studies with VitD supplementation trials have yielded conflicting results [9, 10] and even the results from large-scale randomized controlled trials with CVD as a primary outcome are still under dispute.

1.2 Sources of Vitamin D

VitD belongs to a group of fat-soluble molecules called secosteroids, which are similar to steroids, but with “broken” rings, and exist in several forms (Figure 1) [9]. Although 5 forms of VitD (D1 through D5) are known, the D2 and D3 are the most studied ones and from now on they will be referred to as VitD (Table 2) [11]. Humans acquire VitD after exposure to sunlight (UVB radiation), from their diet, and from dietary supplements (Table 3) [5]. VitD2 (Ergocalciferol) is principally synthesized in plants and invertebrates and is consumed nutritionally as supplements or fortified foods. VitD3 (Cholecalciferol) is mainly produced by vertebrates and commonly consumed in the form of oily fish. It is also synthesized in human skin by the photochemical cleavage of cutaneous 7-dehydrocholesterol by the solar ultraviolet radiation and it is the major source in man amounting to 80%-90% under normal conditions. For that reason its designation as a vitamin is a misnomer [12]. Total-body sun exposure to 1 minimal erythemal dose while wearing a bathing suit provides the equivalent of 250 to 500 μg (10,000 to 20,000 IU) of VitD per day [13]. Oily fish such as salmon, mackerel, herring and sardines, eggs and dairy products are rich sources of VitD, but the dietary supply of VitD is minor compared to cutaneous formation [14]. However nutritional supplementation (food fortification or use of supplements) can become an important source of VitD when required. Although the promotion of a healthier lifestyle with more outdoor activities and rational sunlight exposure combined with optimal nutrition is definitely preferred for maintenance, this is not enough to treat hypovitaminosis D. There are limitations in the sunlight exposure due to the potential adverse effects like skin cancer leading to inadequate skin vit D synthesis. Also intake of VitD supplements may be limited by poor adherence (particularly in individuals of low-socioeconomic status) or by the fear of potential overdosing. On the other hand, systematic VitD food fortification may be an effective intervention to improve VitD status in the general population. This has already been introduced by countries such as the US, Canada and Finland. The main VitD fortified foods currently practiced in these countries are listed in Table 4 [15].

Overall, both endogenous and consumed VitD is stored in the fat tissue and is released into the circulation bound to a vitamin D-binding protein [5]. The major

circulating metabolite of VitD is serum 25-hydroxyvitamin D (25(OH)VitD), reflecting the input from cutaneous synthesis and dietary intake. The serum 1,25-dihydroxyvitamin D ($1,25(OH)_2$VitD) which is the active metabolite is synthesised according to the needs from the second hydroxylation in the kidneys independent on the circulating 25(OH)VitD [5]. The concentration of 25(OH)VitD is about 1000 times greater than that of $1,25(OH)_2$VitD, and 25(OH)VitD has a longer half-life of approximately 2-3 weeks, while that of $1,25(OH)_2$VitD is less than four hours due to their different metabolic pathway rate control [16]. Therefore, it is important to emphasize that the most reliable method for estimating VitD status in humans is to measure serum 25(OH)VitD, rather than $1,25(OH)_2$VitD, since there is a cut off point above which the extrarenal cells can incorporate 25(OH)VitD and convert it to 1,25(OH)VitD for their autocrine actions. Additionally in the case of VitD deficiency, PTH secretion increases, and renal conversion to $1,25(OH)_2$VitD is activated [16].

Figure 1. Vitamin D's group of fat-soluble molecules called secosteroids [9]

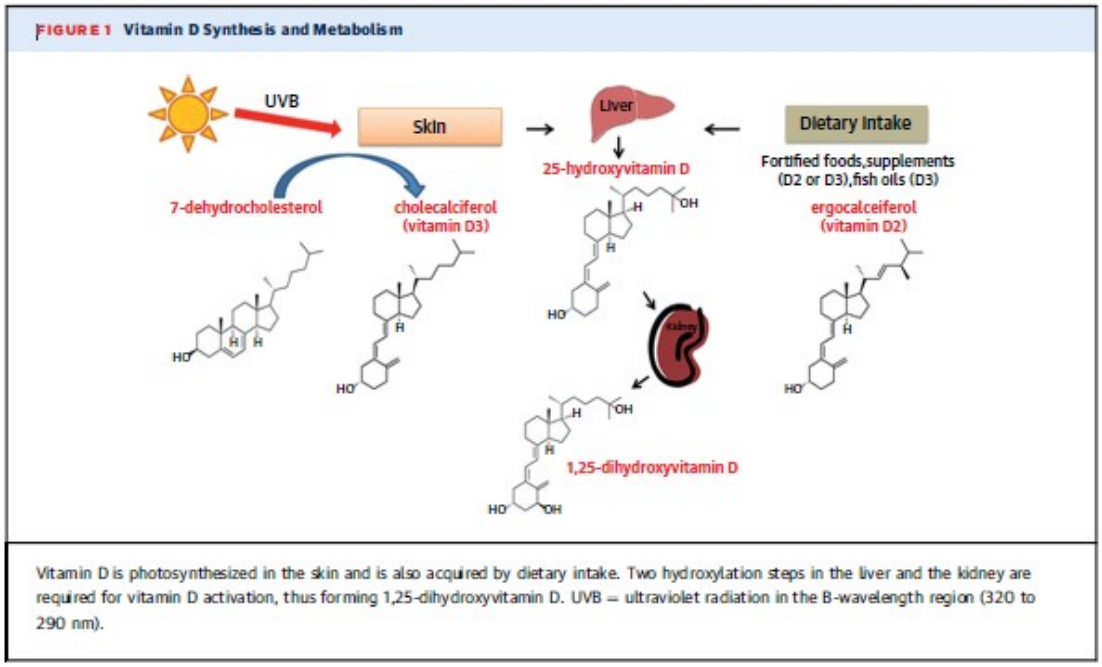


Table 2. Classification of VitD group of molecules [11]

Class	Chemical Composition	Significance
Vitamin D_1	Combination of ergocalciferol and lumisterol	
Vitamin D_2	Ergocalciferol: made from ergosterol or pre–vitamin D_2	Made by invertebrates, fungus, and plants in response to ultraviolet irradiation; not made by vertebrates
Vitamin D_3	Cholecalciferol: made from 7-dehydrocholesterol or pre–vitamin D_3	Made in the skin as a response to ultraviolet B radiation after reacting with 7-dehydrocholesterol
Vitamin D_4	Dihydroergocalciferol: vitamin D_2 without 22,23 double bond	Ineffective form of vitamin D
Vitamin D_5	Sitocalciferol: made from 7-dehydrositosterol	May have antitumor properties

Table 3. Dietary Sources of Vitamins D2 and D3 [5].

Source	Vitamin D Content
Natural sources	
Salmon	
Fresh, wild (3.5 oz)	About 600–1000 IU of vitamin D_3
Fresh, farmed (3.5 oz)	About 100–250 IU of vitamin D_3 or D_2
Canned (3.5 oz)	About 300–600 IU of vitamin D_3
Sardines, canned (3.5 oz)	About 300 IU of vitamin D_3
Mackerel, canned (3.5 oz)	About 250 IU of vitamin D_3
Tuna, canned (3.6 oz)	About 230 IU of vitamin D_3
Cod liver oil (1 tsp)	About 400–1000 IU of vitamin D_3
Shiitake mushrooms	
Fresh (3.5 oz)	About 100 IU of vitamin D_2
Sun-dried (3.5 oz)	About 1600 IU of vitamin D_2
Egg yolk	About 20 IU of vitamin D_3 or D_2
Exposure to sunlight, ultraviolet B radiation (0.5 minimal erythemal dose)†	About 3000 IU of vitamin D_3

Table 4. Vitamin D food fortification in the United States, Canada and Finland [15]

Food (serving)	United States	Canada	Finland
VITAMIN D PER SERVING IN μg (1 μg = 40 INTERNATIONAL UNITS)			
	Mass fortification (usually mandatory)		
Fluid cow's milk (250 ml or 1 cup)	2.5–5.0†	2.5–5.0††	2.5
Margarine/Fat spread (10 g)		1.5–3.0††	2.0
	Fortification of selected brands		
Yogurt	1.5–5.0 per 170 g	1.0 per 100 g	0.5–1.0 per 100 g
Cheese slice (16 g)	1.5		
Orange juice (125 ml or 1/2 cup)	1.25	1.25	1.25
Plant-based milk such as soy, oat or almond (250 ml or 1 cup)	1.5–3.0	1.5–3.0	1.9–3.75
Margarine 10 g	0.75–5.0		
Bread (100 g)	2.25		1.7
Cereals, ready-to-eat (1/2–3/4 cup)	1–2.5	1.0	3.0 per 100 g

†*FDA in 2016 permitted voluntary "doubling" of mandatory vitamin D in milk.*
††*Health Canada will require doubling of mandatory amounts by 2020.*

1.3 Synthesis and Metabolism of Vitamin D

Sunlight exposure and UVB radiation lead to the conversion of 7-dehydrocholesterol (7-DHC) in the skin to previtamin D3. The 7-DHC is present in all skin layers but 65% is found in the epidermis. Once previtamin D3 is synthesized in the skin, it can undergo either a photoconversion to lumisterol, tachysterol, and 7-DHC or a heat-induced membrane-enhanced isomerization to vitamin D3 [5, 16] (Figures 2 [5] and 3 [17]). The cutaneous production of previtamin D3 is regulated so as to avoid sun-induced VitD intoxication. At times of prolonged exposure to UVB radiation the solar photoproducts tachysterol and lumisterol are produced, which are inactive on calcium metabolism. VitD3 itself is also sensitive to solar irradiation and is, thereby, inactivated to suprasterol 1 and 2 and to 5,6-trans-vitamin D3 [5]. Once formed, VitD3 comes out of the keratinocyte plasma membrane and is led into dermal capillaries bound to the vitamin D binding protein (VDBP). Ingested VitD is incorporated into chylomicrons, released into the lymphatic system and then into venous circulation, where it binds to VDBP and lipoproteins that transport it to the liver [5, 18]. VitD has no biological activity without a two-step hydroxylation process. The first step, in the liver, requires P450 enzymes such as CYP2R1 and CYP27A1 (25-hydroxylases) to form the major circulating form 25(OH)VitD. This form is biologically inactive and requires the second hydroxylation step, in the kidneys, to

be converted to the biologically active form 1,25-dihydroxyvitamin D ($1,25(OH)_2$VitD) by the P450 enzyme, CYP27B1 (1α,25-hydroxylase). The 25(OH)VitD bound to VDBP is filtered in the kidneys and is reabsorbed in the proximal renal tubules by megalin cubilin receptors [19] (Figures 2 and 3). The renal 1α-hydroxylation is closely regulated: parathyroid hormone (PTH), hypocalcemia, and hypophosphatemia enhance it, whereas hyperphosphatemia, fibroblast growth factor-23 (FGF23), and $1,25(OH)_2$VitD itself inhibit it [5, 20]. In particular, $1,25(OH)_2$VitD decreases its own synthesis through negative feedback: it decreases the synthesis and secretion of PTH by the parathyroid glands and it also increases the expression of 25-hydroxyvitamin D-24-hydroxylase (24-OHase) to catabolize $1,25(OH)_2$VitD to the water-soluble, biologically inactive calcitroic acid, which is excreted in the bile [5] (Figures 2 and 3).

The biological functions of VitD are exerted through the interaction of the steroid hormone $1,25(OH)_2$VitD with a single vitamin D receptor (VDR) in the cell nucleus, that functions as a ligand-dependent transcription factor [21]. This receptor as well as CYP27B1 are expressed widely in several tissues [2] (Figure 4). The mechanism involves the creation of a heterodimeric complex with an X-retinoic acid receptor ($1,25(OH)_2$VitD/VDR/RXR). Once this cluster is connected to specific nucleotide sequences in the DNA known as vitamin D-responsive elements (VDRE), a variety of transcription factors can attach to this complex, and according to the needs result in either up- or down-regulation of the relevant gene's activity (Figure 4) [6, 22]. It is estimated that 200 to 2000 genes have VDREs or are influenced indirectly, possibly by epigenetics, and control a multitude of genes across the genome [21, 22]. In addition, $1,25(OH)_2$VitD can also act through intracellular signal transduction pathways, activated after binding to the cytoplasmic membrane of the cell (Figure 4). VDRs have been detected in most of the body's cells, including those of smooth muscle fibers, α-pancreatic cells, small and large intestine, brain, skin, prostate gland, gonads, breast, lymphocytes and macrophages [18, 22]. Many of these organs and cells, not only have a VDR but are also capable of producing $1,25(OH)_2$VitD, through the local action of CYP27B1 and which then acts locally in an autocrine manner. This production depends on the availability of circulating 25(OH)VitD, indicating the biological importance of sufficient blood levels of this VitD metabolite [23, 24]. The estimated 2000 genes that are directly or indirectly regulated by $1,25(OH)_2$VitD have a wide range of proven biological actions, among which inhibiting

cellular proliferation and inducing terminal differentiation or apoptosis, inhibiting angiogenesis, stimulating insulin production, inhibiting renin production and stimulating macrophage cathelicidin production (Figure 4) [18, 21, 22, 25]. This observation has led to the hypothesis that VitD must be involved in the pathogenesis of various chronic diseases such as various types of cancers, diabetes mellitus (type 1 and 2), CVD and various autoimmune and infectious diseases [6].

Figure 2. Synthesis and Metabolism of Vitamin D and Role in the Regulation of Calcium, Phosphorus, and Bone Metabolism [5]

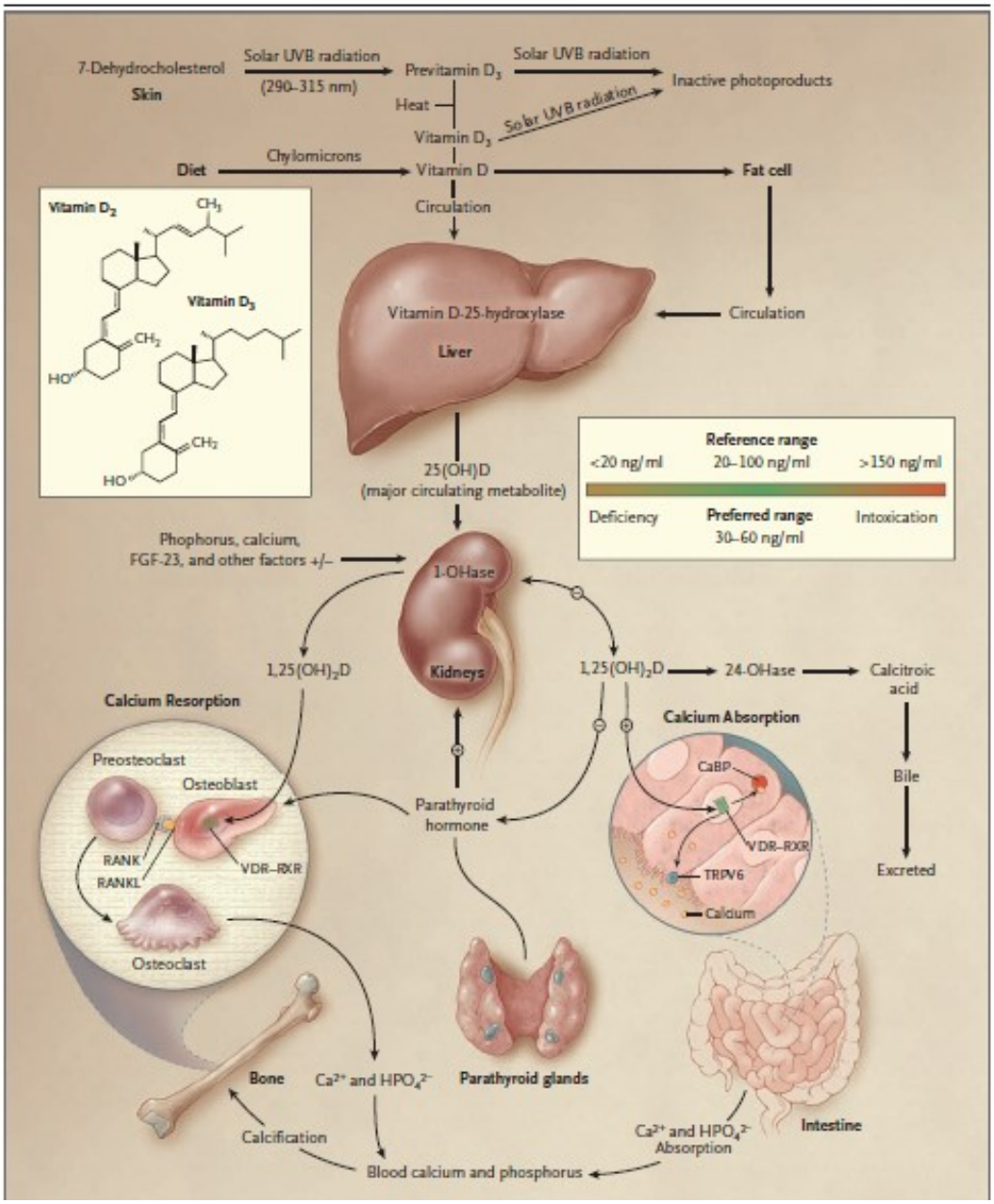
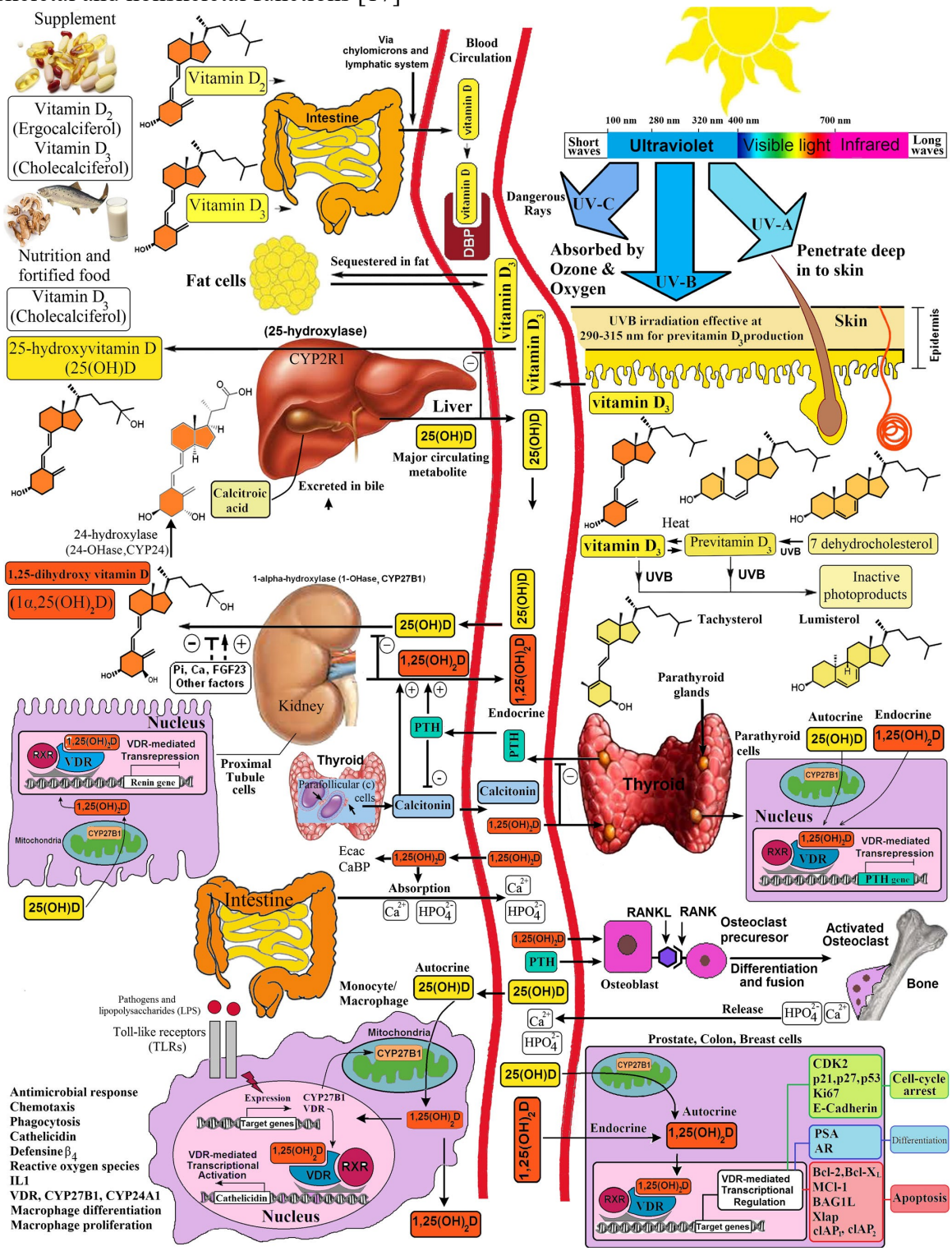
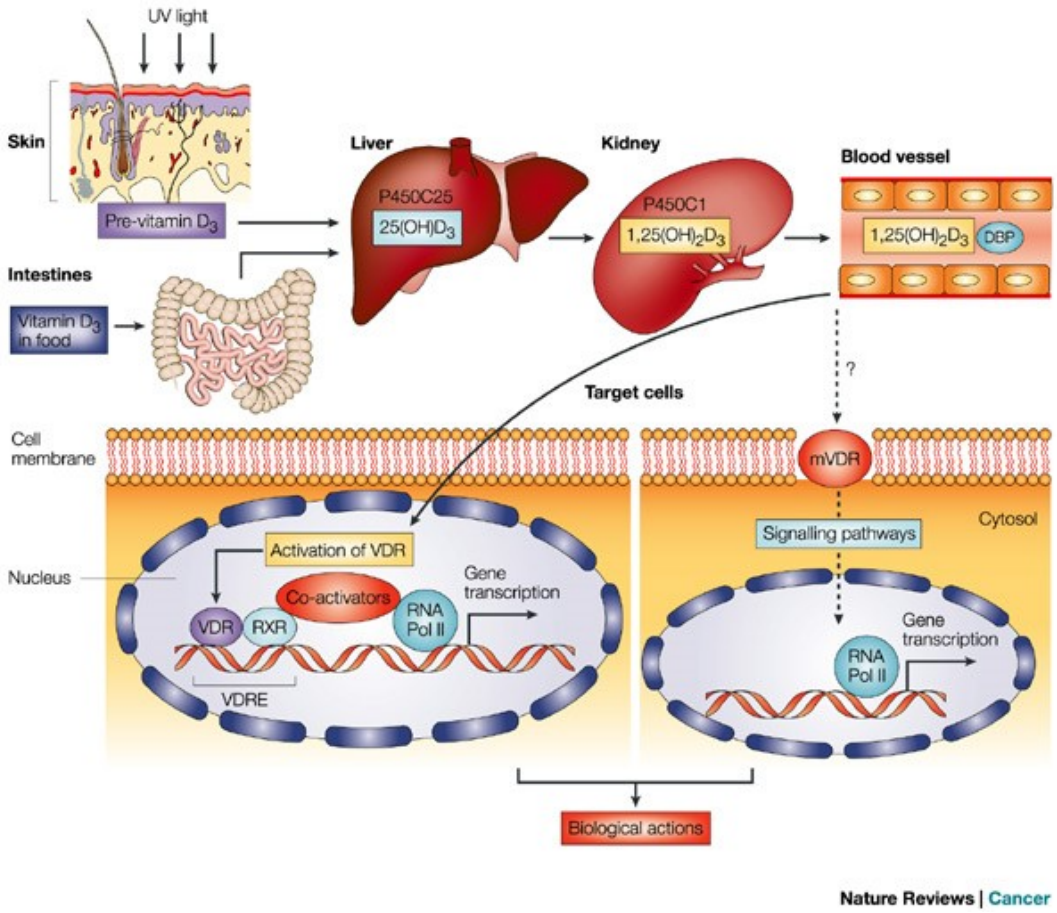


Figure 3. Schematic representation of the synthesis and metabolism of vitamin D for skeletal and nonskeletal functions [17]



1-OHase: 25-hydroxyvitamin D-1a-hydroxylase, 24-OHase: 25-hydroxyvitamin D-24-hydroxylase, 25(OH)D: 25-hydroxyvitamin D, 1,25(OH)2D:1,25-dihydroxyvitamin D, CaBP: calcium-binding protein, CYP27B1: cytochrome P450-27B1, DBP: vitamin D-binding protein, ECaC: epithelial calcium channel, FGF-23: fibroblast growth factor 23, PTH: parathyroid hormone, RANK: receptor activator of the NF-kB, RANKL: receptor activator of the NF-kB ligand, RXR: retinoic acid receptor, TLR2/1: Toll-like receptor 2/1, VDR: vitamin D receptor, vitamin D: vitamin D2 or vitamin D3

Figure 4. Interaction of $1,25(OH)_2$VitD with the vitamin D receptor (VDR) in cell nucleus



1.4 Definition of Vitamin D status

The "gold-standard" method to determine VitD status is measurement of serum 25(OH)VitD concentration through tandem mass spectrometry [9], but other methods like chemiluminescent microparticle immunoassay or ELISA are more widely used. Although $1,25(OH)_2$VitD is the biologically active form, it is not used for VitD status estimation because it is often normal or even elevated in children and adults who are VitD deficient and has very short half-life (hours) compared to 25(OH)D that has 2-3 weeks [11].

In 2011 the Institute of Medicine (IOM) [26] and the Endocrine Society [27] released separate guidelines for VitD requirements and categories of VitD status (Figure 5) [9], for reasons discussed below.

Figure 5. Categories of Vitamin D Status and VitD requirements according to the Institute of Medicine Versus the Endocrine Society [9]

<table>
<tr><td></td><td colspan="2">Vitamin D Status</td><td></td><td></td><td></td></tr>
<tr><td>Institute of Medicine</td><td colspan="2">25-OH D (ng/ml)</td><td>Endocrine Society</td><td>Treatment*</td><td>Maintenance†</td></tr>
<tr><td>Deficient</td><td><12</td><td rowspan="2"><20</td><td rowspan="2">Deficient</td><td rowspan="2">50,000 IU/week for 6–8 weeks</td><td rowspan="2">600–2,000 IU/day</td></tr>
<tr><td>Insufficient</td><td>12–19</td></tr>
<tr><td rowspan="2">Sufficient</td><td>20–29</td><td>20–29</td><td>Insufficient</td><td colspan="2" rowspan="3">≥400 IU/day</td></tr>
<tr><td>30–49</td><td rowspan="2">≥30</td><td rowspan="2">Sufficient</td></tr>
<tr><td>High</td><td>>50</td></tr>
</table>

Generally, in various cross-sectional studies 25(OH)VitD levels ranging from 12 to 40 ng/mL is the threshold below which PTH increases. VitD supplementation suppresses PTH only when 25(OH)VitD levels are <20 ng/mL, and $1,25(OH)_2$VitD levels are almost always normalized with serum 25(OH)VitD levels >20 ng/mL [9]. Also fracture risk starts to increase in the elderly at 25(OH)VitD levels <20 ng/mL, but intestinal calcium absorption is found to be optimal at serum 25(OH)VitD levels near 30 ng/mL [9]. VitD intoxication is observed with serum 25(OH)VitD levels >150 ng/mL [5]. Based on these findings, almost all experts recognize the importance of diagnosing and treating severe VitD deficiency (25(OH)VitD <12 ng/mL), and most would consider treating subjects with 25(OH)VitD levels <20 ng/mL.

However, there is no consensus on whether 25(OH)VitD levels of 30 ng/mL or higher compared with 20 ng/mL provide additional health benefits in the general population. Notably, the IOM report [26] focuses only on bone health (calcium absorption, bone mineral density, and osteomalacia/rickets) and found no evidence that a serum 25(OH)VitD concentration >20 ng/mL had any beneficial effects at a population level. On the other hand, the Endocrine Society [27], after having taken into consideration the new available evidence on skeletal and extraskeletal properties of VitD and the lack of toxicity after VitD supplementation at recommended doses, concluded that serum 25(OH)VitD

levels of 30 ng/mL should be attained to avoid other risks related to an inadequate VitD status. Therefore, the Endocrine Society recommended that VitD deficiency be defined as a 25(OH)VitD level <20 ng/mL, VitD insufficiency as 21 to 29 ng/mL, and vitamin D sufficiency as >30 ng/mL for adults and children [27]. It suggested that maintenance of a 25(OH)VitD level of 40 to 60 ng/mL is ideal and that up to 100 ng/mL is safe [27].

Interestingly, there is accumulating evidence that circulating 25(OH)VitD levels <16-24 ng/mL are nonlinearly related to an increased risk of musculoskeletal diseases and probably also to an increased CVD risk (Table 5) [28].

Nevertheless, both IOM and Endocrine Society agree that VitD is essential for skeletal health and that there is no unequivocal evidence of an association between low VitD levels and CVD or overall mortality. Also, both of them do not recommend screening the general population routinely for VitD deficiency.

In **children and adolescents** normal and abnormal serum concentrations of 25(OH)VitD have been classified by various scientific societies and institutions, also without consensus. The American Academy of Pediatrics (AAP) concluded that serum 25(OH)VitD concentrations represent respectively: severe deficiency for values <5 ng/mL, deficiency 5-15 ng/mL, insufficiency 16-20 ng/mL, sufficiency 21-100 ng/mL, excess 101-150 ng/mL and intoxication >150 ng/mL [29, 30]. Similarly, the Institute of Medicine (IOM) Committee (2011) stated that serum 25(OH)VitD levels of 20 ng/mL meet the requirements in almost 100% of the population, but highlighted that 25(OH)VitD levels >50 ng/mL should raise concerns about potential adverse effects as for some outcomes U-shaped associations were observed, with risks at both low and high levels [26]. At present, the AAP, IOM and European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [31] suggest 20 ng/mL as the cut-off for VitD deficiency. On the contrary, the British Paediatric and Adolescent Bone Group argues that the definition of VitD status should be based only on skeletal effects and propose 10 ng/mL as cut-off for deficiency and 10–20 ng/mL as cut-off for VitD insufficiency [32]. In the same line, the "Global Consensus Recommendations on Prevention and Management of Nutritional Rickets" has more recently (2016) classified VitD status as: sufficiency for serum 25(OH)VitD >20 ng/mL, insufficiency 12–20 ng/mL, deficiency <12 ng/mL, while toxicity has been defined as serum 25(OH)VitD >100 ng/mL along with hypercalcemia, hypercalciuria and suppressed PTH [3]. On the other hand, the Endocrine Society [27] and

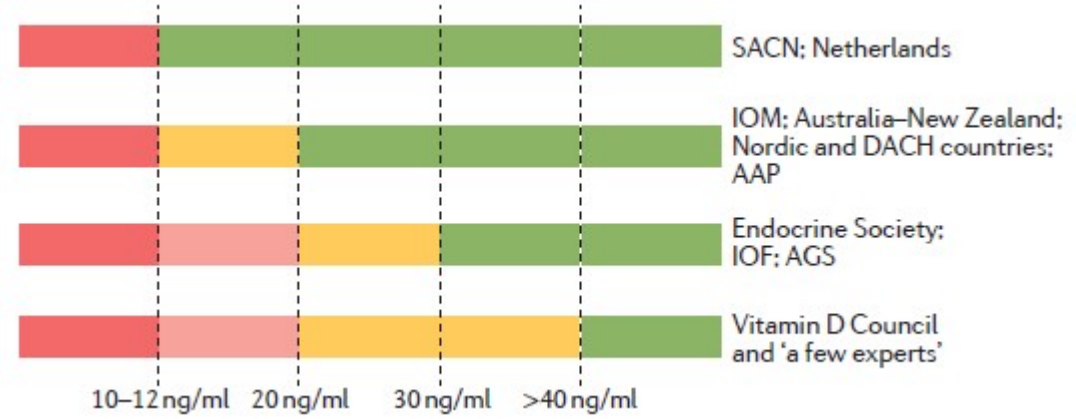
the Society for Adolescent Health and Medicine [33] consider 25(OH)VitD levels 20–29.9 ng/mL as VitD insufficiency, and establish the cut-off for VitD sufficiency at ≥30 ng/mL. Figure 6 shows a schematic representation of how different agencies and countries interpret serum levels of 25(OH)VitD.

Table 5. Suggested dose-response relationship of circulating 25(OH)VitD with musculoskeletal and cardiovascular disease [28].

25(OH)VitD concentration	**Musculoskeletal system**	**Cardiovascular system**
<5 ng/mL	Rickets ↑↑, osteomalacia ↑↑ Elderly people: falls ↑↑, fractures ↑↑	CVD events ↑ (?)
5 – 10 ng/mL	Rickets ↑↑, osteomalacia ↑↑ Elderly people: falls ↑↑, fractures ↑↑	CVD events ↑ (?)
10 – 20 ng/mL	Elderly people: falls ↑, fractures ↑	CVD surrogate parameters probably adversely affected
20 – 40 ng/mL	Adequate muscle and bone function	Adequate cardiovascular function
>40 ng/mL	Elderly people: falls ↑, fractures ↑	CVD events ↑ (?)

CVD: cardiovascular disease events; (?): probably; ↑: elevated; ↑↑: markedly elevated.

Figure 6. Recommendations for interpreting serum levels of 25(OH)VitD [34]



Red: severe deficiency (danger), has to be corrected without exception
Yellow: mild deficiency (modest concern), intervention is desirable
Green: sufficient supply, does not benefit from additional supplementation
AAP: American Academy of Pediatrics, AGS: American Geriatrics Society, DACH: Deutschland (Germany), Austria and Confoederatio Helvetica (Switzerland), IOF: International Osteoporosis Foundation, IOM: Institute of Medicine, SACN: Scientific Advisory Committee on Nutrition.

1.5 Risk Factors for Vitamin D deficiency/insufficiency

There are many risk factors for VitD deficiency, including reduced skin synthesis and absorption of VitD, decreased bioavailability, increased catabolism and acquired or heritable disorders of VitD metabolism and responsiveness, as summarised in Table 6 [5] .

Table 6. Causes and effects of VitD deficiency [5]

Cause	Effect
Reduced skin synthesis	
Sunscreen use: absorption of UVB radiation by sunscreen	Reduces vitamin D3 synthesis — SPF 8 by 92.5%, SPF 15 by 99%
Skin pigment: absorption of UVB radiation by melanin	Reduces vitamin D3 synthesis by as much as 99%
Aging: reduction of 7-dehydrocholesterol in the skin	Reduces vitamin D3 synthesis by about 75% in a 70-year-old
Season, latitude, and time of day: number of solar UVB photons reaching the earth depending on zenith angle of the sun (the more oblique the angle, the fewer UVB photons reach the earth)	Above about 35 degrees north latitude, little or no vitamin D3 can be produced from November to February
Patients with skin grafts for burns: marked reduction of 7-dehydrocholesterol in the skin	Decreases the amount of vitamin D3 the skin can produce
Decreased bioavailability	
Malabsorption: reduction in fat absorption, resulting from cystic fibrosis, celiac disease, Whipple's disease, Crohn's disease, bypass surgery, medications that reduce cholesterol absorption, and other causes	Impairs the body's ability to absorb VitD
Obesity: sequestration of VitD in body fat†	Reduces availability of vitamin D
Increased catabolism	
Anticonvulsants, glucocorticoids, HAART (AIDS treatment), and antirejection medications: binding to the steroid and xenobiotic receptor or the pregnane X receptor	Activates the destruction of 25(OH)VitD and $1,25(OH)_2$VitD to inactive calcitroic acid
Breast-feeding	
Poor VitD content in human milk	Increases infant risk of VitD deficiency when breast milk is the sole source of nutrition
Decreased synthesis of 25(OH)VitD	
Liver failure	
Mild-to-moderate dysfunction	Causes malabsorption of VitD, but production of 25(OH)VitD is possible
Dysfunction of 90% or more	Results in inability to make sufficient 25(OH)VitD

Increased urinary loss of 25(OH)VitD	
Nephrotic syndrome: loss of 25(OH)VitD bound to vitamin D–binding protein in urine	Results in substantial loss of 25(OH)VitD to urine
Decreased synthesis of $1,25(OH)_2$VitD	
Chronic kidney disease	
Stages 2 and 3 (estimated glomerular filtration rate, 31 to 89 ml/min/1.73 m^2)	
Hyperphosphatemia increases fibroblast growth factor 23, which decreases 25-hydroxyvitamin D-1á-hydroxylase activity	Causes decreased fractional excretion of phosphorus and decreased serum levels of $1,25(OH)_2$VitD
Stages 4 and 5 (estimated glomerular filtration rate <30 ml/min/1.73 m^2)	
Inability to produce adequate amounts of $1,25(OH)_2$VitD	Causes hypocalcemia, secondary hyperparathyroidism, and renal bone disease
Heritable disorders — rickets	
Pseudovitamin D deficiency rickets (vitamin D–dependent rickets type 1): mutation of the renal 25-hydroxyvitamin D-1á-hydroxylase gene (*CYP27B1)*	Causes reduced or no renal synthesis of $1,25(OH)_2$VitD
Vitamin D–resistant rickets (vitamin D–dependent rickets type 2): mutation of the vitamin D receptor gene 1	Causes partial or complete resistance to $1,25(OH)_2$VitD action, resulting in elevated levels of $1,25(OH)_2$VitD
Vitamin D–dependent rickets type 3: overproduction of hormoneresponsive-element binding proteins	Prevents the action of $1,25(OH)_2$VitD in transcription, causing target-cell resistance and elevated levels of $1,25(OH)_2$VitD
Autosomal dominant hypophosphatemic rickets: mutation of the gene for fibroblast growth factor 23, preventing or reducing its breakdown	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25(OH)VitD-1α-hydroxylase activity, resulting in low-normal or low levels of $1,25(OH)_2$VitD
X-linked hypophosphatemic rickets: mutation of the *PHEX* gene, leading to elevated levels of fibroblast growth factor 23 and other phosphatonins	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25(OH)VitD-1α-hydroxylase activity, resulting in low-normal or low levels of $1,25(OH)_2$VitD
Acquired disorders	
Tumor-induced osteomalacia: tumor secretion of fibroblast growth factor 23 and possibly other phosphatonins	Causes phosphaturia, decreased intestinal absorption of phosphorus, hypophosphatemia, and decreased renal 25(OH)VitD-1α-hydroxylase activity, resulting in low-normal or low levels of $1,25(OH)_2$VitD
Primary hyperparathyroidism: increase in levels of parathyroid hormone, causing increased conversion of 25(OH)VitD to $1,25(OH)_2$VitD	Decreases 25(OH)VitD levels and increases $1,25(OH)_2$VitD levels that are high-normal or elevated
Granulomatous disorders, sarcoidosis, tuberculosis, and other conditions, including some lymphomas: conversion by macrophages of 25(OH)VitD to $1,25(OH)_2$VitD	Decreases 25(OH)VitD levels and increases $1,25(OH)_2$VitD levels
Hyperthyroidism: enhanced metabolism of 25(OH)VitD	Reduces levels of 25(OH)VitD

Traditional risk groups for VitD deficiency include the elderly, institutionalized populations, obese persons, race/ethnic groups with darker skin colouring living in the Northern hemisphere. Also children and adolescents as well as pregnant and lactating women and their infants, especially when there is multiparity, short spacing between pregnancies, non-white maternal skin and exclusive breast feeding are also potentially at risk for VitD deficiency. Finally, patients with chronic diseases and those receiving specific therapies (corticosteroids, several antiepileptic, antifungal, and antiviral medications) are high risk populations for hypovitaminosis D [5, 17].

1.6 Vitamin D sufficient production/intake

The major source of VitD for adults and children is sunlight exposure. It is estimated that in a fair skinned healthy adult, 20 to 30 minutes of sunlight exposure on the face and forearms at midday can generate the equivalent of around 2000 IU of VitD [35], while exposure in a bathing suit to an amount of sunlight that causes a minimal erythema (light pinkness to the skin 24 h after exposure; 1 MED) is equivalent to receiving about 20,000 IU of VitD [5]. Notably, cutaneous VitD synthesis is influenced by various factors such as skin pigmentation, sunscreen use, latitude, altitude, season, time of day, air pollution and age. Coloured people who have natural sunscreen protection from their increased melanin pigment, that absorbs the vitaminD_3-producing UVB radiation, are more than 90% less efficient in producing VitD in their skin and need a twofold to 10-fold increased sunlight exposure time or frequency to get the same level of VitD synthesis as fair skinned individuals [36]. Moreover, a sunscreen with a sun protection factor of 30 applied properly reduces the ability of the skin to produce VitD by as much as 95% to 99% [17]. Also the zenith angle of the sun which occurs between approximately 10 AM and 3 PM has the highest impact on the cutaneous production of VitD. A more oblique angle, as it happens during winter and early morning and late afternoon, results in the absorption of solar UVB radiation by the ozone layer. This is why above the approximately 33° latitude minimum VitD quantities are produced in the skin during winter [37, 38]. Therefore, hypovitaminosis D is more usual in northern latitudes and its incidence is higher in late winter/spring and considerably less in summer [5]. In addition, air pollution with increased

ozone and nitrogen dioxide levels also absorb VitD-producing UVB photons but it is often overlooked as a VitD deficiency risk factor for people living in urban areas [39]. In addition, behaviours and dress codes that limit sun exposure for religious or cultural reasons, like strict adherence to the use of a veil, headscarf, or other sun concealing type of clothing, place those individuals to high VitD deficiency risk [9]. Similarly, exposure to sunlight through glass markedly decreases VitD skin synthesis, because glass absorbs all UVB radiation [40].

The elderly are also at high risk for low 25(OH)VitD levels and consequently for clinical complications associated with it. With increasing age, cutaneous VitD production decreases by more than 2-fold, because of atrophic skin changes that lead to a reduced amount of its precursor 7-dehydrocholesterol (7-DHC) [41, 42]. In addition major lifestyle changes co-exist, such as limited solar exposure due to less outdoor activities, changes in clothing and probably less efficient dietary menu habits with lower VitD content.

Another vulnerable population for hypovitaminosis D is the overweight and obese people. This is mainly attributed to their sedentary lifestyle, with much more time spent in front of TV screens and computer games, instead of having outdoor physical activities and exposure to sunlight. Other possible reported mechanisms include the sequestration of the fat-soluble VitD in adipose tissue, a lower dietary VitD intake, its reduced intestinal absorption, or even an altered vitamin D metabolism [43].

Diseases obstructing one of the steps of VitD metabolite activation may cause a deficiency (i.e., severe liver or renal failure) [27]. Also VitD deficiency due to poor intestinal absorption is seen in patients suffering from untreated celiac disease, cystic fibrosis, inflammatory bowel disease, short bowel syndrome, patients with percutaneous gastrostomy, or bariatric patients. Moreover, skin diseases and extended hospitalization lead to long times spent indoors and inadequate sun exposure [40]. Finally, several drugs are known to interfere with VitD metabolism and lead to decreased 25(OH)VitD levels. Indeed, people on anticonvulsant medications, systemic glucocorticoids, antifungals and medications for AIDS should receive VitD supplementation at least two to three times more than their respective age group's recommendations [27].

Some paediatric populations are also at increased risk for VitD deficiency, with age-related factors playing a distinct role in each age group. At birth, the newborn's VitD status depends mostly on the maternal VitD status. Low maternal VitD levels during pregnancy

have been associated with adverse neonatal outcomes, including small for gestational age and preterm births [44, 45]. In infancy, exclusively breast fed babies without adequate sun exposure or VitD supplementation are at highest risk [5], since although human milk is the best nutrition for infants, its content in VitD is low and insufficient as it is influenced by maternal sun exposure, skin pigmentation, clothing, latitude, season and diet [46]. Even if a lactating mother is receiving 400 IU VitD/day, the VitD content of milk is found less than 80 IU/L [47]. However, the optimal supplement dose for breast-feeding mothers who seek to give VitD to their infant via breast-feeding is not clearly defined and is not yet recommended [46]. On the other hand, infant formulas contain about 40–120 IU/100 kcal (400 IU/L) of VitD [48]. Subsequently, according to AAP recommendations, every breast-fed should be supplemented with at least 400 IU of VitD/day, and similarly for the formula-fed one until it is able to consume 1 L/day of fortified milk [46]. Especially preterm infants are more prone to hypovitaminosis D, since VitD stores at birth can be lower than those of full-term infants. As a result, preterm infants are substantially dependent on exogenous sources of VitD [49].

During childhood and adolescence, low VitD status is also a consequence of a poor and unbalanced diet, even in developed countries, with frequent consumption of fast and junk food being a relevant risk factor [50].

Important risk factors for VitD deficiency are schematically shown in Figure 7 [17] and pediatric groups at risk for developing hypovitaminosis D are summarized in Table 7 [50].

Figure 7. Vitamin D deficiency risk factors [17]

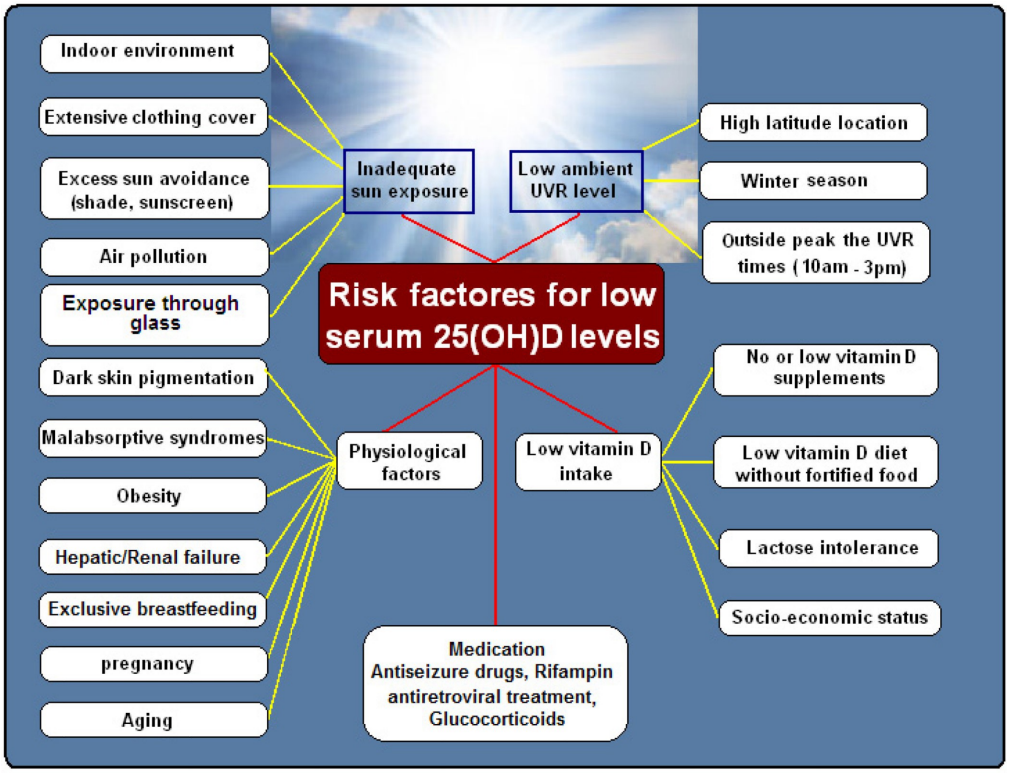


Table 7. Paediatric groups at-risk for vitamin D deficiency [50].

At-risk group	Mechanism of vitamin D deficiency
Newborns born to mothers with vitamin D insufficiency/deficiency	Insufficient stores
Preterm infants	Insufficient stores
Exclusively breastfed Infants	Decreased intake
Infants without vitamin D supplementation	Decreased intake
Children with darker skin	Decreased skin synthesis
Children living at higher latitudes during winter and spring	Decreased skin synthesis
Children with restricted sunlight exposure	Decreased skin synthesis
Obese children	Increased sequestration in adipose tissue
Children with severe liver failure	Decreased synthesis of 25(OH)VitD
Children with severe renal failure	Decreased synthesis of 1,25(OH)2VitD
Children with chronic diseases reducing fat absorption	Decreased intestinal absorption
Children with skin diseases	Decreased skin synthesis
Children under chronic treatment with systemic glucocorticoids	Enhanced inactivation of 25(OH)VitD by up-regulation of 24-hydroxylase activity
Children under chronic treatment with anticonvulsants (carbamazepine, phenytoin, topiramate, phenobarbitone)	Increased hepatic vitamin D catabolism
Migrant children and adopted children	Multiple mechanisms

Interestingly, more recent data associate genetic and epigenetic factors with increased risk for hypovitaminosis D, beyond environmental determinants of 25(OH)VitD levels. The specific genetic determinants of 25(OH)VitD levels are only beginning to be identified and represent a complex trait for which family studies have estimated heritability ranging from 43% to 80% [51]. Further elucidation of these genetic characteristics could potentially help identify those at risk for VitD deficiency/insufficiency [52].

Several single nucleotide polymorphisms (SNPs) of VitD related genes have been associated with low VitD serum levels [53]; and this may explain the wide inter-individual variability in VitD sensitivity, with consequences to disease risk. The VDR gene**,** located on chromosome 12q13-14, exhibits also several polymorphic regions [54]. Of note, a recent identification of 141 SNPs in the VDR from a cohort study of white participants (Cardiovascular Health Study) also revealed that lower serum 25(OH)VitD levels were associated with clinical outcomes according to genetic differences in the VDR (Hazard Ratios for the risk of the composite outcome of 1.40 for those who had 1 minor allele at rs7968585 (in VDR) and 1.82 for those with 2 minor alleles) [17]. Furthermore, two common polymorphisms at the VitD binding protein (VDBP) gene on chromosome 4q12-q13 also affect circulating 25(OH)VitD levels [55]. Three other SNPs in VitD pathway genes (rs2282679, rs2298849, and rs10877012) were significantly associated with VitD deficiency in African-Americans [56]. Two recent genome-wide association studies (GWASs), reported a 49% increased risk of VitD deficiency associated with the rs2282679 minor allele also in white individuals [57].

Also recent epigenomic findings in GWAS confirmed 3 of the 4 genes (DHCR7, CYP2R1, and CYP24A1), which play crucial roles in VitD metabolism [58]. DHCR7 is a novel gene identified in 2 recent GWASs [59, 60], encoding the enzyme 7-DHC reductase, which converts 7-DHC to cholesterol, thereby removing the substrate from the VitD synthetic pathway. CYP24A1 gene which encodes 25(OH)VitD–24-hydroxylase, a mitochondrial protein that initiates the degradation of $1,25(OH)_2$VitD, has been identified as a candidate gene for VitD insufficiency in one GWAS but not in the other [57, 61]. These epigenomic findings suggest that individuals with VitD deficiency are more likely to have reduced synthesis and increased catabolism of 25(OH)VitD and $1,25(OH)_2$VitD [58].

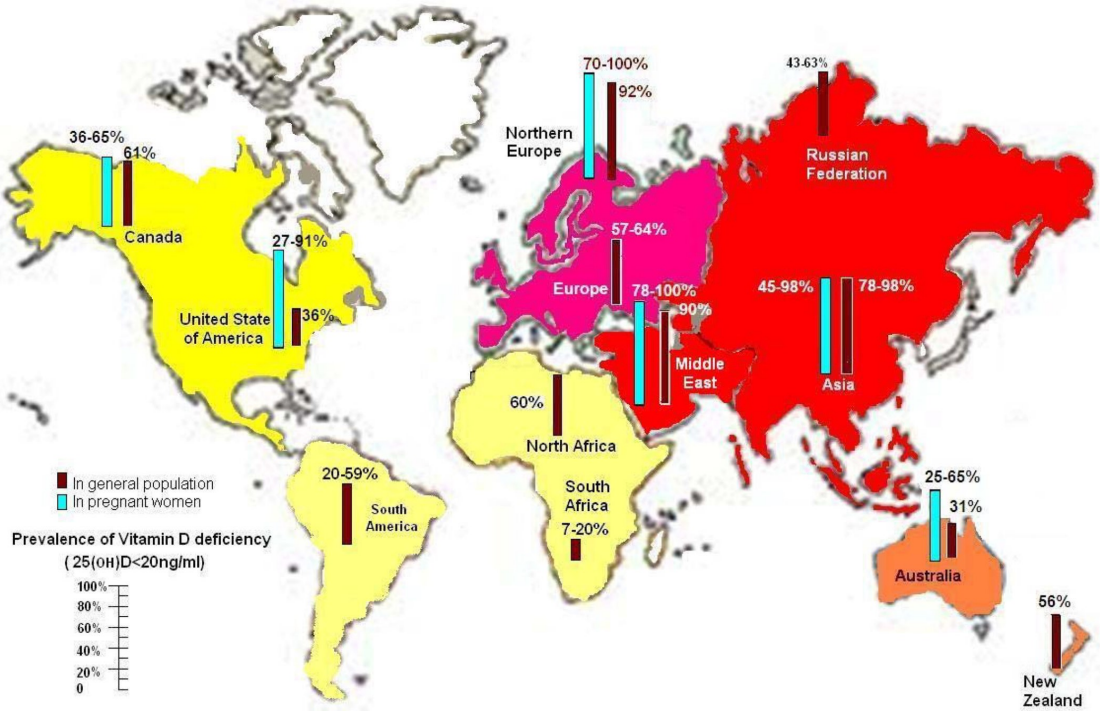
Genomic and epigenomic data interpretation has helped to better understand the complex nature of heritable VitD deficiency risk factors. However, a recent GWAS of

prospectively collected 25(OH)VitD data (from 5 studies, 5575 individuals) reported that known GWAS-associated SNPs explain only approximately 5.2% of the observed variance in circulating 25(OH)VitD levels [52]. Therefore, it is concluded that combined genetic and environmental interactions and their effects determine circulating 25(OH)VitD levels.

1.7 Incidence of Vitamin D deficiency / insufficiency

Several studies have investigated the prevalence of low serum 25(OH)VitD levels in general populations throughout the world [62-67]. It was estimated that the prevalence of VitD deficiency is approximately 30–50% in the general population [68] and that serum 25(OH)VitD concentrations and VitD supply are insufficient to meet the VitD requirements in significant parts worldwide, even in sunny countries and not only in at-risk groups [15] (Figure 8) [17]. There exist, of course, regional differences in the burden of VitD deficiency. According to recent surveys, serum 25(OH)VitD concentrations <12 ng/mL and <20 ng/mL are documented in 13.0 and 40.4% of the general population in Europe, in 6.7 and 26.0% of the general population in the US, respectively [64, 65]. The prevalence of serum 25(OH)VitD levels <20 ng/mL was almost in one-third of the US population, with in more than 70% of non-Hispanic black individuals and in more than 40% of Hispanic/Mexican individuals [59]. Australian adults, had also high rates of VitD deficiency/insufficiency with 31% having serum 25(OH)VitD <20 ng/mL and 73% <30 ng/mL in one study [69]. Even more increased hypovitaminosis D rates seem to be found in many low and lower-middle income countries, like in India, Tunisia and Mongolia where more than 20% of the entire population has 25(OH)VitD concentrations <12 ng/mL [63]. In the Middle East and Asia, VitD deficiency is highly prevalent as well [70, 71].

Figure 8. Reported incidence of vitamin D deficiency defined as a 25(OH)VitD level <20 ng/ml around the globe in pregnant women and general population [17].



Across **Europe particularly,** prevalence estimates of VitD deficiency are so widespread that meet the criteria of a pandemic [64]. Using the Institute of Medicine's definition of VitD deficiency (i.e. 25(OH)VitD <12 ng/mL) [26], an overall of 13% across all ages from childhood, teenage, adult, and older adult population from all Europe (n=55,844, southern to mid and up to northern European member states), had VitD deficiency [64]. Among those tested in the extended winter and summer periods, VitD deficiency's prevalence was 17.7% and 8.2%, respectively [64] .

Using the alternately suggested definition of VitD deficiency as per the Endocrine Society (i.e. 25(OH)VitD <20 ng/mL) [27], the prevalence reached 40.4% [64]. However, there was a considerable variation depending on season, latitude, age group, and ethnic mix of the study populations.

As expected, there was also considerable variation in the prevalence of VitD deficiency among the European Union countries, depending on latitude. Interestingly

however, in studies with adults and older adults, the prevalence of VitD deficiency appeared to be dependent on age group. VitD deficiency was much less in the more northerly latitude countries such as Norway, Iceland, and Finland, whereas more mid-latitude countries such as the United Kingdom, Ireland, Netherlands, and Germany had a higher prevalence, even accounting for ethnicity [64]. Also VitD deficiency's prevalence in extended winter compared with extended summer was also much lower in the northerly latitude countries, which may be attributed to higher rates of food fortification policies, high consumption of fatty fish and cod liver oil and/or VitD supplement use in these countries [64, 67]. Notably, in the **Southern Europe and the Eastern Mediterranean regions** there was a high prevalence of hypovitaminosis D, despite abundant sunshine. A systematic review showed that more than one-third of the studies reported mean 25(OH)VitD levels <20 ng/mL and ~10% <10 ng/mL [66]. An observational cross-sectional study in Greek adults from urban and rural regions found mean serum 25(OH)VitD at 20± 8 ng/mL, with VitD deficiency highly prevalent among healthy Greek men and women, reaching 87.7% of the population [72]. The lower VitD status in Greece, Italy and Spain could be attributed to increased skin pigmentation, sunshine-avoiding behaviours, and air pollution, all of which reduce sun-induced VitD production in the skin [69]. Overall dark-skinned ethnic subgroups had much higher (3- to 71-fold) prevalence of serum 25(OH)VitD <12 ng/mL than did white populations [64].

Moreover, contrary to ethnic and age-grouping differences [64], studies showed no sex differences in the prevalence of VitD deficiency in both young and older European populations. Serum 25(OH)VitD levels <12 ng/mL were found on average to be 13.1% and 12.9% for males and females respectively [64].

Several studies have evaluated VitD status in **childhood and adolescence** and demonstrate widespread hypovitaminosis D around the world as well [73-79] (Table 8) [40]. In the United States, large US National Health and Nutrition Examination Survey (NHANES) studies estimated that 50% of children aged 1 to 5 years and 70% of children aged 6 to 11 years were VitD deficient or insufficient [78]. Recent studies reported that also adolescents and young adults are at risk for VitD deficiency [80], probably because of increased obesity incidence, decreased milk consumption, and increased sunscreen use [17]. More limited data are available for the European pediatric population [73-76].

Interestingly childhood population studies showed that the relatively mid-latitude countries (47–60ºN) had a higher prevalence range of low VitD status of 5–20% than did southern countries (<41ºN) with 4.2–6.9% [64]. A recent study among Greek schoolchildren (9–13 years old) showed that the prevalence of 25(OH)VitD serum levels <12 and <20 ng/mL was 5.2 and 52.5%, respectively [66]. Despite Greece's southerly latitude, the prevalence of hypovitaminosis D was similar or even greater than that reported among other European children and adolescents [66]. Notably, girls had a higher prevalence of 25(OH)VitD <12 (7.2 versus 3.2%) and 20 ng/mL (57.0 versus 48.0%) than boys.

Children in urban/semi-urban regions had higher prevalence of 25(OH)VitD <20 ng/mL than children in rural regions, particularly during spring months (74.6 versus 47.2%). The highest prevalence rates of 25(OH)VitD <12 and <20 ng/mL (9.1 and 73.1%, respectively) were observed during spring, whereas the lowest (1.5 and 31.9%, respectively) during autumn [66].

Furthermore, other researchers examined VitD status during adolescence worldwide [76, 77, 81]. In particular, European studies showed that **teenagers** aged 15–18 years, irrespective of latitude, seemed to exhibit a higher prevalence of VitD deficiency ranging from 12–40% than did other age groups, with those of children 1–6 years being at 4–7%, 7–14 years at 1–8% and older adults at 9–24%, respectively [64]. Similarly, in our area of North West of Greece a study showed a high percentage (47%) of the subjects aged 15-18 years who had 25(OH)VitD <10 ng/ml in winter and the prevalence being higher in the girls in agreement with other reports. In the younger ages (13-14 years) the prevalence was much less (13-14%) while in the summer they all had levels >10 ng/ml [82].

In conclusion, VitD deficiency is evident throughout the world at prevalence rates of a new "pandemic". Focused actions are required both from a public health and a clinical perspective.

Table 8. Recent epidemiological studies assessing vitamin D status in children and adolescents [40]

First author	Year	Country	Latitude	n	Age (years)	<20 ng/mL	20–29.9 ng/mL	≥30 ng/mL
Absoud M	2011	UK	53–59° N	1,102	4–18	35 %	–	–
Sioen I	2012	Belgium	51.1° N	357	4–11	58 %	40 %	2 %
Vierucci F	2013	Italy	43–44° N	652	2–21	46 %	34 %	20 %
Gonzàlez-Gross M	2012	Europe	Multicenter study	1,006	12.5–17.5	42 %	39 %	19 %
Dong Y	2010	USA	33° N	559	14–18	29 %	28 %	43 %
Kumar J	2009	USA	NHANES 01-04	6,275	1–21	9 %a	61 %b	30 %
Mansbach JM	2009	USA	NHANES 01-06	3,951	1–11	18 %	51 %	31 %
Maguire JL	2013	Canada	43.4° N	1,898	1–5	6 %	24 %	70 %
Santos BR	2012	Brazil	25–30° S	234	7–18	36 %	54 %	10 %
Kim SH	2012	S. Korea	34–38° N	2,062	10–18	68 %	28 %	4 %
Poopedi	2011	S. Africa	MA 26° S	385	10	7 %	19 %	74 %

1.8 Clinical manifestations of Vitamin D deficiency / insufficiency

Hypovitaminosis D has been associated with several health outcomes both in children/adolescents and adults, including musculoskeletal (rickets, osteomalacia, osteopenia, osteoporosis, bone fractures and muscle weakness) and non-skeletal complications [5]. Non-skeletal effects include a wide spectrum of diseases: cardiovascular diseases and risk factors [83, 84] such as cognitive heart failure [28], impaired systolic and diastolic function [85], myocardial infarction [86], peripheral vascular disease [87], non-valvular atrial fibrillation [88], as well as common obesity [89], type 1 [16] and type 2 diabetes [90, 91] and insulin resistance, metabolic syndrome [92] and hypertension [93]. In addition, it has also been associated with respiratory tract infections and tuberculosis and also with autoimmune diseases as is rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases etc. Moreover hypovitaminosis D is believed to be implicated in malignant diseases [94], neuropsychiatric diseases as schizophrenia and depression, cognitive deficits [95] and into overall mortality [96].

1.8.1 Effect of Vitamin D on the Cardiovascular System

Seasonal variation of CVD was observed many years ago, with higher mortality from ischaemic heart disease occurring in winter and at higher latitudes [97-99]. Later clinical and epidemiological studies have linked low VitD status to CVD risk factors such as hypertension, diabetes, obesity and dyslipidemia [100, 101] and VitD deficiency was proposed to affect cardiovascular health through many direct and indirect mechanisms (Figure 9). The direct mechanisms of vitD involvement include: A) preservation of heart muscle and vascular system functions through: (1) inhibition of vascular smooth-muscle cells proliferation, intima-media thickening and metalloproteinase activity; (2) enhancement of vasodilatory action of endothelial nitric oxide synthase; (3) prevention of the development of atherosclerotic plaques (inhibition of macrophages conversion into foam cells); (4) prevention of calcium deposition in vessels; (5) endothelium protection against advanced glycation end products (AGEs), (6) anti-thrombotic activity [9, 102-104] and B) anti-inflammatory effects (modulation of inflammatory markers [105] and metalloproteinase-9 (MMP-9) [106]). VitD's indirect effects on cardiovascular system include: (1) downregulation of PTH [107], (2) suppression of the renin-angiotensin-aldosterone system [108], (3) improved insulin sensitivity and secretion and beneficial effect on lipid profile (Figure 10) [109].

Chronic VitD deficiency leads to PTH overproduction, mainly due to decreased intestinal calcium absorption and increased calcium mobilization from bone. Increased PTH levels and hypercalcemia can also have adverse cardiovascular effects including left ventricular hypertrophy (LVH), valvular calcification, myocardial calcification, blood clot formation, cardiac arrhythmia and arterial hypertension [110] (Figure 11) [111].

Overall VitD has been recognized as a powerful molecule which can hypothetically prevent from various processes implicated in the pathogenesis of cardiovascular system dysfunctions [11] (Figure 11, Table 9).

Figure 9. Effect of VitD on cardiovascular system [109]

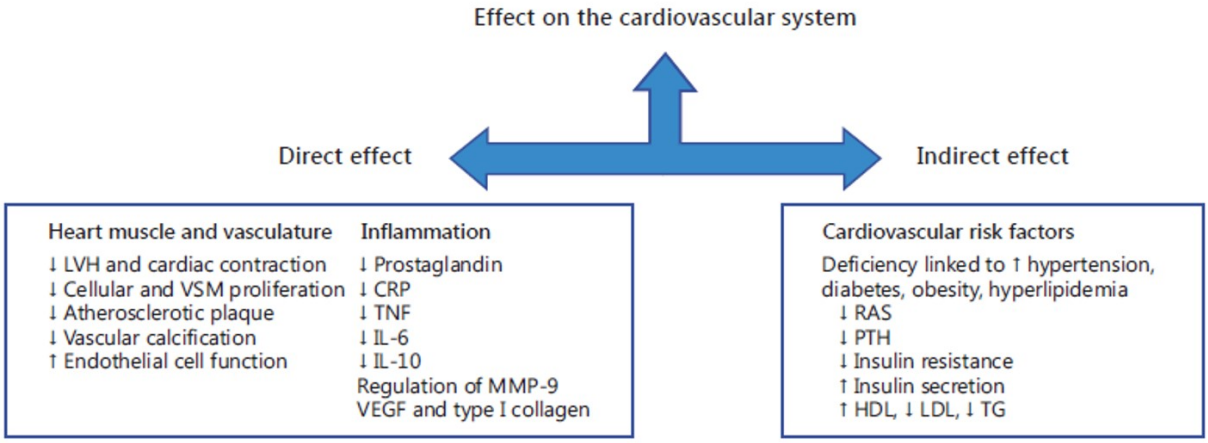
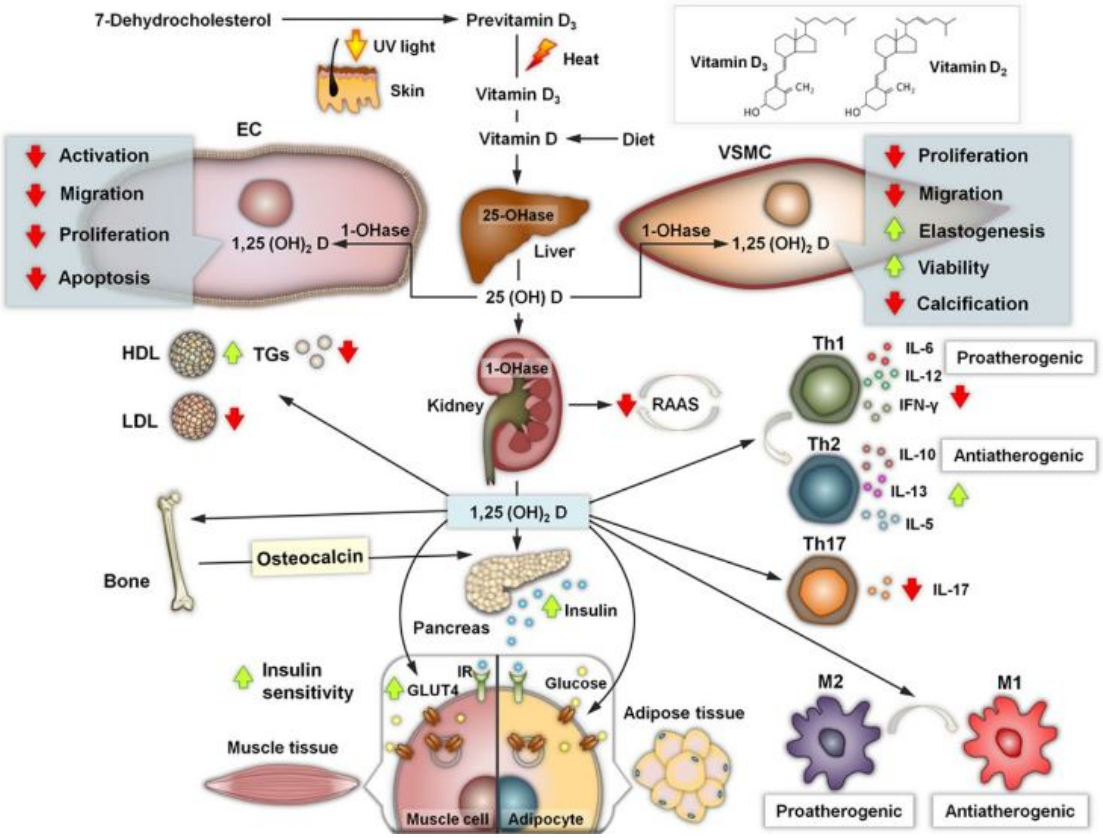


Figure 10. Representation of VitD's actions in cells and tissues implicated directly and indirectly in the atherosclerotic process [109].



EC: endothelial cell, VSMC: vascular smooth muscle cell, Glut-4: glucose transporter 4, M1/2: macrophage-monocyte

Figure 11. Potential mechanisms through which VitD deficiency and high calcium levels may lead to CVD manifestation [111].

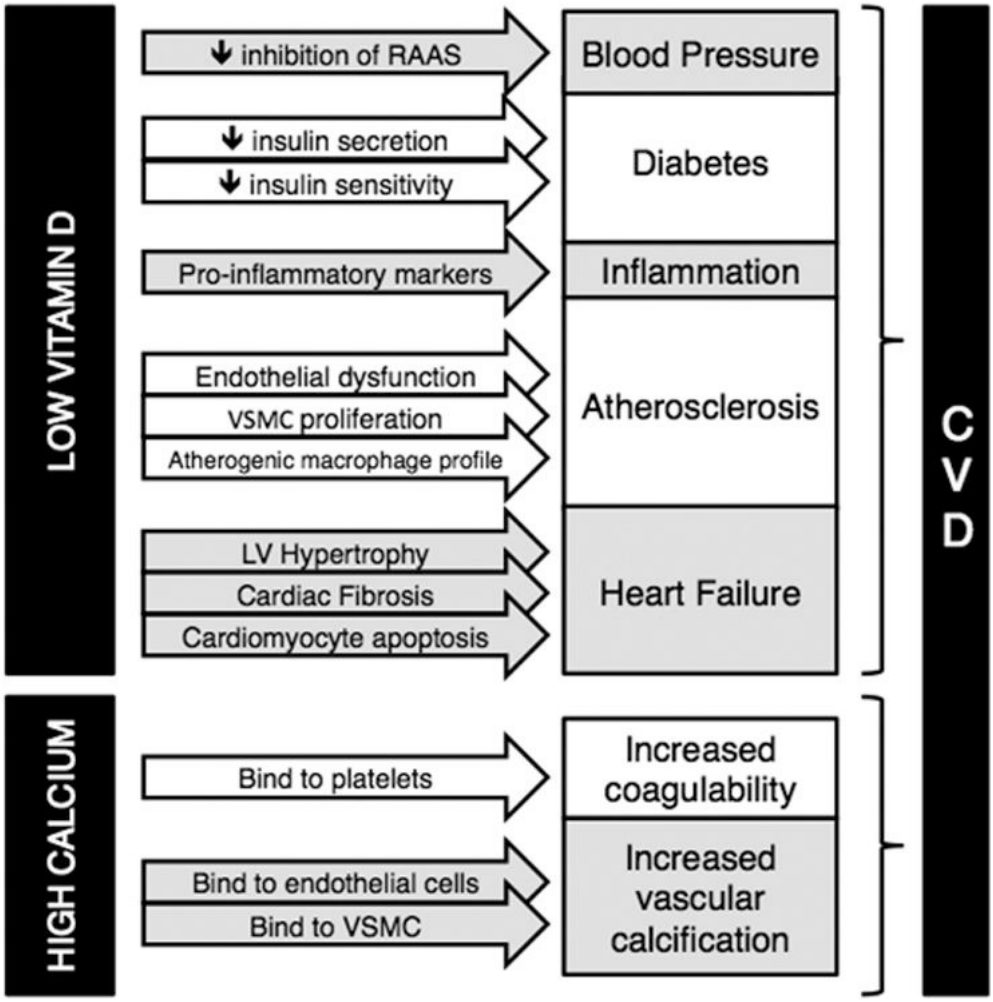


Table 9. Potential mechanisms through which VitD deficiency may affect CVD related pathologies

Pathology	Proposed Mechanism of Action
Hypertensive vascular disease	● Increased intracellular calcium leading to decreased renin activity ● Calcitriol suppression of renin promoter gene ● Alteration of the sensitivity of vascular smooth muscle cells
Peripheral vascular disease	● Increased calcification
Diabetes mellitus	● Immunomodulatory effects by reducing tumor necrosis factor–α, parathyroid hormone, and interleukin-10 ● Decreased insulin receptor expression, leading to peripheral resistance of insulin ● Effect on intracellular calcium levels leading to decreased insulin secretion
Lipid metabolism	● Increase peripheral insulin resistance, contributing to high lipid profile ● Statins may increase vitamin D levels by increasing 7-dehydrocholesterol ● Increased vessel free radicals lead to oxidation of low-density lipoprotein and increased engulfment by macrophages, an early sign of atherosclerosis
Coronary artery disease	● Indirect effect through risk factor modification ● Altering endothelial function ● Increased coronary artery calcification
Heart failure	● Direct effect on myocardial contractility ● Regulation of brain natriuretic peptide secretion ● Reduction of left ventricular hypertrophy with effects on extracellular remodeling ● Regulation of inflammatory cytokines ● Secondary hyperparathyroidism, which leads to vasodilatation and positive inotropic stimulation
Arrhythmias	● Direct myocardial substrate modification ● Indirectly via calcium levels and metabolism at a cellular level

1.8.2 Effect of Vitamin D on the Redox / Detoxification Metabolism

Normal cell functions and survival are preserved by maintaining a highly reduced internal environment. A possible increase in the levels of intracellular reactive oxygen species (ROS) causes an imbalance in the oxidant/anti-oxidant status, which in turn leads to redox stress-induced impairment of cell homeostasis [112].

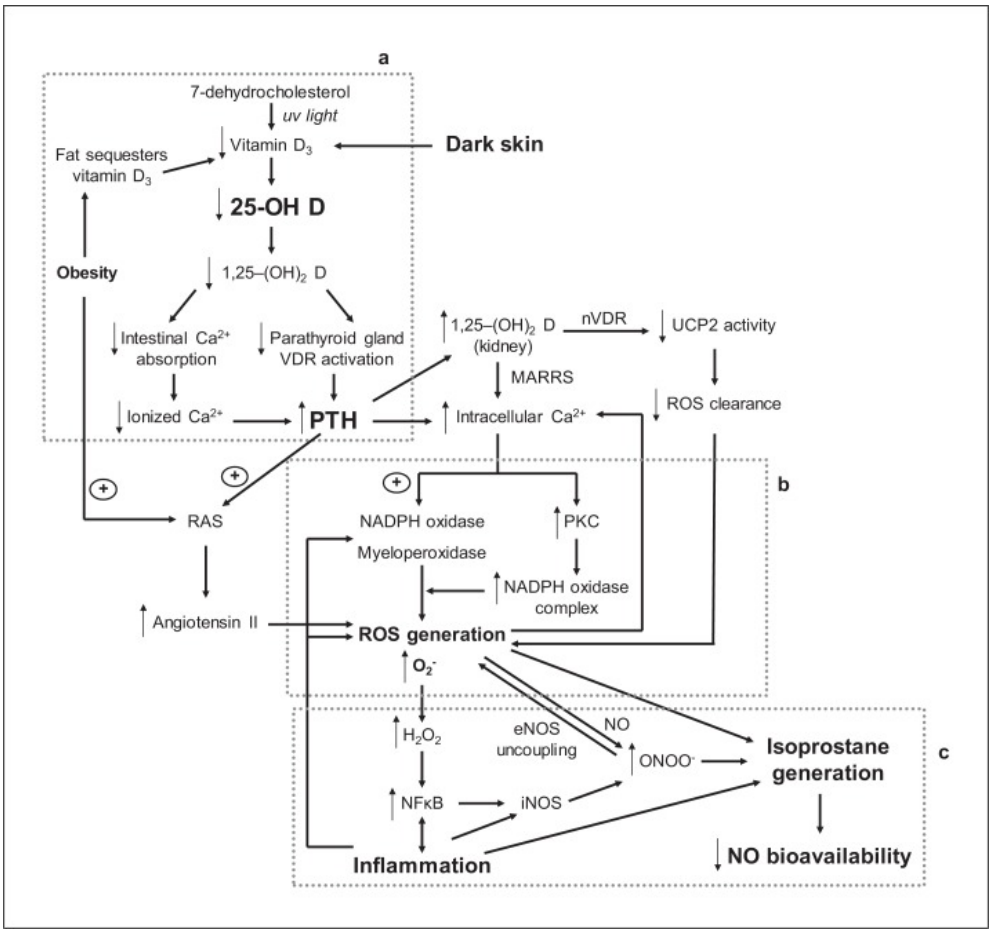
VitD is found able to regulate the expression of many antioxidant systems. For example the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the major enzyme system that generates superoxide anion (the main ROS) may also be modulated by VitD [113]. NADPH oxidase may be activated by the receptor for advanced glycation-end products (RAGE). A RAGE-induced inflammation is observed in several pathological conditions associated with VitD deficiency, such as diabetes, cardiovascular diseases, neurodegenerative disorders and cancer [114, 115].

VitD may also exert protective effects on endothelial cell's dysfunction, which are inflammatory processes that precede atherosclerosis, through stimulation of nitric oxide (NO) production and may counteract superoxide anion generation, possibly by controlling at the level of phospho-active extracellular signal-regulated kinases [116]. VitD and PTH potentially influence NO bioavailability as depicted in Figure 12 [117]. In the setting of VitD deficiency, increased PTH activates $1,25(OH)_2$VitD production in the kidneys, increasing intracellular calcium which, in turn, promotes ROS production [118]. Angiotensin II induces superoxide formation which augments inflammation and attenuates NO production and endothelium-dependent vasodilation [119]. Mounting evidence indicates that VitD regulates the renin-angiotensin system. Compared with VitD-sufficient individuals, those with VitD deficiency and insufficiency had greater plasma angiotensin II levels and a trend for higher plasma renin activity [120].

VitD may also control the expression of the nuclear factor-erythroid-2-related factor 2 (Nrf2), a transcription factor able to activate genes encoding for antioxidant and detoxifying enzymes [114]. Notably, Nrf2 is able to induce the up-regulation of VDR and RXR, in order to enhance cell sensitivity to low VitD levels [121]. In addition, VitD is found to regulate the expression of Klotho, a trans-membrane protein with antioxidant action, which is released as a humoral factor able to influence several signaling pathways

and cellular processes [122]. VitD deficiency may possibly lead to reduced expression of Nrf2 and Klotho and consequently to the disruption of redox system homeostasis. The loss of this control may be the onset of numerous age-related disorders [123].

Figure 12. Proposed model linking low 25(OH)VitD and raised PTH to greater oxidative stress and lower NO bioavailability [117]



a Dark skin and adipocytes decrease VitD. Low 25(OH)VitD is initially associated with low 1,25(OH)$_2$VitD decreasing serum ionized calcium (Ca 2+), which consequently increases PTH release.

b Raised PTH activates renal 1α -hydroxylation of 25(OH)VitD to produce 1,25(OH)$_2$VitD in the kidneys, rennin-angiotensin system (RAS), and intracellular calcium overload. The paradoxically increased circulating 1,25(OH)$_2$VitD augments intracellular calcium in adipocytes by activating the putative membrane vitamin D receptor (MARRS). Angiotensin II and calcium influx generate reactive oxygen species (ROS) by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase, and protein kinase C that stabilizes NADPH oxidase. 1,25(OH)$_2$VitD binds to nuclear VDR downregulating uncoupling protein 2 (UCP2) activity, which decreases ROS clearance.

c Raised superoxide radicals (O_2^-) consume NO producing peroxynitrite ($ONOO^-$), which in turn causes endothelial NO synthase (eNOS) uncoupling. O_2^-also promotes inflammation via overwhelming superoxide dismutase to release hydrogen peroxide (H_2O_2) that activates nuclear factor kappa beta (NF-êB) enhancing $ONOO^-$. Overall, inflammatory stress and oxidative stress generate lipid peroxidation, such as isoprostanes, leading to NO destruction and, as a consequence, decreased NO bioavailability.

Normal cell function and are guaranteed by the maintenance of highly reduced internal environment of cells [124]. In VitD deficient subjects the oxidant/anti-oxidant status seems to lean in favor of oxidative stress [124], while correcting hypovitaminosis D may counteract the negative effects caused by oxidative stress as found in epithelial cells [125].

Higher BMI leads to lower 25(OH)VitD levels probably due to the sedentary lifestyleof subjects, the reduced 25(OH)VitD skin synthesis and VitD sequestration in their excessive fat tissue, as analysed below [126]. MetS has been associated with increased oxidative stress, which plays a crucial role in the formation, progression and rupture of atherosclerotic plaques [127]. Oxidation of low dencity lipoprotein cholesterol (LDL-C) by free radicals and formation of oxidised LDL (ox-LDL) particles happens in the very early but critical steps of atherosclerosis [128].

VitD deficiency has been associated with increased oxidative stress also in obese individuals, patients with chronic diseases and the elderly [129]. Some data suggest that VitD deficiency may promote vascular oxidative stress and induce hypertension as well as changes in cardiac gene expression [130]. VitD deficiency may also play a possible role in the link between oxidative stress and diabetes development and progression [131].

Notably, to date there is no unique method which can accurately measure oxidative stress. Paraoxonase activity and arylesterase activity (i.e. a PON-1 activity more closely related to PON-1 mass) are used as indicators and are found to be inversely associated with oxidative stress, since they inhibit LDL oxidation and protect against cardiovascular diseases [132]. Another useful and validated oxidative stress marker is isoprostanes, especially the biologically active 8-iso-PGF_{2a}, which are produced from the random oxidation of tissue phospholipids by oxygen radicals [133].

Some studies in **adults** indicated that VitD supplementation has been associated with improvement of oxidative stress and inflammation [125], though not consistently. However, there is little data on the association between VitD and oxidative stress markers assessed in this study (ox-LDL, PON-1 activities, and urinary 8-isoprostanes). In particular, a recent study in adults found that serum ox-LDL levels were significantly higher in type 2 diabetic patients with hypovitaminosis D compared with those with normal VitD status [134]. On the other hand, a study in VitD deficient but otherwise healthy persons and matched controls showed that ox-LDL and LDL levels did not differ

between groups and there was no association between VitD and ox-LDL levels [124]. In the same study, treatment with 50,000 IU VitD/week per os for 8 weeks was not associated with changes in ox-LDL levels [124]. Regarding PON-1 activity, a previous study showed that supplementing asymptomatic VitD deficient people with 300,000 IU VitD intra-muscularly (IM) monthly for 3 months was not associated with changes in serum paraoxonase activity compared with VitD sufficient controls [135]. Observational studies also show conflicting results about the relationship between VitD and isoprostane levels. An analysis of the Framingham Offspring Study showed that plasma 25(OH)VitD concentration was inversely associated with urinary isoprostanes [136]. Similar results were obtained in a study in type 2 diabetic patients with hypovitaminosis D [134]. Another cross-sectional study though did not support an association of 25(OH)VitD levels with plasma isoprostanes in African-Americans probably due to the relatively modest sample size [117]. Of note, data from interventional studies are lacking. In particular, one study in type 2 diabetic patients showed that treatment with 5000 IU VitD/day per os for 12 weeks versus placebo was not associated with improvement in plasma 8-isoprostanes [137].

Other studies in obese VitD deficient subjects showed that VitD supplementation in combination with exercise could possibly ameliorate oxidative stress as assessed by urinary 8-isoprostane, hydrogen peroxide, tumor necrosis factor-alpha and other factors [138, 139]. Studies in patients with chronic diseases found that VitD administration was associated with improvement of several oxidative stress markers. In particular, patients with diabetes showed reductions in plasma nitric oxide (NO), glutathione (GSH) and malondialdehyde (MDA) levels and increase in total antioxidant status (TAS) [140, 141]. Furthermore, women with polycystic ovary syndrome exhibited decreases in MDA and GSH levels and an increase in TAS [142, 143], while patients with non-alcoholic fatty liver disease experienced a decrease in MDA levels and an increase in TAS [144]. Moreover, VitD deficient individuals exhibited reduction in MDA levels and increase in TAS, while in other studies a decrease in total oxidant status (TOS) and fibrinogen, but not ox-LDL levels were found [124, 135]. On the contrary, in type 2 diabetic patients VitD administration was not associated with improvement of oxidative stress markers (superoxide dismutase or plasma 8-isoprostanes) [137]. To date, however, there is lack of data regarding the effect of VitD supplementation on oxidative stress markers in patients with MetS.

Moreover, some data have indicated that VitD deficiency may be implicated in oxidative stress development even in **youth**, while VitD supplementation is reported to have favorable effect on their antioxidant system [145]. However there is considerable inadequacy of data regarding this mater in childhood and adolescence.

1.8.3 Other non-skeletal consequences of Vitamin D deficiency / insufficiency

During last decades even more studies exploring effects of VitD beyond its well-known effects on the musculo-skeletal system have come about. Except the vast majority of observational studies that have reported low 25(OH)VitD levels to be associated with an increased risk of adverse health outcomes, randomized control trials (RCTs) the gold-standard for exploring any causal link, have been carried out. Also within the last few years, many systematic reviews have been published, including meta-analyses (MAs) with results from RCTs on non-skeletal outcomes in response to VitD supplementation [146-148]. Overall most MAs and the individual RCTs on risk of cardiovascular diseases, type 2 diabetes, weight-loss, and malignant diseases give inconsistent results. Only 14 out of the 54 MAs suggest beneficial effects of VitD supplementation on different extra-skeletal outcomes among which 1 MAs on depression, 2 MAs on blood pressure, 3 MAs on respiratory tract infections, and 8 MAs on mortality [96]. Despite that and as researchers claim, those findings do not challenge the formulated hypothesis based on the findings from observational studies for adverse health outcomes from low 25(OH)VitD levels [96]. However others claim that the inverse association between 25(OH)VitD levels and various health outcomes, could be the result of disease process causing low 25(OH)VitD levels rather than low 25(OH)VitD levels causing the disease i.e., low 25(OH)VitD levels being a marker of ill health [147].

From now onwards the focus will be on VitD and cardiovascular diseases and other risk factors both in adults and children/adolescents.

1.9 Vitamin D and Cardiovascular Diseases

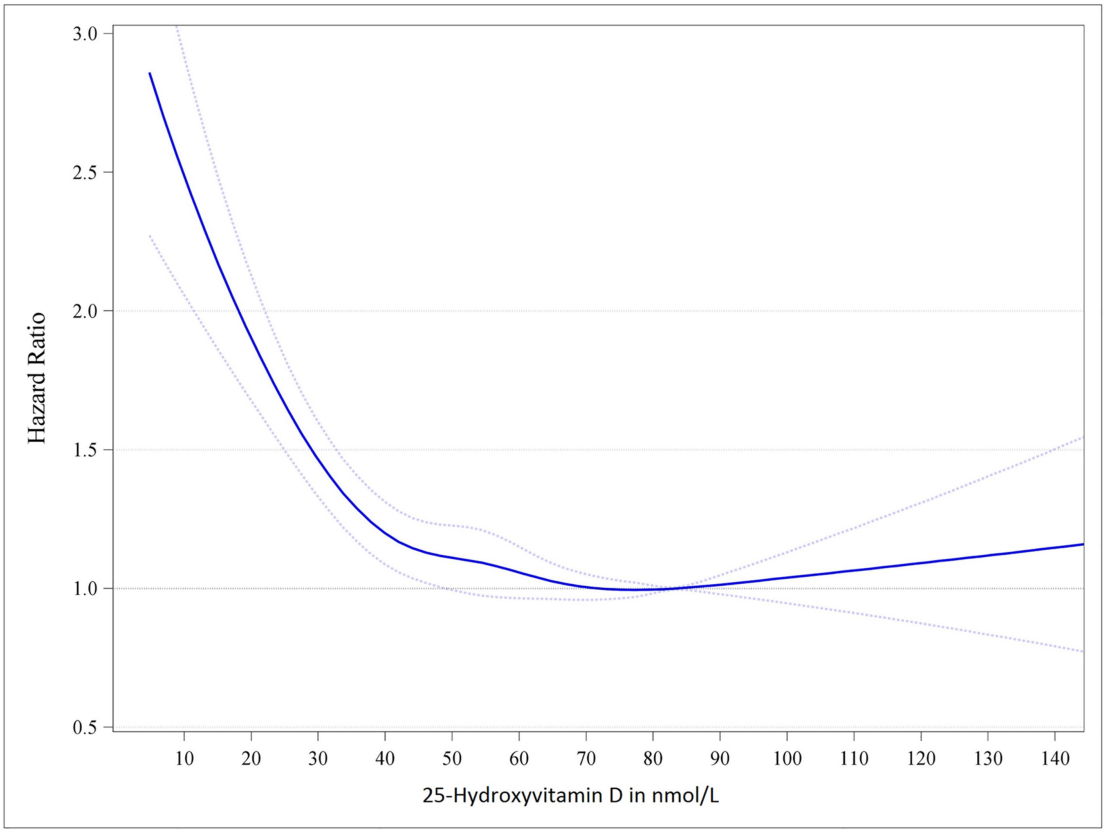
Cardiovascular diseases (CVD) are a leading cause of mortality worldwide, accounting for approximately 30% of all deaths [7]. Thus, identifying modifiable risk factors and treatments remains a high priority for CVD prevention.

Around one billion people worldwide have either VitD deficiency or insufficiency [5] and observational studies give strong evidence in linking low 25(OH)VitD serum levels with poor cardiovascular outcomes and risk of CVDs [84, 86, 149, 150]. Also MAs of data from observational studies have consistently shown an increased risk of CVD in subjects with hypovitaminosis D [150, 151]. Notably, VitD deficiency has been associated with increased risk of cardiovascular mortality [150, 152], myocardial infarction (MI) [153, 154], coronary heart disease (CHD) [155, 156], stroke [157-159], and heart failure (HF) [160]. In addition low VitD status has been linked with several CVD risk factors both in adulthood and childhood/adolescence, including hypertension, diabetes, dyslipidemia, inflammation/oxidative stress and obesity/metabolic syndrome, which will be extensively analyzed below. In this context VitD has been considered as an attractive interventional target, since increasing serum 25(OH)VitD levels with supplementation is relatively inexpensive.

However, not only low but also high serum 25(OH)VitD levels have been associated with increased incidence of cardiovascular outcomes, including CVD, stroke, and acute myocardial mortality. In a 2015 analysis of the Copenhagen vitamin D (CopD) study (n=247,574) [161], serum 25(OH)VitD was non-linearly associated with CVD mortality in a “reverse J-shaped” pattern [161]. The lowest CVD mortality risk occurred at 28 ng/ml (70 nmol/L), a level lower than the Endocrine Society's guidelines for VitD sufficiency of 30 ng/ml [27] but higher than the level deemed adequate for health by the IOM (20 ng/ml) [26]. These results were consistent with another study which found increased mortality risk at high VitD levels >50 ng/ml (125 nmol/L) [162].

In general morbidity and mortality from all causes were lowest at 25(OH)VitD levels between 20 and 36 ng/mL (50 and 90 nmol/L) and increased again above this range (Figure 13) [163].

Figure 13. Dose-response trend of hazard ratios of death from all causes by standardized serum 25(OH)VitD [163]



With regard to cardiovascular mortality a MA of prospective cohort studies, involving 757,304 participants, reported a U-shaped association also with dietary calcium intake. Total daily dietary calcium intake (i.e. from food sources) that were either higher or lower than 800 mg were associated with increasing risk of cardiovascular mortality [164]. In another prospective cohort study with cardiac surgical patients, a U-shaped association between circulating 25(OH)VitD levels and the risk of major adverse cardiac and cerebrovascular events has been reported. Risk was highest at both circulating 25(OH)VitD levels <12 ng/mL and >40 ng/mL [28]. A recent RCT in advanced heart failure provided further evidence for adverse VitD effects in CVD patients [165].

Although results from observational epidemiological studies were encouraging, RCTs of VitD supplementation have not so far reached into conclusions for beneficial or not cardiovascular effects [166-175]. None of the MAs have shown either beneficial or harmful effects from estimates in response to the interventions on risk of CVDs, in terms

of any CV events, myocardial infarctions (MI), stroke/cerebrovascular disease, or CV death [96]. However, most published trials have either had a relatively small sample size or did not include CVD outcomes as a pre-specified outcome. Only the recently published study by Scragg et al. had CVD as primary outcome. Researchers randomized 5110 participants from the general population (42% females) with baseline deseasonalized 25(OH)VitD concentration of 26.4 ng/ml to receive either placebo (n=2552) or vitamin D3 (n=2558) with an initial dose of 200,000 IU, followed a month later by monthly doses of 100,000 IU, for a median of 3.3 years. Their results did not show any beneficial effects of VitD supplementation on the risk of CVD [176]. In their systematic review, Wang et al. showed a statistically nonsignificant reduction in cardiovascular disease with moderate doses of VitD (approximately 1,000 IU/d) [171]. Also Mao et al. showed that neither VitD supplementation nor calcium supplementation had an effect on major cardiovascular events, myocardial infarction, or stroke [173]. A 2015 meta-analysis of 13 RCTs of oral VitD supplementation in adults with chronic kidney disease, found no significant effect on all-cause mortality, cardiovascular mortality, or serious cardiovascular adverse events [177]. Another meta-analysis of 21 VitD trials with outcomes of cardiac failure, MI and stroke, found no statistically significant difference in those supplemented with VitD compared to placebo [178]. Ford et al., despite their belief that there is insufficient evidence to support VitD supplementation for the reduction of cardiovascular events, they highlighted the possibility that VitD supplementation might still have an effect on heart failure [169].

Other studies with selected populations have also been carried out. The Vitamin D Treating Patients with Chronic Heart Failure (VINDICATE) study was a randomized, placebo-controlled, double blind trial of VitD supplementation to patients with HF and VitD deficiency [179]. They found that while one year of 4,000 IU/day of VitD did not improve 6-minute walk distance, their primary outcome was that those receiving VitD supplementation showed a 6.1% improvement in ejection fraction and a decrease in left ventricular end diastolic diameter. While this sample was relatively small (n=229) as not to be generalized (participants were all male HF patients), it does provide some new encouraging evidence on the potential effects of VitD supplementation. On the contrary, a recent MA of 7 RCTs investigating whether VitD supplementation has protective effects in patients with chronic HF, found that while VitD may decrease PTH levels and some

inflammatory markers, no statistically significant improvement in the left ventricular function could be detected [180]. Here again the MA was limited by the small sample sizes and relatively short follow-up duration of the included trials; while the VINDICATE trial was both larger in size and had a longer follow-up compared to all trials included in the MA.

More specifically designed large-scale VitD supplementation trials have been conducted or are currently underway. The VITamin D and OmegA-3 TriaL (VITAL), a placebo-controlled, double-blind 2×2 factorial trial of over 25,875 multi-ethnic participants randomized to 2,000 IU/day of vitamin D3 and omega-3 fatty acid supplements for 5 years has been completed. VITAL study showed that supplementation with VitD (2000 IU/day) did not result in a lower incidence of cardiovascular events (including myocardial infarction, stroke, or death from cardiovascular causes) than placebo. Additionally, no excess risks of hypercalcemia or other adverse events were identified [181]. Another completed study is the Vitamin D Assessment (ViDA) study, a randomised, double-blind, placebo-controlled trial, to evaluate the efficacy of monthly VitD supplementation (100,000 IU or placebo) in reducing the incidence of a range of acute and chronic diseases and intermediate outcomes. The results showed no beneficial effect of VitD supplementation on incidence of cardiovascular disease, falls, non-vertebral fractures and all cancer types. But VitD supplementation was beneficial for persistence with taking statins in participants on long-term statin therapy, and also in bone mineral density and arterial function in participants with low 25(OH)VitD levels, and in lung function among ever smokers (especially if VitD deficient) [182]. EVITA (Effect of vitamin D on all-cause mortality in heart failure) study examined the effect of VitD supplementation (4000 IU VitD daily or matching placebo) on all-cause mortality in 400 heart failure patients (HF) with 25(OH)VitD levels <30 ng/mL for 3 years. The results showed that a daily VitD dose of 4000 IU not only did not reduce mortality in patients with advanced HF but was associated with a greater need for mechanical circulatory support implants. Hence these data indicate caution regarding long-term supplementation with moderately high VitD doses [165]. The D-Health Trial, a placebo-controlled trial with 21,315 participants randomized to 60,000 IU/month of vitamin D3 for 5 years with primary outcome all-cause mortality and secondary outcomes total cancer incidence and colorectal cancer incidence is still going on [183].

These studies have their limitations. Most trials are studying older populations, and the results may not be generalizable to younger individuals. Furthermore, only one trial had a low VitD status (25(OH)VitD <30 ng/ml) as an enrollment criterion. If VitD intervention is effective, the effect would likely be most pronounced in VitD deficient individuals; thus benefits may not be seen in populations that started with adequate VitD levels.

Overall, based on the most recently published data (2019) MA of RCTs have demonstrated that CVD risk markers, such as lipid parameters, inflammation markers, blood pressure, and arterial stiffness, are largely unaffected by VitD supplementation. Similar results have been obtained regarding CVD events and mortality from MA of RCTs, even in subgroups with 25(OH)VitD concentrations <20 ng/mL. Likewise, Mendelian randomization studies have indicated that the genetic reduction of the 25(OH)VitD concentration does not increase CVD risk [184].

In **children and adolescents** there are no studies with cardiovascular disease events as primary outcomes, since CVD events occur most frequently during or after the fifth decade of life. However, there is evidence that pathological precursors of CVD originate in childhood and they may be affected by VitD deficiency. In particular VitD deficiency has been associated with CVD risk factors that promote vascular stiffness and calcification, leading to the development of atherosclerosis, as a degenerative vascular process that starts in childhood [185-187]. Atherosclerosis is observed as lipid accumulation in the intima arteries of the youth. Approximately at 3 years of age, almost all children with atherosclerosis have at least some degree of aortic fatty streaks [188], which can increase after the 8 years of age [189] and form atherosclerotic plaques in the coronary arteries during adolescence [190]. Later, in adulthood, this might cause myocardial infarction or stroke [104]. Observational studies have showed that children with VitD deficiency or insufficiency had higher risk of an elevated blood glucose level, increased risk of hypertension, and increased risk of metabolic syndrome, a prelude to type 2 diabetes [78, 191-193]. These among others CVD risk factors that may be adversely affected by VitD deficiency, will extensively be analyzed below.

1.10 Vitamin D deficiency and Cardiovascular Disease Risk Factors

1.10.1 Vitamin D and Blood Pressure

Hypertension is a very common and major chronic health problem in developed countries, with a prevalence of approximately 29% in adults [194] and with an estimated 1.6 billion cases of hypertension expected in 2025 [195]. Environmental studies have shown that the prevalence of hypertension is lower in sunny regions, while it increases with increasing distance from the equator [196]. Thereafter a possible link between VitD and hypertension has been extensively investigated. Strong observational data associate low 25(OH)VitD levels with an increase in blood pressure and an increased risk of hypertension [197-199]. An analysis of NHANES III 1988–1994 of 12,644 participants aged >20 years showed an inverse association between VitD levels and blood pressure [200]. Similar results were obtained from analysis of NHANES 2003–2006 of 7228 participants [201].

Laboratory and animal experiments proposed a cause-and-effect relationship, on the basis of the key modulating effects of VitD on the renin-angiotensin-aldosterone (RAAS) axis. In vitro studies using a juxtaglomerular cell model showed that VitD suppresses both renin gene expression via a vitamin D–responsive element in the promoter of the renin gene and the expression of the angiotensinogen gene by blocking the nuclear factor κB (NF-κB) pathway [108, 202]. Hypovitaminosis D was associated with hypertension possibly through RAAS activation [108, 203], whereas sufficient levels may afford “endogenous” proximal inhibition [9]. Moreover, human studies have shown increased levels of renin and angiotensin II in subjects with VitD deficiency [203, 204]. Also, VitD may have a direct vascular effect, as implied by the presence of 1α-hydroxylase activity in vascular smooth muscle and endothelial cells and the presence of VDRs in endothelial cells and macrophages [205]. Subsequently local mechanisms of action on the vessel wall have been suggested to be of importance for hypertension manifestation in VitD-deficient patients, leading to arterial stiffness [206] and endothelial dysfunction [207]. Also, hyperparathyroidism may be associated with increased blood pressure levels since a

positive correlation has been shown between PTH and angiotensin II/aldosterone [208, 209].

Prospective studies show conflicting results, with some reporting that 25(OH)VitD levels could serve as a predictor of future hypertension, while others do not confirm this speculation [199, 210]. Results from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study (a prospective cohort study of 3316 patients) showed that lower 25(OH)VitD and $1,25(OH)_2$VitD levels were independently associated with up-regulated circulating RAAS [211]. Forman et al have also demonstrated an inverse association between VitD and risk of incident hypertension from two prospective cohort studies including 39,000 men from the Health Professionals' Follow-Up Study and 78,729 women from the Nurses' Health Study. Their results, combining men and women with measured 25(OH)VitD levels, showed a pooled relative risk of 3.18 (95% confidence interval 1.39–7.29) [198]. On the contrary the recently published Kailuan study, involving 2456 underground miners, reported that lower 25(OH)VitD levels were not related to a greater risk of incident hypertension [212]. However, a meta-analysis by the same authors, which included 7 prospective studies with 53,375 participants, showed a significant association between VitD deficiency and incident hypertension [212].

Data from interventional studies and especially from RCTs investigating the effect of VitD supplementation on blood pressure are also conflicting. A meta-analysis by Wu et al. of 4 RCTs found a reduction of systolic blood pressure (SBP) by –2.44 mm Hg, but no effect on diastolic blood pressure (DBP) [213]. However, a meta-analysis by Witham et al. of 11 RCTs showed that administration of VitD and ultraviolet A and B radiation was associated with a non-significant SBP reduction by –3.5 mm Hg and a significant DBP reduction by –3.1 mm Hg [214]. No beneficial effects of VitD supplementation were reported in other more recent MAs of RCTs on blood pressure (BP) [93, 167, 172]. Of note however is that some of those studies included patients with mild hypertension, or pregnant women or healthy normotensive persons. Furthermore only a small number of the trials have actually examined effects in patients with low VitD levels [215]. Overall two of nine published MA and eight of 59 individual RCTs included in the MAs, showed beneficial effect of VitD supplementation on BP [96], whereas one MA found an increase in SBP in obese adults in response to VitD supplementation [216]. These results do not provide substantial support to the hypothesis raised by observational studies of an effective role of

VitD sufficiency in blood pressure regulation. However larger RCTs targeting only to hypertensive patients with profound VitD deficiency are needed which may help in reaching safer conclusions [217].

For **children and adolescents,** the data are sparse and again the results from observational and interventional studies are controversial like those of adults. According to most evidence serum 25(OH)VitD levels seems to be negatively associated with blood pressure [78, 218, 219], despite being disputed by some [220, 221]. Kumar et al., in a representative sample of one- to 21-years-olds from NHANES 2001-2004 (n=6,275), found an association between 25(OH)VitD deficiency (chosen cutoff <15 ng/mL) and high systolic BP, since those with VitD deficiency were found to have at 2.4 times greater risk of developing hypertension [78]. Ganji et al. who studied 5,867 adolescents aged 12-19 years through three cycles at NHANES (2001-2002; 2003-2004, and 2005-2006) found also an inverse association between serum 25(OH)VitD and systolic BP [59]. Those data are in line with those by Williams et al. (2011), who in a cross-sectional study involving 5,617 adolescents at NHANES (2003-2006) found an inverse linear relationship between 25(OH)VitD and systolic BP [222]. Moreover, a population‐based study of 1,441 Peruvian adolescents aged 13–15 years old, reported that 25(OH)VitD deficiency was associated with elevated diastolic BP and mean arterial pressure [223]. A clinical trial by Parikh et al., in 701 American adolescents aged 14 to 18 years old, found significant correlations between 25(OH)VitD levels and systolic BP [224]. Pacifico *et al*. reported that increasing serum 25(OH)VitD levels, resulted in a significant decrease of median systolic and diastolic BP [192].

Interestingly, however, a more recent analysis from NHANES 2007-2010 (n=2,908, aged 8-18 years) showed that 25(OH)VitD was not associated with systolic BP when adjusting for BMI [221]. But an earlier retrospective cross-sectional study by Kao et al. involving 229 children and adolescents (aged 3-18 years) showed lower 25(OH)VitD serum levels to be associated with systolic and diastolic BP, even after adjustment for BMI or total fat mass [219]. Also Petersen et al. found that each 10 mmol/L increase in serum 25(OH)VitD was associated with lower diastolic BP, independently of the fat mass index [53].

The proposed possible underlying mechanisms for the antihypertensive and vasoprotective effects of 25(OH)VitD in children and adolescents are similar to those of adults. Shortly, they include RAS system down regulation, PTH suppression and proinflammatory cytokine and prostaglandin production by sufficient VitD levels [108, 214].

Results from the few interventional studies however in the youth are conflicting. The recently published ODIN Project that assigned adolescents (n=110 white, healthy, 81% normal weight, 14-18 years) to receive either placebo or VitD (400 or 800 IU/day) for 20 weeks found no effect on systolic or diastolic BP [225]. Also another study which examined 271 adolescents with type 1 diabetes mellitus (mean age of 15.7 ± 1.4 years) and suboptimal VitD levels (<15 ng/mL) who were treated for 12 to 24 weeks with a VitD analog ($VitD_3$) at doses of 1000 or 2000 IU daily did not find any significant impact on systolic or diastolic BP [226]. On the contrary, Dong et al. who randomly assigned 49 normotensive black boys and girls (aged 16.3±1.4 years) to either 400 IU/d; n=24 used as controls or 2000 IU/d; n=25 as experimental concluded that daily 2000 IU VitD supplementation may be effective in counteracting the progression of aortic stiffness in black youth since carotid-femoral PWV decreased from baseline in the higher dose and not in the lower one [227]. In line, Kelishadi et al. found that improving VitD status was associated with a decrease in mean arterial pressure in children with metabolic syndrome [228]. A MA by Dolinsky et al. reasoned that although cross-sectional studies indicate a potential relationship between 25(OH)VitD levels and systolic BP, still there is not enough evidence to prove that VitD supplementation could yield cardio-metabolic benefit in children and adolescent populations [229]. It is still a challenge for future trial designs to select appropriate target serum 25(OH)VitD levels, doses for VitD supplementation as well as young populations at risk to test for any possible cardiovascular improvements after such interventions.

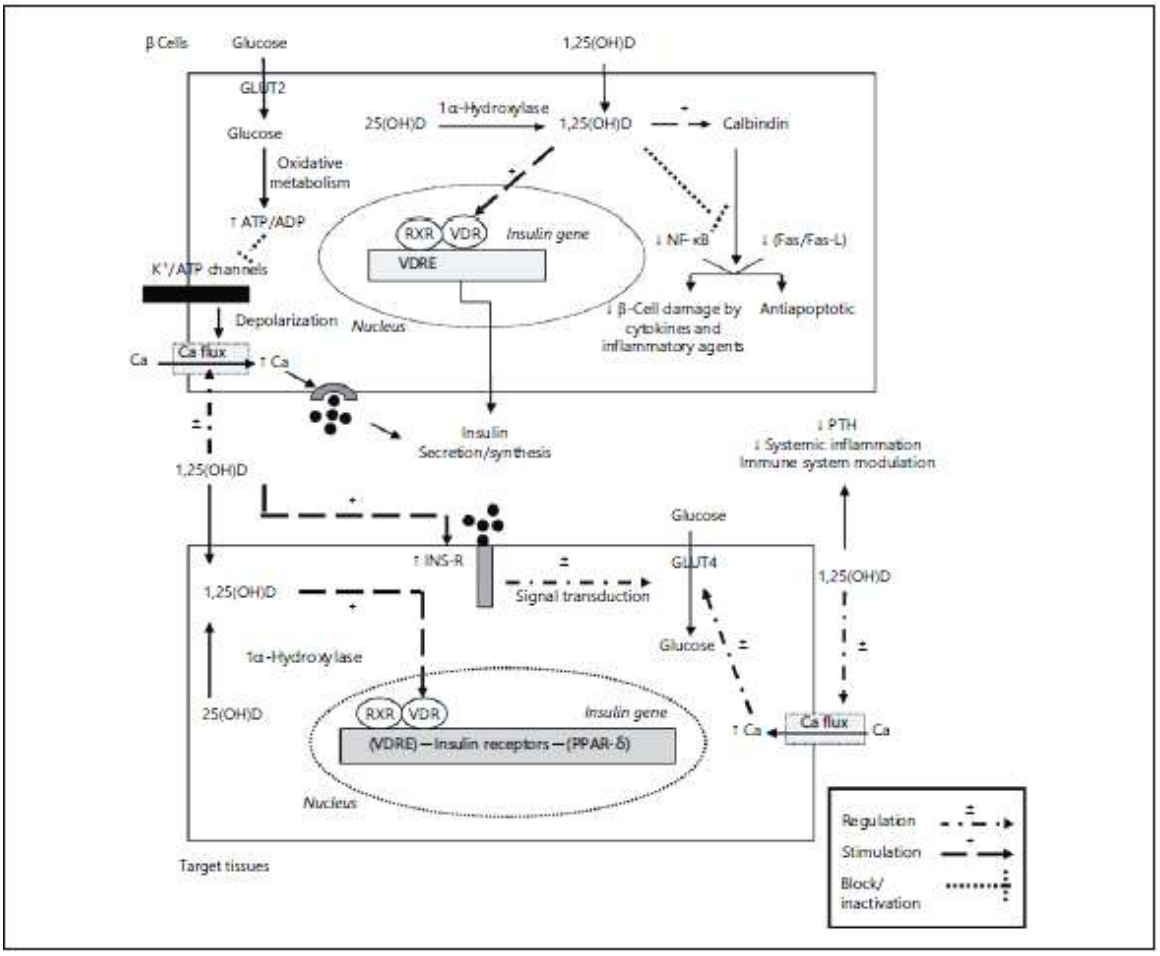
1.10.2 Vitamin D and Carbohydrate Metabolism

A possible link between hypovitaminosis D and abnormal glucose metabolism was first raised when a seasonal variation in plasma glucose and insulin was observed in normal individuals [230]. Interestingly the first evidence for a relationship between VitD and glucose homeostasis was reported when lower serum $1,25(OH)_2$VitD levels were found in diabetic rats compared to the control animals, while insulin treatment led to restoration of $1,25(OH)_2$VitD to a normal level [231]. Later a low VitD status has been noticed in patients with type 2 diabetes mellitus (T2DM) compared to healthy controls [232].

Impaired pancreatic β cell function and insulin resistance are usually the underlying circumstances that lead to T2DM development. Nowadays there is evidence that VitD may influence both of these mechanisms based on the discovery of VDRs and the expression of 1α-hydroxylase enzymes in the pancreatic β cell along with the existence of a VitD response element in the human insulin gene promoter [233, 234]. In particular, VitD is reported to affect glucose homeostasis in both direct and indirect manners. Direct effects may be mediated by the binding of the active form $1,25(OH)_2$VitD to the VDR, which is expressed in β cells [235], and the VitD response element (VDRE) in the human insulin gene promoter [234], that lead to the transcriptional activation of the human insulin gene and regulate insulin synthesis and secretion [236]. Alternatively, the activation of VitD may occur within the β cell by the 25(OH) D-1a-hydroxylase (CYP27B1), which is expressed in β cells [233]. Insulin secretion may also be indirectly influenced by VitD, which regulates calcium flux through the cell membrane and plays a role in the synthesis and regulation of calbindin, a vitamin D-dependent Ca-binding protein in pancreatic β cells [237]. Another indirect effect of VitD is β cells protection from fatal immune attacks or programmed cell death. In particular, $1,25(OH)_2$VitD may counteract apoptotic pathways and the inflammatory effect induced by cytokines through inactivation of NF-κB antiapoptotic protein and suppression of Fas receptor expression [238, 239]. Furthermore researchers proposed that low VitD levels may play a role in enhancing insulin resistance at target tissues [240]. Elevated PTH found in VitD deficiency may affect both insulin sensitivity and secretion by regulating glucose uptake and inhibiting insulin transport

signaling in the target tissues, primarily by increasing intracellular calcium concentration [241]. Additionally, VitD adequate levels may counteract chronic inflammation which worsens insulin resistance through several mechanisms, including modulation of the release of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), regulation of the activity of NF-κB, regulation of genes encoding pro-inflammatory cytokines and downregulation of Toll-like receptor (TLR)2 and TLR4 expression [242-244]. Figure 14 illustrates the putative role of VitD on insulin synthesis, release and β-cell function [109].

Figure 14. Vitamin D and its role in glucose homoeostasis and pancreatic β -cell function.



Numerous observational studies have shown lower 25(OH)VitD levels in patients with T2DM compared with the general population, as well as an inverse association between 25(OH)VitD levels and fasting plasma glucose, impaired glucose tolerance, and glycated haemoglobin (HbA1c) levels [100, 245-250]. Prospective studies assessing the

association between 25(OH)VitD and diabetes risk are very limited. One study conducted over a 10-year period found a negative association between basal 25(OH)VitD serum levels and the risk of future hyperglycaemia and insulin resistance [251]. Also a recent systematic review and MA showed that for each 10-nmol/l increase in 25(OH)VitD levels there was a 4% lower risk of T2DM development [252]. On the contrary, another MA including data from four RCTs showed no effects of VitD supplementation on risk of incident T2DM [253]. Moreover, despite the overall view from observational studies in favour of a negative impact of hypovitaminosis D on glucose homeostasis and β-cell function, trials in humans on potential effects of VitD supplementation on indices of glucose metabolism have not demonstrated a clear beneficial effect either. Recent MAs on results from RCTs showed no significant improvement on carbohydrate metabolism parameters (including HbA1c levels) in those treated with VitD compared with placebo [147, 254, 255]. However, when restricting study participants to those with diabetes or impaired glucose tolerance, one of the MA showed a small but significant improvement in fasting glucose levels and a small improvement in insulin resistance [254]. Also the Calcium and Vitamin D for Diabetes Mellitus (CaDDM) RCT found that in adults at risk of T2DM, short-term supplementation with cholecalciferol improved β cell function and had a marginal effect on attenuating the rise in HbA_1c [256]. In conclusion, there is a discrepancy between findings from observational studies and RCTs on effects of VitD supplementation on T2DM risk development. However, there are several limitations in the interpretation of interventional studies results, including heterogeneity of techniques, study designs, subject characteristics and the therapeutic regimen used. In addition, the number of trials is relatively small, the majority of subjects included in the trials did not have low 25(OH)VitD levels and most of the available studies used indirect methods to measure the effect of VitD on insulin sensitivity. Hence, the findings from observational studies on an increased risk among those with VitD insufficiency/deficiency cannot be considered as being refuted by data from RCTs.

Based on the available observational studies, little is known about the association between insulin sensitivity/resistance, glucose intolerance and VitD deficiency in **children and adolescents**. Some studies have failed to detect any association between VitD and insulin resistance in obese children/adolescents [59, 192, 228, 257-263]. Other studies

however report an inverse association between hypovitaminosis D and measures of insulin resistance and glycaemia in the same ages [192, 193, 220, 264-271]. Reis et al in a cross-sectional analysis of 3,577 fasting, non-pregnant US adolescents aged 12–19 years without diagnosed diabetes, who participated in the 2001–2004 NHANES found a doubling of the odds ratio for elevated fasting glucose among adolescents in the lowest quartile of serum VitD compared with the highest quartile, independent of adiposity [191]. Similarly Kumar et al. in a representative sample of 1- to 21-year-old children and adolescents with non-alcoholic fatty liver disease (n=6,275), found that those with VitD deficiency or insufficiency showed a 2.5 times higher risk of high blood glucose, which may precede T2DM, and four times greater risk of developing metabolic syndrome [78]. In line, a more recent cross-sectional descriptive study involving 2,314 adolescents aged 12–18 years extracted from the Korean NHANES 2010-2014 reported that VitD deficiency was associated with a 2.07-fold higher risk of elevated fasting blood glucose (≥100 mg/dL) [272].

There remains however a lack of prospective RCT assessing the effect of VitD supplementation on glucose metabolism indices in the youth, while the available results are still contradictory. More analytically, Belenchia et al. conducted an RCT in obese adolescent (n=35, aged: 14.1±2.8 y, BMI: 39.8±6.1, 25(OH)VitD: 19.6±7.1 ng/mL) and randomly assigned them to receive either VitD (4000 IU/d) or placebo [273]. After 6 mo, adolescents who received VitD had a significant reduction in fasting plasma insulin and significant improvements in 2 widely used surrogate markers of insulin resistance and sensitivity (Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative insulin sensitivity check index (QUICKI), respectively), as well as a third more recently proposed marker, the ratio of leptin to adiponectin. However no significant differences were observed between-groups in fasting glucose levels and glycosylated hemoglobin [273]. In a study designed by Ashraf and cols. obese adolescents (average age 14.9±1.8 years) with VitD deficiency (<20 ng/ml) were given 50,000 IU of VitD per week for 8 weeks to investigate effects on glucose parameters. While HOMA-IR and whole body sensitivity index (WBISI) did not improve on the follow up oral glucose tolerance test (OGTT), fasting glucose showed statistical improvement in cases when compared to controls [269]. Moreover, other researchers failed to find any improvement in every glucose metabolism parameter tested after VitD supplementation. In particular, Javed et al.

reported no correlation between 25(OH)VitD concentrations and insulin action (Si) or pancreatic β-cell function as assessed by the disposition index (DI). After randomly assigning obese adolescents with relatively good VitD status to receive either 400 IU/d (n=25) or 2000 IU/d (n=26) of VitD found no change in Si or DI in either of the treatment groups [274]. Similarly, in a dose titration study (400 IU to 4000 IU) of 323 normal to obese early pubertal children (age=9-13 years) VitD supplementation had no significant positive impact on glucose and insulin parameters over a 12 week period [275]. Also, a more recent randomized trial with African American obese children (n=29; 22 female, 13–17 y, with 25(OH)VitD<20 ng/ml) to receive either 50,000 IU VitD/week or a placebo for 12 weeks, showed no significant changes in insulin secretion and sensitivity in either group [276]. Similarly, another recent study in 20 obese, insulin resistant and VitD deficient adolescents demonstrated that using a one time high dose of $VitD_2$ (300,000 IU) "stoss therapy" did not have any beneficial effect on insulin sensitivity (WBISI) and secretory indices (insulinogenic index {IGI}) [277]. These reports, in addition to other equivocal studies across diverse populations varying in age, race, VitD status, degree of glucose tolerance, weight status, and doses used may explain the controversial results of VitD replacement on glycemic control. However, the interesting finding in most of those studies was the negative association of the baseline 25(OH)VitD levels with insulin and insulin resistant parameters. Whether obese adolescents with VitD deficiency and impaired glucose metabolism would respond differently to VitD supplementation remains unclear and warrants further studies. More specially designed studies with homogeneous populations and larger numbers may lead to more clear results

1.10.3 Vitamin D and Adipokines

In recent years, visceral adipose tissue has drawn attention due to the synthesis and release of a number of adipokines from adipocytes some of which are proinflammatory and atherogenic and others, which have anti-inflammatory, protective effects [278]. In MetS patients, it has been found that their increased visceral adipose tissue disturbs adipokine secretion and leads to a low-grade chronic inflammatory state mediated by the infiltration of macrophages into adipose tissue [278].

Leptin and adiponectin are 2 adipocyte-derived cytokines with opposite effects on inflammation and insulin resistance. Subjects with obesity present with hyperleptinemia, due to leptin resistance, which in turn predisposes to insulin resistance [279]. More specifically, elevated leptin levels for a long time in persons with obesity may result in decreased responsiveness of pancreatic β–cell receptors, leading to increased insulin secretion. The resulting hyperinsulinemia may in turn exacerbate obesity and further increase leptin levels, resulting in a diabetogenic positive vicious cycle [280]. Moreover, high leptin levels observed in obesity may contribute to hypertension development [281].

In contrast, adiponectin has anti-atherogenic, antidiabetic, and anti-inflammatory properties that are directly involved in obesity-related disorders. Adiponectin downregulates the expression and release of a number of proinflammatory immune mediators [282] and has strong insulin-sensitizing properties [283]. Adiponectin levels in plasma are inversely associated with visceral fat [284] and are, therefore, lower in more obese individuals [285]. Indeed, clinical studies implicate hypoadiponectinemia in the pathogenesis of T2DM [286], coronary artery disease (CAD) [287], and hypertension [288].

Recently, the leptin/adiponectin ratio has been proposed as a marker of insulin resistance. Although leptin or adiponectin were separately associated with the risk of MetS, T2DM, and CAD, the association of the L/A ratio with T2DM development risk was stronger than with leptin or adiponectin alone [289].

Visfatin is a newly discovered adipokine, the concentration of which is found increased in obesity [290]. It is mainly expressed in visceral adipose tissue and is an endocrine, autocrine and paracrine peptide with many functions including enhancement of cell proliferation, biosynthesis of nicotinamide mono- and dinucleotide and has also hypoglycaemic effects [290].

Interestingly recent data indicated that 25(OH)VitD may interfere with the regulation of the adipoinsular axis too. However more analytically, a recent systematic review and meta-analysis regarding **adult** studies concluded that the inverse association between 25(OH)VitD and serum leptin levels found in most observational studies, was not confirmed in interventional studies [291]. In particular, 25(OH)VitD concentration correlated negatively with leptin levels in pre-diabetic subjects, [292] in type 2 diabetic patients [293], in obese women [294], in women with breast cancer [295] and in chronic

kidney disease patients [296]. This association is possibly explained by accumulation of the fat-soluble VitD in adipocytes that subsequently prompts leptin secretion [297]. Another hypothesis is that low 1,25$(OH)_2$VitD could reduce calcium serum levels and induce secondary hyperparathyroidism, which in turn stimulates lipogenesis and leads to increased leptin secretion by adipocytes [298]. Interventional studies showed conflicting results. Some studies showed that VitD supplementation led to increases in serum leptin levels [297, 299] and one showed that it decreased leptin concentration [300]. A meta-analysis of randomized controlled trials concluded that VitD supplementation did not affect leptin levels [301] and so did a secondary analysis of the D-Health trial [302].

Regarding the association of 25(OH)VitD with adiponectin levels, previous studies have shown equivocal findings. Some showed a positive correlation between 25(OH)VitD and adiponectin [293, 303] while another did not confirm this relation [304]. Interventional studies generally provided little evidence of an effect of VitD supplementation on adiponectin levels [301, 302, 305], while one showed an increase in adiponectin levels [300] and another only a marginal rise [306]. The inconsistencies between these different studies may be due to the fact that adipokine levels are affected by genetic factors, degree of tissue adiposity, maturity of adipocytes, age at diagnosis and severity of associated conditions [307]. Overall, these results should be clarified by larger studies.

Only a few studies have investigated the relationship between 25(OH)VitD levels and the leptin to adiponectin ratio. Observational studies reported an inverse relation of 25(OH)VitD and leptin to adiponectin ratio in type 2 diabetic patients [293] and in women with polycystic ovary syndrome [308]. And interventional studies showed that VitD supplementation reduced the leptin to adiponectin ratio [273, 306, 309].

Similarly, there are only few data regarding the possible 25(OH)VitD and visfatin association, with quite opposite results. Two studies found a negative relationship between 25(OH)VitD and visfatin levels [292, 310], while another did not find any correlation between them [311].

Results are also inadequate from in **children and adolescents**. Some of them in children/adolescents with obesity have found a positive correlation between 25(OH)VitD levels and adiponectin compared with controls [312, 313]. And others showed an inverse correlation of 25(OH)VitD levels with the leptin/adiponectin ratio [309]. An RCT showed

that serum leptin/adiponectin ratio was significantly lower in the VitD supplemented group compared with the placebo [273].

However, there are no reports to our knowledge of an association between 25(OH)VitD and leptin levels alone in children and adolescents. We also found no relevant data in the literature regarding the alleged relationship between 25(OH)VitD and visfatin in this age group.

Despite the consensus achieved with regard to the need to treat VitD insufficiency in obese patients, there is no common point of view on the dosage and duration of cholecalciferol administration appropriate for VitD supplementation. Currently available data on the treatment of VitD insufficiency in obese children and adolescents are contradictory; however, in the overwhelming majority of cases these data allow not only an increase in calcifediol levels but also a positive effect on the secretion of adipokines as well as on carbohydrate and lipid metabolism.

1.10.4 Vitamin D and Lipids

As mentioned earlier, VitD is structurally related to cholesterol since photosynthesis of cholecalciferol is achieved by irradiation of cutaneous 7-dehydrocholesterol, which is a precursor of cholesterol. Interestingly, epidemiologic studies have shown a seasonal variation in plasma lipid levels and lipoprotein composition, whereby higher total cholesterol and LDL-C levels are observed in the winter and reach their nadir during the summer. These cyclical changes remain pronounced despite adjusting for dietary or physical activity changes [314]. Data also suggested an inverse association between circulating levels of 25(OH)VitD and an atherogenic lipid profile [83]. As is well known, blood cholesterol levels are strong predictors of cardiovascular risk, with elevated LDL-C and decreased high density lipoprotein cholesterol (HDL-C) cholesterol levels being independent risk factors for adverse cardiovascular events. In addition, increased concentrations of small, dense LDL particles (sdLDL) are regarded as highly atherogenic [315].

Cross-sectional analyses almost unequivocally confirm an association between optimal VitD status and a favorable lipid profile, but the results are not consistent. A large

cross-sectional analysis (n=108,711) showed that the “optimal” 25(OH)VitD group relative to the “deficient” group displayed lower total cholesterol (TCHOL), lower LDL-C, higher HDL-C and lower triglycerides [316]. A former also large study (n=15,088), based on NHANES III, found too that mean 25(OH)VitD levels were lower in subjects with hypertriglyceridemia and hypercholesterolemia [100]. The association between serum 25(OH)VitD and lipids is also reported from other studies, with albeit inconsistent results. Jorde et al. (n=10,105) found highly significant positive associations between serum 25(OH)VitD and serum TCHOL, HDL-C and LDL-C, and significant negative associations between serum 25(OH)VitD and both LDL-C/HDL-C ratio and triglycerides [317]. The same authors when reviewed 22 other cross-sectional studies showed that serum 25(OH)VitD was positively associated with HDL-C, resulting in a favorable LDL-C (or total cholesterol) to HDL-C ratio, and negatively associated with triglycerides [318]. Thus, in other studies, Hypponen et al. (n= 6,810) [319] and Lee et al. (n=3,069) [320] found a negative association between serum 25(OH)VitD and triglycerides, but the association between serum 25(OH)VitD and HDL-C was not significant after adjustment for confounders. Conversely, other smaller studies showed no significant association between serum 25(OH)VitD and triglycerides [321, 322], a negative relation with serum LDL-C [319] or both TCHOL and LDL-C [322], or no significant association with lipids at all [251]. Notably, a longitudinal analysis (n=6,260) showed that increasing 25(OH)VitD levels from the deficient to sufficient range had a neutral effect on the lipid profile: increased total and HDL cholesterol, but did not change LDL-C and triglycerides levels [316]. Those researchers concluded that a higher level of 25(OH)VitD may simply be a passive marker of better cardiovascular health. Other prospective studies have shown an inverse association between VitD status and triglycerides [323, 324]. Recently, using the filaggrin genotype as an instrumental variable to estimate the causal effect of vitamin D on serum lipids (a mendelian randomization approach), Skaaby et al. showed a 23.8% higher HDL-C level and a 30.5% lower serum level of triglycerides per doubling of VitD [325]. But again, this is no proof of causality, which has to come from intervention studies.

Emerging CVD risk factors usually seen in MetS patients are increased as is the atherogenic small dense LDL-C (sdLDL-C) [326, 327], and the elevated lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activity [328], both of which have been associated with increased CVD risk [329, 330]. Some data indicate a possible relationship between

25(OH)VitD serum levels and these emerging CVD risk factors, though not consistently [331, 332], while an interventional study of a 12 month supplementation in elderly people gave little evidence of any effect on leptin and adipokine levels except for interleukin-6 (IL-6) [302]. A small recent study in patients with obstructive sleep apnoea and increased body mass index (BMI=30.4 kg/m^2) who received 4000 IU/day VitD or placebo per os for 15 weeks showed significant decreases in both LDL-C and $LpPLA_2$ [332]. However, a lot more data are needed to get unequivocal results.

The mechanisms by which VitD may affect lipid metabolism are largely unknown. For triglycerides, one hypothesis could be a vitamin-D-induced increase in calcium absorption, leading to reduced formation of calcium-fatty soaps in the gut and thereby increased absorption of fat [333]. At least in subjects with low serum 25(OH)VitD levels, VitD supplementation leads to increased $1,25(OH)_2D$ formation, which again may stimulate lipogenesis and inhibit lipolysis [334]. In particular, the suppression of parathyroid hormone (PTH) secretion by VitD supplementation may be related to the possible effect on lipids, since PTH can reduce lipolysis [334]. Also a recent study has shown that betatrophin, which is primarily involved in lipid metabolism through an inhibition of lipoprotein lipase, was negatively correlated with VitD and positively with TCHOL, triglycerides and LDL-C levels [335]. Alternatively, VitD may increase calcium level, thereby reducing hepatic triglyceride formation and secretion [336]. Finally, VitD's effect on lipids may be indirectly mediated by its' effect on insulin secretion and sensitivity [337]. Since the higher the triglyceride levels, the smaller the LDL size [327], VitD deficiency could theoretically contribute to increased sdLDL-C concentrations in an indirect manner, i.e. by increasing triglycerides levels.

On the other hand, the interventional studies gave divergent results, with some showing a positive and some a negative effect of VitD supplementation on lipid profile. Regarding serum triglyceride concentrations, which appear to be strongly associated with serum 25(OH)VitD, Jorde et al in a study of overweight and obese subjects found no effect on serum triglycerides after one year's supplementation of 20,000–40,000 IU VitD/week [317]. On the contrary Zittermann et al. in their study with 200 overweight subjects found a significant 13% decrease in the serum triglycerides on a daily dose of 3320 IU for 1 year [139]. Another RCT randomized 151 VitD deficient adults with elevated risk for CVD to receive either 50,000 IU of VitD weekly for 8 weeks or placebo. The results showed that

correcting VitD deficiency in the short-term did not improve the lipid profile (TCHOL, LDL-C, HDL-C, triglycerides and small LDL particle number), while raised serum calcium and decreased serum PTH levels, leading to an increase in LDL-C [338]. A subgroup analysis of 1,191 women participating in the Women's Health Initiative (WHI) study found no effect of VitD supplementation on lipids over a 5-year period [339], while at the same time highlights many pitfalls common to prior studies that preclude reaching a definitive conclusion. MAs of RCT's examining VitD's supplementation effect on lipids showed again contradictory results. One MA of 12 RCTs (n=1,346) showed that VitD supplementation provided a statistically significant increase in LDL-C, a tendency towards an increase in TCHOL, and non-significant reductions in HDL-C and triglycerides. The effect of VitD supplementation on serum LDL-C levels seemed to be enhanced in obese subjects and in studies with relatively shorter durations, while studies with longer durations only showed a significant reduction in HDL-C levels [340]. Another MA reported that out of 19 relevant RCTs identified, benefits of VitD supplementation on lipid profile parameters were observed only in one, while in all the others either no effects or adverse outcomes were detected [341]. On the contrary, a more recent MA reviewing 38 newer RCTs reported that in all those studies, VitD supplementation significantly decreased TCHOL, LDL-C, triglycerides and increased HDL-C together with lowering BP, PTH and serum CRP [10]. Those findings led to conclusions that VitD supplementation may act to protect against CVD through improving risk factors including high blood pressure, elevated PTH, dyslipidemia, and inflammation. Attempts to explain these inconsistent findings have been made and the most popular suggest that high serum VitD concentrations may not be the cause of good health but its outcome instead, as healthy people are more likely to stay outdoors longer and have better eating habits and healthier lifestyles in general. Thus VitD may have no real effect on lipid metabolism per se, since better 25(OH)VitD serum levels may just co-exist with a favorable lipid profile in these persons. Another suggestion is based on the fact that VitD is fat-soluble, and may be sequestrated in adipose tissue, which effectively traps it and is lowering circulating levels of 25(OH)VitD. This phenomenon may explain the associations between 25(OH)VitD levels and the adverse lipid profile in obese subjects and the failure of 25(OH)VitD repletion to improve the lipid profile. Another possibility is that the lipid profile influences vitamin D levels and not the converse; dyslipidemia itself may lower VitD levels [316].

Finally, VitD absorbed through the gut may have different effects on lipid metabolism from VitD synthesized in the skin. Intestinal epithelium possesses 25-hydroxylase and 1-β-hydroxylase activity. Therefore, oral vitamin D can be locally converted to the active 1,25-dihydroxyvitamin D metabolite and induce autocrine signals within enterocytes [342].

VitD status has also been associated with dyslipidemia during **childhood and adolescence**, but the literature again gives discrepant results. Some studies advocate an association between VitD status and lipids [229, 258, 343, 344], while others have not found such relationship [269, 345]. Serum 25(OH)VitD concentrations have been positively correlated with HDL-C in many [59, 78, 228, 258, 268, 343, 346, 347], but not in all [220, 269, 345] cross-sectional studies. A prospective cohort study also reported an association of high 25(OH)VitD levels in childhood with high HDL-C levels in adolescence [268]. The exact mechanism of the positive association between 25(OH)VitD levels and HDL-C is unclear. However it has been proposed that VitD may influence HDL-C via several mechanisms including effect on apolipoprotein A-1 production [348], a major component in the lipoprotein of HDL-C [349], or an effect on cholesterol turnover or its transport. Moreover, other studies reported that 25(OH)VitD levels were either inversely [192, 262, 350] or not at all [258, 345, 351] associated with TCHOL, positively [269] or negatively [262, 350] associated with LDL-C, and even after adjusting for potential confounders such as BMI negatively associated with triglyceride levels [262, 352]. On the other hand, some studies have demonstrated no association between VitD and dyslipidemia at all [219, 220, 260, 267, 347]. These inconsistencies are likely related to differences in study subjects with regard to age, ethnicity/race, pubertal stage, geographic location, dietary habits, physical activity, and assays for measuring 25(OH)VitD. Notably, however, researchers highlight that the results on VitD and blood lipids may be confused with the relationship between this vitamin and obesity [353], suggesting that the most important determinant of low 25(OH)VitD levels is the increased body size. Thus, cardiometabolic risk factors in overweight and obese children may be just a reflection of insulin resistance due to the impact of overall adiposity, independent of 25(OH)VitD [192]. However, a retrospective study showed that in overweight or obese subjects VitD deficiency was significantly associated with higher mean lipid levels, compared with those with adequate levels. At the same time, in the underweight and normal weight subjects no

differences in their lipid levels were observed between the adequate and VitD deficient groups [354].

Results from RCTs on effect of VitD supplementation on lipids in children/adolescents are sparse and inconsistent and similar to adults. In one study with obese adolescents (n=58, 12-18 years), 12 weeks of supplementation with VitD 2000 IU/day did not have an effect on the their lipid profile [355]. In contrast, an increase in HDL-C was noted in healthy 10–14 year old Iranian children who received 1000 IU VitD/day for one month [356]. In line, another study in obese African American children aged 13–17 y, with 25(OH)VitD <20 ng/ml, who were randomized to receive either 50,000 IU VitD/week or placebo for 12 weeks, an improvement of serum lipids as a whole was found in the supplementation group, but only changes in HDL-C were correlated with post-supplementation serum VitD status [276]. Another interventional study evaluated the effect of VitD supplementation (300,000 IU) versus placebo/week for 12 weeks, on metabolic syndrome components of 50 participants, aged 10 to16 years. In the baseline no significant difference was observed in the characteristics of the two groups**.** After the trial the serum triglyceride levels were found significantly decreased in the treatment group, whereas TCHOL, LDL-C, and HDL-C did not significantly differ from those of the control group [228]. Overall, the controversy is still unresolved and more clearly focused research is needed to reach safe conclusions.

1.10.5 Vitamin D and Obesity

According to the World Health Organization (WHO) overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. For adults, WHO defines overweight as having a BMI≥25 kg/m^2 and obesity≥30 kg/m^2 and has estimated that in 2016 39% of adults worldwide being overweight and 13% obese. Obesity is a recognized risk factor associated with mortality, probably due to the link between obesity and the risk of developing diabetes, hypertension, atherogenic dyslipidaemia and CVD [357]. It has been speculated that the link between obesity and CVD may be via insulin resistance [358]. More specifically, abdominal obesity, with visceral as opposed to subcutaneous fat accumulation, appears to be critical in the development of insulin

resistance [359], and is currently considered as an active endocrine organ. Intra-abdominal obesity, which is a classifying characteristic of the metabolic syndrome, promotes insulin resistance, probably by secreting metabolically active substances (adipokines) and making available an increased quantity of free fatty acids [360].

Obesity has been associated with low levels of 25(OH)VitD in a large number of observational studies [361]. The largest MA (3,867 obese individuals and 9,342 healthy subjects), identified a higher prevalence of VitD in the obese group [362]. However the exact nature of this association has yet to be determined. Different mechanisms have been proposed to explain the low VitD status in obese individuals. At first, following endogenous synthesis or dietary intake, VitD is readily stored in adipose tissue, since it is a fat-soluble molecule [363]. As the body pool of fat is larger in obese compared with non-obese individuals, VitD may be diluted or sequestered in the larger body pool of fat resulting in lower plasma levels [364]. Another study showed that following endogenous synthesis, the release of VitD from the skin into the circulation is decreased in obesity [365]. Finally, it has been suggested that obese subjects have decreased VitD skin synthesis due to less sunlight exposure, attributed to clothing habits and/or less involvement in outdoor activities (reduced mobility) [366].

In contrast, a number of other studies have suggested that low 25(OH)VitD levels may predispose to obesity. The VDR is expressed in the adipose tissue which has the ability to locally synthesize $1,25(OH)_2$VitD [367]. Studies have also suggested that VitD may regulate adipose tissue mass, differentiation and metabolism in ways that might contribute to obesity [368]. Furthermore, secondary hyperparathyroidism is a well-known consequence of VitD insufficiency, and promotes calcium influx into the adipocytes. In adipocytes, intracellular calcium may enhance lipogenesis, and PTH excess may thereby promote weight gain [369]. However, a recently published bi-directional Mendelian randomization analysis of multiple cohorts showed that a higher BMI leads to lower plasma 25(OH)VitD levels whereas low 25(OH)VitD levels did not appear to lead to a high BMI [353].

MAs reporting effects of interventional trials, showed overall no effects of VitD supplementation on changes in body weight, fat mass (FM), percentage FM (%FM), or lean body mass [216, 370-372]. Only one MA by Chandler et al. showed a beneficial effect on weight loss of supplementation with calcium and VitD compared to placebo [372]. Also

a very recent MA reported that cholecalciferol supplementation deceased BMI and waist circumference, but did not statistically affect weight loss [373]. In summary, although an inverse association between body weight and 25(OH)VitD levels seems to be well documented, it is still under dispute whether low 25(OH)VitD levels has a causal effect to weight gain. Also the up to date evidence is not in support of a beneficial effect of VitD supplementation on weight loss.

The diagnosis and definition of obesity in **children/adolescents** is challenging. Obesity diagnoses in these age groups are usually determined by calculation of BMI values that are plotted on age-and sex-specific growth charts [374]. According to the Centers for Disease Control (CDC) overweight is most commonly defined at BMI 85-95 percentile and obesity as greater than or equal to the 95th percentile [375]. The WHO overweight definition is the 85^{th}–97^{th} percentile and obesity greater than or equal to the 97^{th} percentile [376]. According to recent WHO data the prevalence of overweight and obesity among children and adolescents aged 5-19 has risen dramatically from just about 4% in 1975 to over 18% in 2016, with over 340 million subjects in this age group being overweight or obese. Together with the high prevalence of obesity and metabolic syndrome in pediatric patients, both children and adolescents in the majority of countries are diagnosed with VitD insufficiency/deficiency. The prevalence of VitD insufficiency in overweight and obese children and adolescents has been explored extensively, but no MA has been carried out. Recent studies worldwide report extremely high incidences of suboptimal VitD status in obese youth: 96.0% in Germany, 78.4% in the United States, and up to 92.0% in the Russian Federation [377]. Obesity is a known risk factor for VitD deficiency [5, 73, 365]. Also 25(OH)VitD concentrations are inversely associated with the severity of obesity [264]. Interestingly one “superfluous” BMI unit is known to induce a 1.15% reduction in the 25(OH)VitD concentration [353]. In particular, an analysis conducted in 58 obese adolescents demonstrated that a 1% increase in fat weight was associated with a 1.15±0.55 nmol/L reduction in serum 25(OH)VitD [378]. However, although some evidence suggests that weight loss may affect VitD serum concentration, the exact nature of this association has not been studied extensively so far in children and adolescents. Preliminary data from an ongoing study on long-term effects of VitD supplementation in VitD deficient obese children, participating in an integrated weight-loss program show that a 12-month

intervention led to a decrease in absolute BMI value, BMI percentile and fat mass content and may prevent bone loss in this population [379]. But still much research is needed in this field regarding children and adolescents to reach safe conclusions.

1.10.6 Vitamin D and the Metabolic Syndrome

Metabolic syndrome (MetS) is a constellation of risk factors for CVD. Its component characteristics are central obesity, disturbed glucose homeostasis and insulin resistance, hypertension and atherogenic dyslipidaemia. Various expert groups have provided definitions of MetS, as summarized in Table 10.

Table 10: Thresholds defining the MetS issued by individual organizations in adults

	WHO 1998 (Alberti 1998)	EGIR (Balkau 1999)	NCEP/ATP III 2001 (NCEP 2002)	AACE (2003) (Einhorn 2003)	IDF consensus 2005 (Zimmet 2005)	IDF consensus (10 to < 16 yr) (Zimmet 2007)
Definition	IGT, IFG, T2DM or lowered insulin sensitivity Plus 2 of the following	Plasma insulin > 75th percentile Plus 2 of the following	3 of the following	IGT or IFG plus any of the following based on clinical judgement	See below	
Europoid waist circumference (cm)	W:H > 0.90 M W:H > 0.85 F or BMI > 30 kg/m^2	⩾ 94 M ⩾ 80 F	⩾ 102 M ⩾ 88 F	BMI ⩾ 25 kg/m^2	⩾ 94 M ⩾ 80 F or BMI > 30 kg/m^2 Plus 2 of the following	> 90th percentile Plus 2 of the following
Triglyceride [mg/dL (mmol/L)]	> 150 (1.7)	> 150 (1.7)	⩾ 150 (1.7)	> 150 (1.7)	> 150 (1.7)	⩾ 150 (1.7)
HDL [mg/dL (mmol/L)]	< 35 (0.91) M < 39 (1.01) F	< 39 (0.91)	< 40 (1.03) M < 50 (1.29) F	< 40 (1.03) M < 50 (1.29) F	< 40 (1.03) M < 50 (1.29) F	< 40 (1.03)
BP (mmHg)	⩾ 140/90	⩾ 140/90 or on treatment	⩾ 130/85	⩾ 130/85	SBP ⩾ 130 or DBP ⩾ 85 or on treatment	SBP ⩾ 130 and/or DBP ⩾ 85
Glucose [mg/dL (mmol/L)]	IGT, IFG or T2DM	IGT or IFG (but not diabetes)	⩾ 100 (5.6) (Grundy) or diabetes	IGT or IFG (but not diabetes)	⩾ 100 (5.6)	⩾ 100 (5.6) or known T2DM
Others	Microalbuminuria ACR > 30 mg/g			Other features of IR[1]		

[1]Includes polycystic ovary syndrome, family history or ethnic group susceptible to type 2 diabetes, sedentary lifestyle and advancing age. ACR: Albumin creatinine ration; BMI: Body mass index; DBP: Diastolic blood pressure; F: Female; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance; IR: Insulin resistance; SBP: Systolic blood pressure; M: Male; T2DM: Type 2 diabetes mellitus; W:H: Waist to hip ratio; WHO: World Health Organization; HDL: High density lipoprotein; IDF: International Diabetes Federation; EGIR: European Group for the Study of Insulin Resistance; NCEP: National Cholesterol Education Program; AACE: American Association of Clinical Endocrinologists; BP: Blood pressure; IR: Insulin resistance.

MetS is very common, affecting about 40% of Americans while the global prevalence can be estimated to be about one quarter of the world population. In other words, over a billion people in the world are now affected with MetS [380]. Interestingly, research implicates hypovitaminosis D in the causation and phenotype of MetS and

appears to be a risk factor for components of the syndrome and its outcome, although the exact mechanism is unclear and the benefits of VitD replacement still unknown. Moreover some researchers question the clinical value of identifying MetS in patients, since it depends on the thresholds of each of the contributing factors. However others argue that it has clinical value [92, 381], although it does not confer additional risk compared to the component risk factors. As MetS is based on related and modifiable CVD risk factors, its identification encourages a holistic approach to address CVD risk rather than focus on the individual aspects of the patients' condition. It is also arguably useful in a research setting when considering the role of possible risk factors, such as low VitD status.

Considerable research has been made on associations between VitD levels and the prevalence of MetS, reasoning for an inverse relationship between serum 25(OH)VitD and MetS. A MA of 28 studies (including 99745 participants-age range: 40.5-74.5 years) deduced that there was a considerable association between high 25(OH)VitD levels and reduced MetS prevalence [382]. Another MA that examined the relationship between serum 25(OH)VitD levels and MetS again in the general adult population, showed a generally linear, inverse relationship between 25(OH)VitD levels and MetS in the cross-sectional studies, but not in the longitudinal ones [383]. Also Song et al. found that compared with the highest quartile serum 25(OH)VitD level group (19.9-55.9 ng/mL), the OR for MetS in the lowest level group (4.2-9.7 ng/mL) was 2.44 (95%CI:1.32-4.48) [384]. Thus, it is clear from these observational surveys that a relationship may exist between 25(OH)VitD levels and MetS without implying a causative effect.

A number of prospective studies have also presented data supporting the suggested theory of an association between low serum 25(OH)VitD concentrations and increased risk of the development of the MetS. Gagnon et al studied 4164 adults (mean age 50 years; 58% women; 92% Europids) and found that the MetS risk was significantly higher in people with 25(OH)VitD in the lower first (<18 ng/mL) and second (18-23 ng/mL) quintiles [385]. Also Kayaniyil et al found a decreased risk of the MetS at follow-up per standard deviation increase in baseline 25(OH)VitD after multiple adjustments [386]. However a very recent mendelian randomization study (n=10 655 participants) found no evidence that genetically determined reduction in 25(OH)VitD conferred an increased risk of MetS and its metabolic traits [387].

Many questions remain that can only be answered by long term interventional studies. Yet no large RCT has been carried out with onset of MetS/diabetes as the primary outcome [92]. A small RCT included 126 individuals with MetS and VitD deficiency who were categorized as obese or nonobese, using a BMI cutoff of 28 kg/m2. At baseline, the obese group had significantly lower serum VitD, fasting plasma insulin and HOMA-IR. After the 1-year intervention (700 IU/day of vit D, or placebo), MetS risk factors did not improve in treated participants, despite the significant increase in serum VitD level in both groups [388]. On the contrary, a recently published study in 160 postmenopausal women aged 50-65 years with VitD deficiency found that isolated supplementation with 1000 IU VitD for 9 months was associated with a reduction in the MetS risk profile. Women undergoing VitD supplementation had a lower risk of MetS, hypertriglyceridemia, and hyperglycemia [389]. In conclusion to date it is still unknown whether VitD replacement may translate to substantial health benefits.

Currently, there are no consensus guidelines or diagnostic criteria for MetS in the **pediatric** population, with more than 40 definitions being proposed [390, 391]. Although the definitions have many similarities, there are important differences between them with respect to cut-off points for various parameters, as summarized in Table 11 [392-397].

Table 11. Thresholds defining the MetS issued by individual organizations in children/adolescents.

Variables	IDF definition age <10 years	IDF definition ages 10–16 years	Cook *et al*.
Defining criteria	Cannot be diagnosed in the age group	Central obesity plus at least 2 out of 4 criteria	≥3 criteria
Central obesity		WC ≥90th percentile or adult cut-off if lower	WC ≥90th percentile
Hypertension		SBP ≥130 mmHg or DBP ≥85 mmHg or treatment with anti-hypertensive medication	BP ≥90th percentile
Hypertriglyceridemia		TG ≥150 mg/dL	TG ≥110 mg/dL
Low HDL		HDL <40 mg/dL	HDL ≤40 mg/dL
Impaired glucose		FPG ≥100 mg/dL or known T2DM	FPG ≥110 mg/dL

Prevalence of MetS in children and adolescents is increasing, as the proportion of the population with obesity continues to rise [398]. Due to the multiple definitions used for MetS in children it is difficult to estimate its exact prevalence with numbers ranging from 0.2% to 38.9% [399]. In a systematic review of 85 studies in children, the median prevalence of MetS in whole populations was 3.3% (range 0–19.2%), in overweight

children was 11.9% (range 2.8–29.3%), and in obese populations was 29.2% (range 10–66%). For non-obese, non-overweight populations, the range was 0–1% [400], whereas the percentage of obese children and adolescents having at least one feature of the MetS is around 90% [394]. Of note, children with MetS have an increased risk of MetS as adults, and possibly an increased risk of T2DM and CVD [401], since MetS is defined by a constellation of physiological, biochemical, clinical, and metabolic factors that directly increase the risk of atherosclerosis and all-cause mortality [402]. Subsequently greater emphasis has been given on the continuum that represents metabolic dysfunction and insulin resistance, and research focus has recently shifted to investigations of the interlinked metabolic irregularities, which constitute the MetS [403].

In this spectrum several cross-sectional and prospective studies have shown an association between low VitD status and increased prevalence of the MetS and individual CVD risk factors [403]. Pacifico et al. examined 452 Caucasian children and adolescents (304 were overweight/obese) and found that serum 25(OH)VitD concentrations were significantly lower in patients with MetS compared with those without, and they decreased as the number, 1, 2, 3, ≥4 of MetS components increased [192]. Also Ganji et al. used data from 3 cycles of NHANES (2001–2002, 2003–2004, and 2005–2006) for 5,867 adolescents, aged 12–19 years, and found that the likelihood of having MetS was significantly higher in the lower first tertile of serum 25(OH)VitD than in the third [59]. More recently the Korean National Health and Nutrition Examination Survey (KHANES) 2008-2010 analyzed data from 2,880 children and adolescents, aged 10-18 years, and showed that the number of subjects with MetS was greater in the low 25(OH)VitD groups than in the high group [267]. Also the Beijing Child and Adolescent Metabolic Syndrome Study (BCAMS study) conducted in 559 Chinese subjects, aged 14-28 years, found that 25(OH)VitD serum levels were significantly lower in participants with MetS compared to their respective counterparts. Moreover participants in the lowest 25(OH)VitD tertile were 2.5 times more likely to exhibit MetS than were those in the highest tertile [404]. On the contrary, a cross-sectional study on Turkish high school students did not find a significant correlation between VitD levels and MetS [405]. However data from interventional trials in youth with MetS, examining the effect of VitD supplementation are very sparse. A study from Kelishadi et al. comprised of 50 participants with MetS, aged 10 to16 years, who were randomly assigned into two groups, one receiving orally VitD (300,000 IU) and the

other placebo for 12 weeks. After the trial, in the VitD group, the continuous value of MetS decreased significantly, both when compared with the baseline and with the placebo group [228]. Given the above findings the role of VitD in the pathogenesis, progression and possible prophylaxis in youth with MetS also needs further examination.

1.11 Vitamin D and Lipid lowering medication

A key element in the treatment and prevention of CVD is the drug treatment of dyslipidemia. Different types of medicines have been used for this reason, depending on the type and severity of dyslipidemia and the achievement of therapeutic goals. Common lipid-lowering medication includes statins as monotherapy or in combination with ezetimibe, fibrates, omega-3 fatty acids and nicotinic acid (which was withdrawn from the market due to side effects).

Statins are a class of lipid-lowering agents that inhibit the enzyme hydroxyl-methyl coenzyme A (HMG-CoA) reductase, a rate limiting enzyme in the synthesis of cholesterol [406]. They are very effective agents in both primary and secondary prevention of CVD [407] and are widely used for those indications. Statins are the drug of choice for elevated LDL-C and the cornerstone of dyslipidemia treatment, providing a safe and effective reduction of LDL-C by 18-85%. They also increase HDL-C by 5-15% and lower triglycerides by 7-30%. Apart from their lipid-lowering action, these agents exert some cholesterol-independent effects, also known as “pleiotropic effects”, such as improvement in endothelial function, stabilization of atherosclerotic plaque and inhibition of vascular inflammation and oxidative stress [408]. It has been proposed that some of these actions may be partly mediated through their effect on VitD metabolism [409, 410].

The idea that there may be a relationship between statins and 25(OH)VitD levels came about when some studies showed that statin use was associated with fewer hip fractures and improved hip bone mineral density [411]. Several statins, such as lovastatin [412], simvastatin [413], atorvastatin [414], and especially rosuvastatin [410, 415] appeared to increase 25(OH)VitD serum levels contrary to the initial concern that statins would impair the formation of steroids dependent on cholesterol synthetic pathway,

including VitD synthesis [416]. On the contrary, more recent studies found no effect of statin use on 25(OH)VitD concentration [417, 418] and so did a systematic review and MA [419]. Another MA was inconclusive, since across RCTs, treatment with statins was associated with a significant increase in serum VitD concentrations, but across studies of non-RCT design, statins treatment was associated with a decrease in VitD concentrations [420]. In particular simvastatin [421-423], atorvastatin [423, 424] and rosuvastatin [424] were not associated with changes in VitD levels.

Several potential mechanisms have been proposed to explain either the increase or the decrease in 25(OH)VitD concentrations after statin therapy reported by the different studies. For the first they suggest that since 25(OH)VitD is catabolised in liver and intestine by CYP3A4 [425], which also extensively metabolises statins (along with CYP3A5), the competition in this common catabolic pathway could be the cause for the increased 25(OH)VitD levels observed in patients under statin treatment. However, increases have also been seen with rosuvastatin which does not use CYP3A4. Another attractive mechanism put out is that when 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase is inhibited by statins, 7-hydrocholesterol levels (the common precursor of cholesterol and 25(OH)VitD) may increase, thereby providing an abundance of substrate for the synthesis of 25(OH)VitD by ultraviolet sun radiation of the skin [426]. On the other hand, others trying to explain the reductions in 25(OH)VitD levels have proposed LDL-C as the major carrier of VitD in the blood, the decrease of which from the statin use would affect the VitD transfer in this lipoprotein fraction [420].

Existing data lead to no definite conclusions (i.e. whether there is a statin-class effect and to what extent), since the studies differed in the populations used, the various statin types with different metabolisms, potencies and bioavailabilities, different doses, and with different baseline 25(OH)VitD levels and follow-up intervals. Moreover, existing studies to date have been limited by their sample size, research design and subject traits (gender, ethnicity, age, etc.) and were mostly underpowered to provide a comprehensive and reliable conclusion.

Ezetimibe selectively inhibits intestinal absorption of cholesterol and is mainly used as an adjunct to statin therapy. Ezetimibe reduces LDL-C by 18% and triglycerides by 8% and increases HDL-C by 1%. The cholesterol-lowering effects are observed when

ezetimibe is administered either as monotherapy or as a statin adjuvant. When added in parallel to statin treatment it provides additional reductions in total cholesterol, LDL-C and triglycerides by 14-45%, and is generally well tolerated.

Its effect on VitD metabolism has not been studied in humans. Animal experiments indicate that ezetimibe may be a moderate inhibitor of the intestinal 25(OH)VitD absorption [427]. The lipid lowering effect of ezetimibe is mediated through a specific inhibition of the Niemann-Pick C1 Like 1 (NPC1L1) cholesterol transporter, which was recently shown to be involved also in 25(OH)VitD intestinal absorption [427]. Moreover, in vitro experiments indicated that ezetimibe inhibited 25(OH)VitD uptake in human colon carcinoma (Caco-2) cells and human embryonic kidney (HEK) cells [427], but in vivo experiments in mice demonstrated a non-significant effect of uptake in proximal intestinal fragments [427]. Similarly, a previous study in rats, also found that the lowering of VitD absorption in the ezetimibe group to be non-significant [428]. Subsequently, it has been assumed that the involvement of NPC1L1 in 25(OH)VitD transportation in vivo may be moderate [427]. Notably, the contribution of intestinal absorption to serum VitD levels, when no supplements are taken, is relatively small since 80-90% of VitD derives from endogenous production in the skin [429]. In line, a recent study reported a negative but non-significant effect on bone mineral density in patients receiving ezetimibe for one year [430]. However no published research data regarding ezetimibe's possible effect on 25(OH)VitD levels in humans, alone or in combination with statins are available.

Fibrates are particularly effective drugs for reducing elevated triglyceride levels. The most commonly used fibrates in clinical practice are fenofibrate and gemfiprozil. The mechanism of action of fibrates involves an increase in the activity of hepatic lipase, which hydrolyzes triglycerides from very low-density lipoprotein (VLDL), thereby drastically reducing triglyceride levels. Their other actions include reducing the liver production of cholesterol and increasing its secretion in the bile. The increase in the concentration of HDL cholesterol observed in the fibrates is mediated by the activated nuclear peroxisome proliferator-activated receptor alpha (PPARa). Typically, fibrates in the usual dosage reduce serum triglycerides by 20-50% and increase HDL-C by 10-35%. LDL-C is usually reduced by 5-20%, but its levels may increase in patients with severe hypertriglyceridemia. Phenofibrate may lower LDL-C levels more effectively than gemfiprazil. Fibrates have

also been shown to improve the size of LDL-C by converting small, dense LDLs (sdLDLs) into larger, less atherogenic LDL molecules.

Nicotinic acid (or niacin) has been used in the treatment of dyslipidemia for 40 years. It is a particularly effective agent in the treatment of patients with multiple lipid disorders, especially with low HDL-C and elevated triglycerides. The action of niacin is due to a decrease in the production and secretion of VLDL cholesterol. It also reduces the release of free fatty acids from adipose tissue into the circulation. When administered at a dose of 1.5-4.5 g/day, niacin reduces LDL-C by 5-25% and triglycerides by 20-50%, while increasing HDL-C by 15-35%. In addition, it is one of the few hypolipidemic agents that also lower lipoprotein (a). Although niacin is a very effective drug for the overall improvement of lipid profile, nicotinic acid (*ER-NA*)/laropiprant (*LRPT*) (ER-NA/LRPT) has been withdrawn from the market due to a plethora of serious albeit nonfatal side effects [431], including skin flashing, gastrointestinal abnormality and multiple dose pharmacological regimen required for optimal results.

Omega-3 fatty acid ethyl esters: EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) **(EPA/DHA)** have a well-documented cardio-protective role, and are useful drugs for the treatment of hypertriglyceridemia, either alone or in combination with statins. The omega-3 fatty acids reduce the production of VLDLs, possibly because DHA and EPA are not efficiently metabolized by the enzymes involved in triglyceride synthesis. DHA and EPA also inhibit esterification of other fatty acids and increase peroxisomal beta-oxidation in the liver. Triglyceride levels are typically reduced by 20-45% and HDL-C moderately increases (5-10%), but less than with fibrates and niacin. LDL-C decreases in normolipidemic individuals, but is often found elevated in hypertriglyceridemic patients. This increase, however, is believed to reflect the conversion of LDL particle size into a larger, less atherogenic form.

To our knowledge, there are no published data concerning possible effects of fibrates, nicotinic acid or omega-3 fatty acids as monotherapy or in combination with other hypolipidemic drugs (as statins) on 25(OH)VitD serum levels. This is a novel field we tried to investigate with the present research, since a possible increase in 25(OH)VitD serum levels by lipid-lowering treatment could be clinically important.

1.12 Prevention and Treatment of Vitamin D deficiency/ insufficiency - Guidelines

Based on the current inconclusive and contradictory study results, VitD supplementation with doses beyond the nutritionally recommended cannot be proposed for the prevention of CVD events [184]. In this section we present the most recent guidelines based on results of a large number of studies that link ranges or thresholds of serum 25(OH)VitD levels with musculoskeletal or extra-skeletal outcomes [34].

The recommended doses, mentioned below, are usually labelled as recommended dietary allowance (RDA), reference nutritional intake (RNI) or 'safe intake' (when data are insufficient to define a RNI), but are all intended to cover the needs of 97.5% of the target population.

The mean recommended intake of VitD for adults is 200 IU per day (25th and 75th percentiles being 200 IU and 400 IU per day, respectively), whereas most recently updated guidelines recommend ≥600 IU per day for adults with minimal exposure to sunlight [34]. Other organizations which are in favor of maintaining serum 25(OH)VitD levels above 20 ng/ml (as shown in Fig 6) recommend an intake of 600 IU or 800 IU per day when exposure to sunlight is limited (Table 14) [34]. In particular, the Endocrine Society recommends a 30 ng/ml 25(OH)VitD target (Fig 6) and accordingly its guidelines recommend a higher intake (up to 2,000 IU per day or higher until the target level is reached) for a long list of individuals at increased risk of VitD deficiency, while at the same time are endorsing the IOM recommendations (Table 12) [432]. Using a full dose–response curve, it has been defined as oral dose of VitD needed to reach a set point in 97% of the target population the >600–800 IU per day [34], although this dose might not be suitable for individuals with severe obesity [433].

Table 12. Guidelines for intake of vitamin D in adults and elderly individuals

Authority and/or country (year)	Recommended intake of vitamin D (IU per day)			
	Age 20 years	Age 50 years	Age 65 years	Age >75 years
*New guidelines**				
IOM (2010)[20]	600	600	600	800
Australia–New Zealand (2013)[23]	600	600	600	800
DACH (2012)[21]	800	800	800	800
Nordic countries (2012)[20]	400	400	400	800
WHO–FAO (2003/2012)[84]	200	200	200	200
UK (SACN; 2016)[28]	400	400	400	400
Netherlands (2012)[22]	400	400	800	800
Belgium (2009)[74]	400	400	400	800
France (Société Française de Nutrition; 2012)[47]	200	200	400–600	400–600
Endocrine Society (2011)[69]	600–2,000	600–2,000	600–2,000	800–2,000
EFSA draft version (2016)[61]	600	600	600	600

This table shows the recommended intake of vitamin D in guidelines updated in the past 10 years (new guidelines). DACH, Deutschland (Germany), Austria and Confoederatio Helvetica (Switzerland); EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; IOM, Institute of Medicine; IU, international units; ND, not determined; SACN, Scientific Advisory Committee on Nutrition.

Infants should receive VitD supplementation from birth until the time in life that exposure to (limited) sunlight is safe and sufficient to maintain a normal VitD status [34]. The American Academy of Pediatrics (AAP) guidelines published in 2008 stated that "breastfed and partially breastfed infants should be supplemented with 400 IU/day of VitD beginning in the first few days of life", and that "supplementation should be continued unless the infant is weaned to at least 1 L/day or 1 qt/day of VitD-fortified formula or whole milk". It was also stated that "All non-breastfed infants, as well as older children who are ingesting less than 1L/day of VitD-fortified formula or milk, should receive a VitD supplement of 400 IU/day" [46]. The IOM proposed a recommended dietary allowance of 400 IU/day of VitD for healthy infants younger than 1 year and 600 IU/day for children from 1 to 18 years [26]. The Endocrine Society stated that 400–1,000 and 600–1,000 IU/day should be continuously provided to children and adolescents at risk during and after the first year of life (1–18 years), respectively. Obese children should receive at least two to three times more VitD to meet their needs [27]. An overview of current guidelines for intake of VitD, generated by several countries and organizations, for infants and children is shown in Table 13 [34].

However the exact duration of VitD supplementation has not been established. A recent expert position statement has recommended VitD supplementation in all children during the first 2 years of life, when growth velocity is particularly high. Similarly, in adolescence which is also a period of fast growth [26, 46]. In children over 2 years, supplementation should be tailored according to the sunlight exposure and the presence of risk factors for VitD deficiency [40].

In order to prevent VitD deficiency there are a few measures we can take. Basically, it is necessary to ameliorate the risk factors by means of increasing sunlight exposure, fortification of the habitual food supply and VitD supplementation.

Humans obtain most of their VitD requirement from sun exposure [5]. Therefore, it is reasonable to consider sensible sun exposure as a good source of VitD [5]. An adult in a bathing suit exposed to 1 minimal erythemal dose (slight pinkness to the skin 24 hours after exposure) is equivalent to taking approximately 20,000 IU (500 μg) of vitamin D2 orally [5]. Thus, exposure of arms and legs to 0.5 minimal erythemal dose is equivalent to ingesting approximately 3000 IU of vitamin D3 [5, 27]. Time of day during sun exposure, season, latitude, and degree of skin pigmentation dictate how much vitamin D3 is produced during sun exposure. Exposure of the arms and legs (abdomen and back when possible) to sunlight 2 to 3 times a week for approximately 25% to 50% of the time it would take to develop a mild sunburn (minimal erythemal dose) will cause the skin to produce enough VitD. For a white person, if 30 minutes of June noontime sun would cause a mild sunburn, then 10 to 15 minutes of exposure followed by good sun protection should be sufficient to produce adequate VitD [5].

Table 13. Guidelines for intake of vitamin D in infants and children/adolescents [34].

Authority and/or country (year)	Recommended intake of vitamin D (IU per day)		
	Age 0–1 years	Age 1–3 years	Age 4–18 years
*New guidelines**			
IOM (2010)[29]	400	600	600
Australia–New Zealand (2013)[23]	400	600	600
DACH (2012)[21]	400	800	800
Nordic countries (2012)[20]	400	400	400
WHO–FAO (2003/2012)[84]	200	200	200
UK (SACN 2016)[28]	340–400	400	400
Netherlands (2012)[22]	400	400	400
Belgium (2009)[74]	400	400	400
France (Société Française de Nutrition; 2012)[47]	800–1,000	400	80,000–100,000 twice a year
Endocrine society (2011)[69]	400–1,000	400–1,000	400–1,000
EFSA draft version (2016)[61]	400	600	600

This table shows the recommended intake of VitD in guidelines updated in the past 10 years (new guidelines). Most of the values are either recommended dietary allowance (RDA) or reference nutrient intake (RNI). However, for infants, the Institute of Medicine (IOM) and the Scientific Advisory Committee on Nutrition (SACN) use adequate intake or 'safe intake', respectively, owing to a lack of sufficient data to define a RDA or a RNI.

DACH, Deutschland (Germany), Austria and Confoederatio Helvetica (Switzerland); EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; IOM, Institute of Medicine; IU, international units; ND, not determined; SACN, Scientific Advisory Committee on Nutrition.

Food sources of VitD are very limited as summarized in Table 3. Foods fortified with VitD (Table 4) usually contain 100 IU per serving. A recent systematic review found that food fortification with VitD (especially in milk) is effective in significantly increasing 25(OH)VitD levels in the population [48]. It is recognized that for every 100 IU of VitD ingested, the blood level of 25(OH)VitD increases by approximately 0.6 to 1 ng/mL [434].

A good way for increasing 25(OH)VitD concentration and treating hypovitaminosis D is by using VitD supplements. An effective strategy to treat VitD deficiency and insufficiency in teenagers and adults is to give them 50,000 IU of vitamin D2 once a week for 6 and 8 weeks, respectively [27, 435]. To prevent recurrence of VitD deficiency in adolescents, administration of 600 to 1000 IU/d is effective [27]. For adults, to prevent recurrence of VitD deficiency, administration of 50,000 IU of vitamin D2 every 2 weeks is effective [5, 27, 432]. This strategy was shown to be effective in maintaining blood levels of 25(OH)VitD at approximately 40 to 60 ng/mL for up to 6 years without any evidence of toxic effects [436]. VitD can be administered daily, weekly, monthly, or every 4 months to

sustain an adequate serum 25(OH)VitD concentration [5]. A bolus of high doses of VitD (up to 300,000 IU) can be initially used in persons with extreme VitD deficiency. Because body fat can sequester VitD, it is now recognized that children and adults who are obese require 2 to 5 times more VitD to treat and prevent VitD deficiency [27].

1.13 Safety issues for Vitamin D – upper intake levels

VitD toxicity due to excessive sun exposure or intake of natural foods is exceptionally rare [57]. However, as with other fat-soluble vitamins, toxicity can be serious and even lethal when children or adults are exposed (repeatedly) to pharmacologic amounts of VitD. VitD intoxication is characterized by hypercalcemia, hypercalciuria, and hyperphosphatemia, which, in turn, are responsible for soft-tissue and vascular calcifications and nephrolithiasis in the long-term. Persons with VitD intoxication usually have markedly elevated 25(OH)VitD serum levels (>150 ng/mL) [5, 27]. However, no consensus has been reached about the dosages causing toxicity or about the upper safe limit of levels of 25(OH)VitD. The tolerable upper level indicated in the IOM guidelines is 2,000 IU per day for infants and 4000 IU for adults [26]. However higher doses of VitD (up to 40,000 IU/day) are still reported to be safe provided that a serum 25(OH)VitD concentration of 200 ng/mL is not exceeded [17]. Of note, polymorphisms in CYP24A1 influence serum concentrations of 25(OH)VitD [437] and some mutations have major effects on the toxicity of smaller dosages of VitD [51, 438].

CHAPTER II.

METRIALS AND METHODS

This study consists of two parts: a cross-sectional and a prospective one.

2.1 Aims of the study

2.1.1 Aims of the Cross-sectional study

This was concerned with the following:

Determination of 25(OH)VitD serum levels in Greek patients, **a)** adults with metabolic syndrome (MetS) and **b)** obese adolescents as well as in matched for age and sex control subjects. In addition, it explored the possible correlation of these levels with those of the parameters used as criteria for MetS diagnosis and of other MetS biochemical characteristics.

2.1.2 Aims of the Prospective study

A) Assessment of the effect of hypolipidemic drugs on the levels of 25(OH)VitD in patients with dyslipidaemia according to the three protocols outlined below.

i) The effect of rosuvastatin 40 mg versus that of the combination of rosuvastatin 10 mg and fenofibrate 200 mg or the combination of rosuvastatin 10 mg and omega-3 fatty acids 2 g on 25(OH)VitD serum levels in patients with mixed dyslipidaemia.

ii) The effect of simvastatin 40 mg versus that of the combination of simvastatin 10 mg with ezetimibe 10 mg on 25(OH)VitD serum levels in patients with hypercholesterolemia.

iii) The effect of rosuvastatin 40 mg versus that of the combination of rosuvastatin 10 mg and fenofibrate 200 mg or the combination of rosuvastatin 10 mg and nicotinic acid/laropiprant 2 g on 25(OH)VitD serum levels in patients with mixed dyslipidaemia.

B) Assessment of the effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters in adults with MetS and adolescents with obesity.

The primary endpoint was concerned with the changes in MetS parameters 3 months after treatment initiation which were:

- Waist circumference
- Blood pressure (systolic and diastolic)
- Fasting serum triglyceride levels
- HDL-C Levels
- Fasting serum glucose levels

The secondary endpoints were concerned with the changes in:

- 25(OH)VitD and PTH serum levels
- Glucose homeostasis (HOMA index: fasting insulin x fasting glucose/405)
- Glycosylated hemoglobin (HbA1c) levels
- LDL-C levels
- LDL-C subclasses (mean LDL-C particle size, sd-LDL-C levels)
- High sensitivity C reacting protein (hsCRP) levels
- Lp-PLA2 activity (lipoprotein-associated phopspholipase)
- Paraoxonase-1 (PON1) and arylesterase (ARYL) activity
- Oxidative stress as measured by urinary 8-isoprostane levels and serum oxidized LDL (oxLDL) levels
- Serum levels of adipokines (leptin, adiponectin and visfatin)

2.2 Study population

2.2.1 Study population of the Cross-sectional study

Determination of 25(OH)VitD serum levels in 52 Greek adult patients with metabolic syndrome (MetS), 69 obese adolescents and in matched for age and sex control subjects (n=58 adults, n=34 adolescents).

Individuals found to be diabetic (1 random measurement of plasma glucose >200 mg/dL plus symptoms of hyperglycemia, 2 measurements of fasting glucose levels >126 mg/dL, or plasma glucose >200 mg/dL after 75 g Oral Glucose Tolerance Test -OGTT-), or with history of CVD were not included in the study. Other exclusion criteria were the presence of thyroid dysfunction (abnormal levels of thyroid stimulating hormone), liver or kidney disease (defined as a positive medical history or a threefold increase in serum aminotransferases and serum creatinine levels of >1.6 mg/dL respectively) and the administration of drugs that may interfere with glucose or lipid metabolism (e.g. statins, fibrates and niacin), as well as with calcium metabolism (e.g. multivitamin preparations or drugs for osteoporosis). Subjects whose HOMA values that exceeded the 75th percentile (i.e. 2.0) were considered to have insulin resistance and were also excluded [439].

A) Hence one hundred ten otherwise healthy **adults**, who had visited the Outpatient Lipid Clinic of the University Hospital of Ioannina, Ioannina, Greece, for a regular check-up, were included in the present study. Of these, 52 fulfilled 3 or more of the American Heart Association (AHA) criteria [397] for the diagnosis of MetS (waist circumference >102 cm in men, >88 cm in women, fasting serum triglycerides >150 mg/dL, HDL-C <40 mg/dL in men, <50 mg/dL in women, blood pressure >130/85 mm Hg, fasting serum glucose >100 mg/dL), while 58 age- and sex-matched individuals with less than 3 criteria for the diagnosis of MetS served as controls.

BP was measured in triplicate in the right arm after patients had rested for 10 minutes at a sitting position. Measurements were performed by trained clinicians using an electronic sphygmomanometer (WatchBP Office, Microlife WatchBP AG, Widnau, Switzerland). Their 25(OH)VitD serum levels in specimens kept at -80^{o}C were determined.

The cut-off values for serum 25(OH)VitD levels in adults that were used in our study were: >30 ng/mL for VitD sufficiency, 20–28 ng/mL for insufficiency and <2 0 ng/mL for deficiency. All specimens were collected during the same season of the year (spring) as to exclude any sunlight effect on VitD levels. All participants were of Greek origin, had similar dietary habits with normal calcium content and comparable levels of sun exposure, since none was institutionalized or homebound or had a special dress code.

B) With regard to **adolescents,** subjects with diabetes, chronic kidney or liver diseases, triglycerides >500 mg/dL and on calcium and/or VitD supplements as well as on lipid-lowering medications or anti-hypertensive drugs were not included.

So one hundred three otherwise healthy adolescents who had attended the Outpatient Paediatric and the Metabolic and Obesity Clinics of the University Hospital of Ioannina, Ioannina, Greece, were included in this study and were tested for their 25(OH)VitD serum levels in specimens kept at -80^{o}C. All adolescents were weighted on a digital scale and height measurement was performed with a stable stadiometer, barefoot and wearing light clothing. Body mass index (BMI) is defined as weight (kilograms)/height (m^2). Per Center for Disease Control and Prevention guidelines, BMI between 5th-84th percentile for age and sex is considered as healthy weight, 85th-94th percentile as overweight and ≥95th percentile as obese. Severe obesity was defined as BMI≥120% of 95th percentile for age and sex or BMI >35 kg/m^2, whichever was lower [270]. Of these adolescents 69 were obese and 34 were normal-weight and served as controls. Blood pressure was measured in a sitting position after a 5 minute rest, using a digital sphygmomanometer (Omron M3 HEM-7131), and the mean of two measurements was recorded. Hypertension was defined as systolic or diastolic blood pressure >95th percentile according to age, sex and percentile of height. For the analysis, all CVD risk factors considered as abnormal were based on the definitions by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP-III) modified for age [394]. These were: waist circumference ≥90th percentile for age and sex, HDL cholesterol ≤40 mg/dL, triglycerides ≥110 mg/dL and fasting glucose ≥100 mg/dL. The pubertal stage was estimated with the Tanner scale.

2.2.2 Study population of the Prospective Study

A) Assessment of the effect of hypolipidemic drugs on the levels of 25(OH)VitD in patients with dyslipidaemia.

i) Assessment of the effect rosuvastatin 40 mg, or a combination of rosuvastatin 10 mg and fenofibrate 200 mg or a combination of rosuvastatin 10 mg and omega-3 fatty acids 2 g on 25(OH)VitD serum levels in patients with mixed dyslipidaemia.

All patients had visited the Outpatient Lipid and Obesity Clinic of the University Hospital of Ioannina, Greece. Eligible were those who had LDL-C >160 mg/dL and triglycerides >200 mg/dL at baseline. Also, patients with hypertension were included in the study if they were on stable medication for at least 3 months before study entry and their blood pressure was adequately controlled (no change in their treatment was allowed during the study period). Exclusion criteria were known coronary heart disease or other atherosclerotic diseases, triglycerides >500 mg/dL, renal disease (serum creatinine levels >1.6 mg/dL or proteinuria >300 mg/d), diabetes (fasting blood glucose >126 mg/dL or treatment with hypoglycemic agents), hypothyroidism (thyroid-stimulating hormone >5 IU/mL), and liver disease (alanine transaminase and/or aspartate transaminase levels >3-fold the upper limit of normal in more than 2 consecutive measurements). Patients currently taking lipid-lowering drugs or having stopped them less than 4 weeks before study entry were also excluded.

All participants were given individualized dietary instructions by a clinical nutritionist, based on each one's basal energy requirements and on an estimation of the participant’s typical activity level according to the NCEP-ATP III guidelines. The treatment groups did not differ in their nutrient intake at baseline, and there were no differences in diet composition between the study groups. All patients were asked to attend the clinic monthly during the treatment in order to assess diet compliance. Patients who remained hyperlipidemic (LDL-C >160 mg/dL plus triglycerides >200 mg/dL) after a dietary intervention period of approximately 3 months were considered eligible for randomization. The final total number of patients included was 60. We followed a simple randomization method using a table of random numbers. Patients were allocated to either open-label 40

mg rosuvastatin (22 patients), or 10 mg rosuvastatin plus micronized 200 mg fenofibrate (21 patients), or 10 mg rosuvastatin plus 2 g omega-3 fatty acids (17 patients) (each gram of the preparation contains approximately 465 mg of eicosapentaenoic acid and 375 mg of docosahexaenoic acid) daily for 3 months. Blood pressure was measured in triplicate in the right arm after patients had rested for 10 minutes at a sitting position. Measurements were performed by trained clinicians using an electronic sphygmomanometer (WatchBP Office, Microlife WatchBP AG, Widnau, Switzerland).

ii) Assessment of the effect of simvastatin 40 mg versus the effect of the combination of simvastatin 10 mg with ezetimibe 10 mg hypercholesterolemia.

Patients with primary hypercholesterolemia attending the Outpatient Lipid and Obesity Clinic of the University Hospital of Ioannina, Ioannina, Greece participated in the study. Inclusion criteria were LDL-C levels above those recommended by the NCEP-ATP III based on each patient risk factors following a 3-month period of lifestyle changes. Patients with hypertension if they were on stable medication for at least 3 months and their blood pressure was adequately controlled (no change in their treatment was allowed during the study) were also included. Exclusion criteria were known CVD, symptomatic carotid artery disease, peripheral arterial disease, abdominal aortic aneurysm, diabetes, triglycerides >500 mg/dL, renal disease (serum creatinine levels >1.6 mg/dL), hypothyroidism (thyroid stimulating hormone >5 IU/mL), liver disease (alanine aminotranferase and/or aspartate aminotranferase levels >3-fold upper limit of normal in 2 consecutive measurements), neoplasia as well as clinical and laboratory evidence of an inflammatory or infectious condition. Patients currently taking lipid-lowering drugs or having stopped them less than 4 weeks before the start of study were excluded. Half of them were randomly allocated to receive either open-label simvastatin 40 mg or simvastatin/ezetimibe 10/10 mg for 12 weeks. Randomization was performed by means of a computer-generated sequence of random numbers. Compliance with study medication was assessed at week 12 by tablet counts; patients were considered compliant if they took 80% to 100% of the prescribed number of tablets. The final number of the study patients was 50 (25 &25).

iii) Assessment of the effect of rosuvastatin 40 mg versus the effect of the combination of rosuvastatin 10 mg and fenofibrate 200 mg or of the combination of rosuvastatin 10 mg and nicotinic acid/laropiprant 2 g on 25(OH)VitD serum levels in patients with mixed dyslipidaemia not responded to conventional doses of statins and not reaching target goal.

For the present analysis the final number of the randomly selected patients was 44 to test for 25(OH)VitD at start and at the end of study. The participating patients in this study had been treated for a minimum of 3 months with a standard statin dose (10-40 mg simvastatin or 10-20 mg atorvastatin or 5-10 mg rosuvastatin) but their LDL-C or non-high-density lipoprotein cholesterol (non-HDL-C) levels had not reached treatment targets. Patients with hypertension and/or diabetes could participate in the study only if they were on stable medication for at least three months and had normalized their blood pressure and glucose serum levels. Exclusion criteria were the presence of chronic kidney or liver disease and triglyceride levels >500 mg/dL. Patients were randomly distributed (without a wash-out phase) to switch to open-label high-dose rosuvastatin (40 mg/day) (n=17) for 3 months or to get on an add-on-statin (rosuvastatin 10 mg) treatment with ERNA/LRPT (1000/20 mg/day for the first four weeks followed by 2000/40 mg/day for the next eight weeks) (n=14) or to get on an add-on-statin (rosuvastatin 10 mg) treatment with micronised fenofibrate (200 mg/day) for three months (n=13). The study design relates to everyday clinical practice, since often patients cannot achieve lipid targets when treated with a conventional statin dose, thereby raising concerns to the attending physician for the appropriate modification of the treatment regimens. Similar dietary advice was given to all patients according to the third report of the National Cholesterol Education Program-Adult Treatment Panel IIII (NCEP-ATP III) guidelines. Moreover, adherence to treatment and lifestyle habits were assessed by tablet count and an appropriate questionnaire, respectively.

All participants in the study were of Greek origin, had similar dietary habits with usual calcium content and comparable amounts of sun exposure, since none was institutionalized or homebound or had a special dress code. In order to exclude the confounding effect of the seasonal variation of serum 25(OH)VitD levels, we chose to analyze all specimens collected from early autumn to late winter. During this period,

sunshine in Greece is of the same duration and therefore we can largely exclude the confounding sunlight effect on 25(OH)VitD levels. Additionally, none of the participants consumed drugs that could interfere with VitD metabolism (ie, osteoporosis treatment) or VitD supplements.

B) Assessment of the effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters in adults with MetS and adolescents with obesity.

Adults with MetS and adolescents with obesity who were followed at the outpatients Lipid and Obesity Clinic and the Pediatric Clinic of the University Hospital of Ioannina were included in the prospective study. The recruitment of the patients was completed within one year. All participants (and/or their caregivers) gave written informed consent before any clinical or laboratory evaluation and any dietary or drug therapeutic intervention. The study protocol was approved by the Scientific Committee of the University Hospital of Ioannina and was conducted following the guidelines outlined in the Declaration of Helsinki. Additionally, the study was registered in NCT01237769 ClinicalTrials.gov.

i) MetS diagnosis (in **adults**) was set by fulfilling 3 or more of the NCEP-ATP III [397] criteria, when they visited the Outpatient Metabolic and Obesity Clinic of the University Hospital of Ioannina, Ioannina, Greece. Eligible adult patients with MetS were randomly allocated (through a computer-generated sequence of random numbers) by sex and age as baseline factors to either only dietary instructions (n=25, Non-Suppl group) or to receive 2000 IU VitD/day (Vitamin D3) along with dietary instructions (n=25, VitD Suppl group) for 3 months. We administered 2000 IU VitD/day, a higher than usual dose but within safety limits, since former studies failed to find any significant changes in CVD risk factors when using usual VitD doses (400-800 IU/day). Supplementation of up to 2000 IU VitD daily has been regarded by the U.S. Food and Drug Administration's nutritional guidelines as more effective and safe [440]. Similarly, Endocrine Society clinical practice guidelines conclude that to raise serum 25(OH)VitD levels above 30 ng/mL, intakes of 1500 to 2000 IU/day may be required [27]. All patients followed a 12-week dietary intervention program according to NCEP ATP III guidelines [397]. The compliance to dietary instructions was assessed by completing food diaries and through discussion during

follow up visits, while compliance with study medication was controlled by tablet count at week 12; patients were considered compliant if they received 80-100% of the prescribed tablets. The final number of compliant patients was 50 and their reassessment was performed after 3 months from starting intervention.

ii) Obese **adolescents** (BMI= 35.0±7.9) with hypovitaminosis D (25(OH)VitD <20 ng/mL) were given 2000 IU VitD per os daily (Vitamin D3) plus dietary instructions and were re-evaluated after 3 months. The clinical examination of their somatometric characteristics was performed based on the same methods and criteria described in the cross-sectional analysis.

In adolescent participants with obesity we chose to administer a dose of 2000 IU VitD/day for 3 months, in order to correct VitD deficiency taking into consideration the following recommendations. The Endocrine Society clinical practice guidelines (2011) recommend that one- to 18-year-old children and adolescents need to be given 600-1000 IU/day (and no more than 4000 IU/day) of VitD supplementation [27]. Especially for adolescents at risk of VitD deficiency or insufficiency, including obese adolescents (>95th BMI), the Society for Adolescent Health and Medicine (2013) proposed to administer at least 1,000 IU/day [92], while according to Belenchia et al. a daily 4000 IU VitD dose, which is the maximum allowable according to the Institute of Medicine, was considered safe and effective in improving VitD nutritional status in obese adolescents [273]. In parallel participants also followed a 12-week dietary intervention programme. That included a moderate carbohydrate and increased protein diet, which provided 40-45% of total energy as carbohydrate (moderate glycemic load), 30% fat (≤10% saturated fat), and 25-30% protein. Two different energy levels were assigned, depending upon age: 6000-7000 kJ (10-14 years old) or 7000-8000 kJ (15-17 years old). The dietary intervention conferred 2000 kJ less than the recommended intake for age and promoted an increase in incidental activity and a decrease in sedentary behavior [441]. The compliance to dietary instructions was assessed by completing food diaries and through discussion during follow up visit, while compliance with study medication was checked by tablet count at week 12; patients were considered compliant if they received 80-100% of the prescribed tablets. The final number assessed was 15.

Exclusion Criteria both for adults and adolescents were the presence of diabetes, chronic kidney or liver disease, triglycerides >500 mg/dL and intake of calcium and/or VitD supplements as well as lipid-lowering medications. Patients with elevated BP who were not on any treatment participated in the study, as well as patients with hypertension who had received stable treatment for at least 3 months and had normalised their BP levels during the intervention.

In order to minimise the season effect on 25(OH)VitD levels, all specimens were collected during March to September, a season during which the duration of sunlight is approximately similar in Greece.

2.3 Laboratory measurements in the Cross-sectional and Prospective Studies

All samples were collected after an overnight fast (approximately 12 hours). The following parameters were determined:

- serum 25(OH)VitD
- serum PTH
- serum glucose, insulin, HbA1c, HOMA index (estimated)
- serum total cholesterol, HDL-C and triglyceride levels, whereas LDL-C levels were assessed using the Friedewald equation (LDL-C=TCHOL-HDL-C-triglycerides/5) and non-HDL was calculated by the equation: non-HDL-C= TCHOL - HDL-C. LDL-C was calculated using the Friedewald formula (provided that triglycerides were <400 mg/dL).
- LDL subfraction profile
- Serum apolipoprotein AI and B (apo AI and apoB)
- Lp-PLA_2 activity in plasma
- high sensitivity C reactive protein (hs-CRP) in serum
- serum PON1 activity and ARYL activity
- urinary 8-isoprostane
- oxidized LDL (oxLDL) in plasma
- serum adipokines (leptin, adiponectin, visfatin)

Serum 25(OH)VitD levels were measured quantitatively by an enzyme immunoassay method using the reagents in the kit purchased from DRG Instruments GmbH (DRG, Marburg, Germany). The method's analytical sensitivity is 1.28 ng/mL and the intra- and inter assay variation is 13% for each at the level of 18 and 16 ng/mL, respectively.

Serum parathyroid hormone (iPTH) was measured by IMMULITE 2500 Intact PTH, a solid-phase, two-site chemiluminescent enzyme-labeled immunometric assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA 90045-6900 USA).

Glucose was measured by the hexokinase method and serum insulin by a microparticle enzyme immunoassay on an AXSYM analyzer (Abbott Diagnostika, Wiesbaden-Delkenheim, Germany) with coefficients of variation of 4.2% to 9.0% respectively. Homeostasis model assessment (HOMA) index was calculated using the equation: fasting insulin (mIU/L) * fasting glucose (mg/dL)/405. The determination of HbA1c was based on a latex agglutination inhibition assay (Randox Laboratories Ltd., Antrim, UK). HbA1c values are expressed as percentage of the total hemoglobin concentration. The sensitivity of the method is 0.25 g/dL of HbA1c and the within run and between runs precision <6.67% and <4.82%, respectively.

Routine laboratory determinations were carried out by automated chemical analysis in the laboratory of the University Hospital of Ioannina using an Olympus AU 600 analyzer (Olympus Diagnostica GmbH, Hamburg, Germany). Total cholesterol, triglycerides and HDL-C were measured enzymatically on an Olympus AU600 Clinical Chemistry analyzer (Olympus Diagnostica, Hamburg, Germany). The Friedewald formula was used to calculate serum LDL-C (when triglycerides were <350 mg/dL; 3.95 mmol/L).

LDL subclass analysis was performed electrophoretically by the use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL System (Quantimetrix, Redondo Beach, CA) according to the manufacturer's instructions [442-445]. Briefly, 25 μL of sample were mixed with 200 Ml of Lipoprint Loading Gel and placed upon the upper part of the 3% polyacrylamide gel. After 30 min of photopolymerization at room temperature, electrophoresis was performed for 60 min with 3 mA for each gel tube. Each electrophoresis chamber involved 2 quality controls (provided by the manufacturer). For quantification, scanning was performed with a ScanMaker 8700 digital scanner (Mikrotek Co, USA) and iMac personal computer (Apple Computer Inc, USA). After scanning, electrophoretic mobility (Rf) and the area under the curve (AUC) were calculated

qualitatively and quantitatively with the Lipoprint LDL system Template and the Lipoware software (Quantimetrix Co, Redondo Beach, CA), respectively. The LDL subfraction was calculated using the Rf between the very low-density lipoprotein (VLDL) fraction (Rf 0.0) and the HDL fraction (Rf 1.0). After electrophoresis, the very low-density lipoprotein (VLDL) remained at the origin [retention factor (R_f)=0.0] and high-density lipoprotein (HDL) migrated to the front (R_f=1.0). In between, several bands can be detected: MID bands C, B, and A, which correspond mainly to intermediate-density lipoprotein (IDL), as well as up to 7 LDL bands. LDL is distributed from Rf 0.32 to Rf 0.64 as 7 bands, whose Rfs are 0.32, 0.38, 0.45, 0.51, 0.56, 0.6 and 0.64 (LDL1 to LDL7, respectively). The LDL-1 and LDL-2 bands correspond to large buoyant LDL particles, whereas the bands LDL-3 to LDL-7 correspond to sdLDL particles. The cholesterol mass (in mg⁄dL) of sdLDL particles, the mean LDL particle size (in nm) and the proportion (%) of the cholesterol mass of sdLDL particles over the total LDL-C mass were determined. The cholesterol concentration (in mg/dL) of each LDL subfraction is determined by multiplying the relative AUC of each subfraction by the TCHOL concentration of the sample (the TCHOL concentration of the sample is measured independently). The proportion of sd-LDL-cholesterol (sd-LDL%) was defined as the percentage of the LDL-cholesterol carried in sd-LDL (i.e. bands 3 to 7). LDL peak particle diameter (LDL-PPD) (nm) was determined using the Rf of the highest peak of the LDL bands according to the following equation proposed: LDL-PPD=(1.429-Rf)*25 [446]. Moreover, the Lipoprint LDL System provides a mean LDL particle size (nm) and uses a size of 26.8 nm as a cut-off point to classify individuals into phenotypes A (absence of sd-LDL particles) and non-A (presence of sd-LDL particles).

Serum Apolipoproteins (apo) A-I and apoB were measured using a Behring Nephelometer BN100 and with reagents (antibodies and calibrators) from Dade Behring Holding GmbH (Liederbach, Germany). The apoA-I and apoB assays were calibrated according to the International Federation of Clinical Chemistry standards.

Serum concentrations of hs-CRP were measured by the high sensitivity CRP method (Dade Behring, Marburg, Germany) based on particle enhanced immunonephelometry; the reference range is 0.175 to 55 mg/L.

Lp-PLA_2 activity was measured with the trichloroacetic acid precipitation procedure in plasma using [^{3}H]-PAF (1-*O*-hexadecyl-2-[^{3}H-acetyl]-*sn*-glycero-3-phosphocholine) (10

Ci/mmol; DuPont-New England Nuclear, Boston, MA) as a substrate at a final concentration of 100 μM. The reaction was performed for 10 min at 37°C. The radioactivity was determined in a liquid scintillation counter (Packard Tri-Card 2100). Lp-PLA_2 activity was expressed as nmol PAF degraded per min per mL of plasma [447].

The paraoxonase-1 (PON-1) activities in serum were measured using paraoxon (paraoxonase activity) or phenylacetate (arylesterase activity), as a substrate. Both PON-1 activities were determined in the presence of 2 mM Ca^{2+} in 100 mM Tris-HCl buffer (pH 8.0) for paraoxon and in 20 mM Tris-HCl buffer (pH 8.0) for phenyl acetate.

Urine levels of 8-isoprostane (8-epiPGF_2a) were determined by a competitive enzyme immunoassay (commercial 8-isoprostane EIA kit, Cayman Chemicals, Ann Arbor, MI), following manufacturer instructions. The 8-epiPGF2a levels in urine were expressed as ng/mmol creatinine.

Plasma levels of oxidized low-density lipoprotein (ox-LDL) were measured by a competitive enzyme-linked immunosorbent assay using a specific murine monoclonal antibody (4E6) according to the instructions provided by the manufacturer (Mercodia, Uppsala, Sweden). The specificity of this method was studied by performing the assay in 5 different plasma samples in which 5 or 15 ng of protein of native LDL or oxLDL were added exogenously. Intra-assay and inter-assay coefficients of variation of the assay are 6.0% and 7.0%, respectively [448]. Low-density lipoprotein (LDL, *d*=1.019-1.063 g/ml) was isolated by sequential ultracentrifugation from pooled fresh plasma [449]. Prior to oxidation, a part of purified LDL was incubated with 0.5 mmol/L pefabloc for 1h at 37ºC, a procedure that completely and irreversibly inactivates the LDL-associated Lp-PLA_2 [450]. After incubation, LDL was dialyzed against two changes of a 200-fold volume of 10 mmol/L PBS for 24 h at 4ºC to remove the excess of pefabloc. LDL with active Lp-PLA_2 or pefabloctreated LDL [containing inactive Lp-PLA_2 and denoted as LDL(−)], at a final concentration of 100 μg protein/mL, was oxidized in the presence of 5 μmol/L CuSO4, for 3 h at 37ºC [451].

Leptin was determined by using the human leptin ELISA kit purchased from BioVendor (Czech Republic) DRG (following manufacturer instructions. Coefficient of variation was less than 7%. Each concentration obtained was determined from the standard curve [452]. Serum levels of total adiponectin were determined by a competitive enzyme immunoassay (ELISA) using the reagents of the adiponectin kit purchased by DRG

Instruments GmbH (Marburg, Germany). The sensitivity of the method was 26 ng/ml and the intra- and inter-assay coefficients of variation were 5.9% & 6.3% at the levels 12 and 8 μg/ml respectively. Visfatin was determined by a "competitive" enzyme immunoassay method using the kit by Phoenix Pharmaceuticals, Inc (USA). The intra-assay CV was <10%.

2.4 Statistical analysis

2.4.1 Statistical analysis in the cross-sectional study:

For investigating the possible associations of the 25(OH)VitD serum levels in the patients (n=52 adults with metabolic syndrome (MetS), n=69 obese adolescents) with those of the parameters used for MetS diagnosis and of other MetS biochemical characteristics and CVD risk factors the following analysis was used:

Preliminary analyses were performed to ensure no violation of the assumptions of normality and linearity. The Kolmogorov-Smirnoff test was used to evaluate whether each variable followed a Gaussian distribution. Data are presented as mean and standard deviation except for non-Gaussian distributed variables, which are presented as median (range). The relationship among study variables were investigated by use of the Pearson product moment correlation coefficient, whereas correlations including at least 1 non-Gaussian distributed variable were performed with the Spearman correlation coefficient. The independent samples *t*-test (or Mann Whitney *U*-test when required) was used to assess differences between groups (MetS, non-MetS for adults and obese, normal weight for adolescents). Stepwise linear multiple regression analyses were performed to explore the relationships between emerging CVD risk factors and a set of independent variables (or predictors) that were significantly correlated with the dependent variable in the univariate analysis after checking for normality and linearity. For adults a p value ≤ 0.05 was considered to be significant whereas for adolescents the p value ≤ 0.03 was considered to be significant due to multiple comparisons (Bonferoni correction). All analyses were carried out with the SPSS 18 software package (SPSS Inc., 1989-2004, Chicago, IL).

2.4.2 Statistical analysis in the in the prospective study:

A) Regarding the assessment of the effect of hypolipidemic drugs on the levels of 25(OH)VitD in patients with dyslipidemia, we performed the analysis described below:

Again preliminary analyses were performed to ensure no violation of the assumptions of normality and linearity. The Kolmogorov-Smirnoff test was used to evaluate whether each variable followed a Gaussian distribution. Data are presented as mean and standard deviation except for non-Gaussian distributed variables, which are presented as median (range). For variables that did not follow the normal distribution, appropriate nonparametric tests were used. The paired samples t test (or the Wilcoxon signed ranks test, when required) was used for assessing the effect of treatment in each group (between baseline and post-treatment values). Analysis of covariance (ANCOVA) or the Kruskal-Wallis test for nonparametric variables, adjusted for baseline values, was used for comparisons between treatment groups. Significance was defined as $p < 0.05$.Analyses were performed using the SPSS 18.0 statistical package for Windows (SPSS Inc, 1989-2004, Chicago, Illinois).

B) Regarding the assessment of the effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters in adults with MetS and adolescents with obesity, we performed the analysis described below:

This was a pilot study and therefore formal power calculations were not performed. Similarly, the evaluation of the distribution of each variable (Gaussian or not) was done with the Kolmogorov-Smirnoff test. For variables with a Gaussian distribution data are presented as mean±standard deviation and for those with a non-Gaussian distribution as median (range, min-max). The paired samples t-test or the Wilcoxon Signed Ranks test was used so as to assess the effect of treatment in each group. For comparisons between treatment groups analysis of covariance (ANCOVA) or the Kruskal-Wallis test for non-parametric variables, adjusted for baseline values was applied. The significance was set at $p \leq 0.01$ for adults and ≤ 0.03 for adolescents due to multiple comparisons (Bonferoni

correction). All analyses were performed through the SPSS 18.0 statistical package for Windows (SPSS Inc., 1989-2004, Chicago, IL).

CHAPTER III.

RESULTS

3.1 Results of the Cross-sectional study

Determination of 25(OH)VitD serum levels in Greek adult patients with metabolic syndrome (MetS) and adolescents with obesity and their respective controls. Furthermore, exploring any possible correlation of these levels with those of the parameters criteria set for MetS diagnosis and of other MetS biochemical characteristics and CDV risk factors.

A) The clinical and laboratory characteristics of **adult** participants are shown in Table 14. There were no differences in the age and sex distribution between the study groups. As anticipated, subjects with MetS exhibited significantly elevated weight, BMI, waist circumference, systolic and diastolic BP, triglycerides, apoB, fasting plasma glucose, insulin and HOMA index, and lower HDL-C and apoAI compared with controls. Total cholesterol, LDL-C and PTH levels did not differ significantly between groups. Subjects with MetS presented with significantly higher hsCRP, sdLDL-C and Lp-PLA_2 and smaller LDL size compared with participants without MetS. The MetS group exhibited significantly lower 25(OH)VitD serum levels compared with controls (11.8 [0.6-48.3] ng/mL; 29.5 [1.5-120.75] nmol/L vs 17.2 [4.8-62.4] ng/mL; 43 [12-156] nmol/L, p=0.027) (Table 14, Figure 15).]. Overall 91.3% of the study participants had VitD insufficiency [25(OH)VitD <30 ng/mL] of which 65% VitD had deficiency [25(OH)VitD) <20 ng/mL]. Furthermore, prevalence of deficiency tended to be higher among MetS patients (65.3%) compared with controls (59.2%).

In the MetS subjects univariate analysis showed that 25(OH)Vit D was inversely associated with triglycerides (r=-0.416, p=0.003), but not with the other diagnostic criteria of MetS (i.e. waist circumference, BP, HDL-C and fasting glucose) (Table 15). In addition, 25(OH)VitD was inversely related to sdLDL-C levels (p=0.03) and PTH (r=-0.376, p=0.04), but not with LDL size, Lp-PLA_2 and hsCRP (Table 15).

In the stepwise multivariate linear regression analysis, sdLDL-C was assigned as the dependent variable and sex, age, smoking, SBP, DBP, fasting glucose, HOMA index, triglycerides, HDL-C, LDL-C, apoB, hsCRP and 25(OH)VitD as independent variables. In

this analysis, sdLDL-C levels were found to be significantly affected only by the triglyceride levels and not by any of the others including 25(OH)VitD concentrations (Table 16).

Table 14. Clinical and laboratory characteristics of adult participants

	Metabolic Syndrome (n=52)	Non-Metabolic Syndrome (n=58)	p
Age (y)	52±10	50±12	NS
Sex (male/female)	24/28	26/32	NS
Smoking (yes/no)	15/37	17/41	NS
Weight (kg)	83±13	78±15	**0.01***
BMI (kg/m^2)	30.2±3.0	28.2±4.6	**0.01***
Waist circumference (cm)	102±8	95±14	**0.007***
SBP (mm Hg)	135±15	124±18	**0.002***
DBP (mm Hg)	88±9	80±10	**0.000***
TCHOL (mg/dL)	236±37	226±46	NS
HDL-C (mg/dL)	49±11	57±12	**0.001***
LDL-C (mg/dL)	152±29	148±39	NS
Triglycerides (mg/dL)	152 (78-350)	97 (37-294)	**0.000***
Non HDL-C (mg/dL)	186±35	169±45	**0.031***
Apo AI (mg/dL)	141±26	154±25	**0.03***
Apo B (mg/dL)	119±17	105±25	**0.04***
sdLDL-C (mg/dL)	9.0 (0.01-66.0)	3.5 (0.01-28.0)	**0.006***
LDL-size (Å)	265.0±7.0	270.0±2.5	**0.001***
Lp-PLA$_2$ activity (nmol·mL^{-1}·min^{-1})	60±16	51±14.6	**0.019***
Fasting glucose (mg/dL)	104±16	92±11	**0.000***
Insulin (μU/mL)	11 (2-57)	8 (2-33)	**0.045***
HOMA index	2.4 (0.5-20)	1.8 (0.4-7)	**0.045***
hsCRP (mg/L)	2.7 (0.2-6.8)	2.1 (0.2-6.2)	**0.045***
25(OH)Vit D (ng/mL)	11.8 (0.6-48.3)	17.2 (4.8-62.4)	**0.027***
PTH (pg/mL)	42 (19-125)	53 (11-96)	NS
Total Ca (mg/dL)	9.7±0.4	9.5±0.3	NS
eGFR (mL/min/1.73m^2) Cockcroft-Gault	111±30	106±21	NS
MDRD	80±15	83±10	NS

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, T-CHOL: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, Apo: apolipoprotein, sdLDL-C: small dense LDL-C, Lp-PLA$_2$: lipoprotein-associated phospholipase A$_2$, HOMA index: homeostasis model assessment insulin resistance index, 25(OH)VitD: 25-hydroxy vitamin D, PTH: parathyroid hormone, hsCRP: high-sensitive C-reactive protein, Ca: calcium, e-GFR: estimated glomerular filtration rate, MDRD: Modification of Diet in Renal Disease

* p value <0.05 was considered to be significant

Figure 15. Serum 25(OH)VitD levels in adults with MetS and Controls

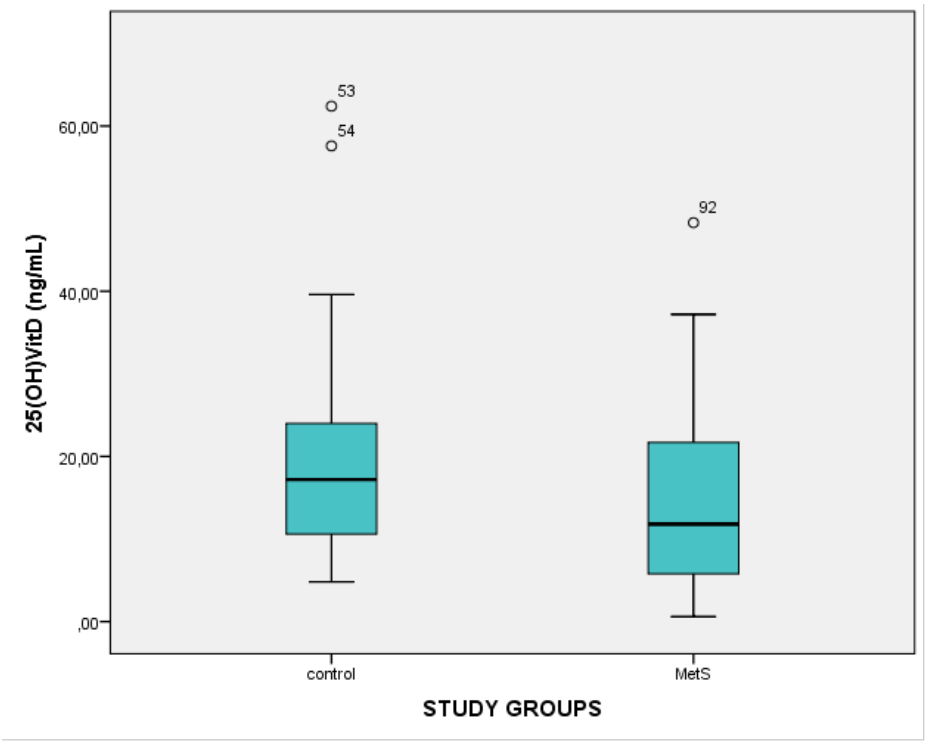


Table 15. Univariate correlations of Log [25(OH)Vit D] levels with metabolic parameters in the MetS adult subjects (n=52).

	r	p
Waist circumference	0.119	0.422
Blood Pressure SBP	0.234	0.109
Blood Pressure DBP	0.009	0.949
Log [Triglycerides]	-0.416	**0.003***
HDL-C	0.127	0.390
Fasting glucose	0.048	0.747
HOMA index	0.083	0.632
Log [sdLDL-C]	-0.305	**0.03***
LDL size	0.275	0.165
Lp-PLA$_2$ activity	0.064	0.746
hsCRP	-0.096	0.562
PTH	-0.376	**0.04***

* p value <0.05 was considered to be significant

Table 16. Multivariate regression analysis for the effect of various parameters on Log [sdLDL-C] levels in adult subjects with MetS (n=52).

	beta	p	95% CI
Sex	0.068	0.779	-0.355 – 0.462
Age	-0.091	0.770	-0.031 – 0.024
Smoking	-0.046	0.875	-0.577 – 0.498
SBP	-0.128	0.710	-0.017 – 0.012
DBP	0.008	0.975	-0.018 – 0.018
Fasting Glucose	-0.121	0.692	-0.022 – 0.015
HOMA index	0.172	0.561	-0.049 – 0.086
Log [Triglycerides]	0.689	**0.019***	0.146 – 1.311
HDL-C	-0.109	0.719	-0.025 – 0.018
LDL-C	0.227	0.619	-0.011 – 0.018
ApoB	0.119	0.781	-0.018 – 0.024
Log [hsCRP]	0.214	0.330	-0.267 – 0.726
Log [25(OH)Vit D]	-0.001	0.996	-0.363 – 0.361

* p value <0.05 was considered to be significant

B) Baseline clinical characteristics and laboratory findings of **adolescent** participants (n=103, 69 obese and 34 normal weight) are shown in Table 17. The 2 groups had significant differences in weight, BMI, WC, SBP, DBP, insulin, HOMA index, triglycerides, HDL-C, leptin levels and leptin/adiponectin ratio. Adolescents with obesity had lower 25(OH)VitD compared with normal weight controls [12.0 (3.0-36.0) versus 34.0 (10.0-69.0) ng/mL respectively, p=0.000]. Overall 74.7% of the study participants had VitD insufficiency [25(OH)VitD <30 ng/mL], of which 52.4% had VitD deficiency [25(OH)VitD) <20 ng/mL] (Figure 16). Furthermore, prevalence of deficiency was higher among obese (73.9%) compared with normal weight adolescents (17.6%) (p=0.001). In the obese adolescents, 25(OH)VitD was inversely related to leptin (r = -0.280, p = 0.037) (Table 18), which persisted after adjustment for BMI (r = -0.340, p = 0.009). In contrast, 25(OH)VitD was not associated with weight, BMI, WC, SBP, DBP, TCHOL, HDL-C, LDL-C, triglycerides, glucose, insulin, HOMA index, leptin/adiponectin ratio, adiponectin and visfatin (Table 18).

Table 17. Baseline characteristics of the adolescent participants

Parameter	Adolescents with obesity (N=69)	Normal Weight (N=34)	p
Sex (Boys/Girls)	31/38	15/19	NS
Age (years)	12.0±2.0	12.6±2.0	NS
Weight (kg)	74±21	47±12	**0.000***
BMI (kg/m^2)	29.7±5.5	19.6±2.3	**0.000***
WC (cm)	92±13	69±7	**0.000***
SBP (mmHg)	123±9	108±9	**0.000***
DBP (mmHg)	71±10	68±7	**0.04***
Glucose (mg/dL)	87±8.0	86±8.0	NS
Insulin (μU/mL)	13 (6-43)	8 (2-16)	**0.000***
HOMA index	2.8 (1.2-9.6)	1.7 (0.4-3.6)	**0.000***
TCHOL (mg/dL)	168±30	169±16	NS
Triglycerides (mg/dL)	103 (50-222)	79 (28-123)	**0.005***
HDL-C (mg/dL)	42±9	50±8	**0.000***
LDL-C (mg/dL)	104±25	102±16	NS
Leptin (ng/mL)	36 (7-105)	8 (1-27)	**0.000***
Adiponectin (μg/mL)	8±5	8±4	NS
Leptin/adiponectin ratio	7.4±5.7	1.5±1.1	**0.000***
Visfatin (ng/mL)	15±8	16±7	NS
25(OH)VitD (ng/mL)	12 (3-36)	34 (10-69)	**0.000***

* p value <0.05 was considered to be significant

Figure 16. Serum 25(OH)VitD levels in obese adolescents and controls

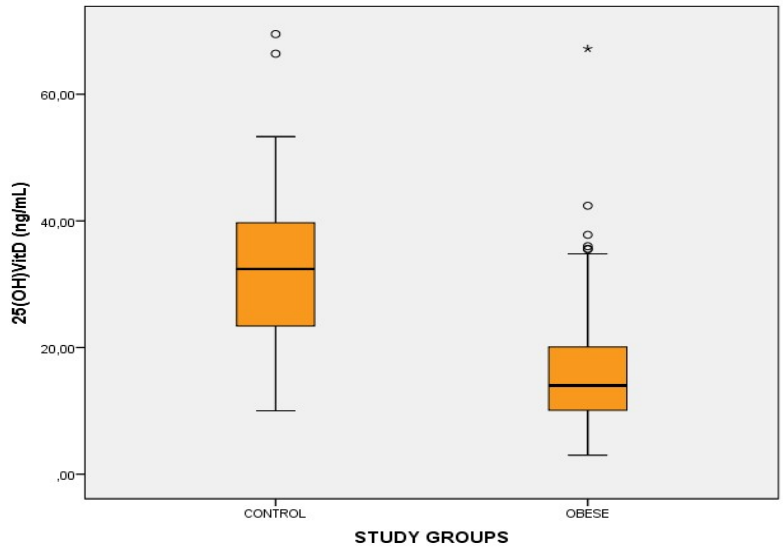


Table 18. Univariate correlations of Log[25(OH)VitD] with metabolic parameters in adolescents with obesity.

Parameter	r	p
Weight	0.149	0.221
BMI	0.196	0.107
WC	0.151	0.259
SBP	0.313	0.089
DBP	0.206	0.092
Glucose	-0.139	0.260
Log(Insulin)	-0.073	0.558
Log(HOMA index)	-0.085	0.493
TCHOL	-0.178	0.147
Log(triglycerides)	-0.010	0.932
HDL-C	-0.120	0.329
LDL-C	-0.205	0.096
Log(Leptin)	-0.280	**0.037***
Adiponectin	-0.281	0.058
Leptin/adiponectin ratio	0.029	0.839
Visfatin	-0.175	0.223

* p value <0.05 was considered to be significant

3.2 Results of the Prospective study

3.2.1 Assessment of the effect of hypolipidemic drugs on the levels of 25(OH)VitD in patients with dyslipidaemia.

i) The effect of rosuvastatin 40 mg (n=22) versus the effect of the combination of rosuvastatin 10 mg and fenofibrate 200 mg (n=21) and of the combination of rosuvastatin 10 mg and omega-3 fatty acids 2 g (n=17) on 25(OH)VitD serum levels in patients with mixed dyslipidaemia.

No significant differences in baseline characteristics were noted between the 3 groups (Table 19).

Table 19. Baseline characteristics of study participants who received rosuvastatin 40 mg (group R) rosuvastatin 10 mg plus micronized fenofibrate 200 mg (group RF) and rosuvastatin 10 mg plus omega-3 fatty acids 2 g (group RN).

Characteristics	Group R	Group RF	Group RN	p
N (males/females)	22 (7/15)	21 (11/10)	17 (5/12)	NS
Age (years)	58±13	54±10	53±15	NS
Current smokers (%)	41	28	53	NS
Body weight (kg)	76±12	84±12	82±16	NS
BMI (kg/m^2)	29±4	31±3	30±3	NS
WC (cm)	98±8	104±9	101±10	NS
SBP (mm Hg)	131±21	127±10	129±10	NS
DBP (mm Hg)	81±10	84±8	82±7	NS
TCHOL (mg/dL)	318±75	300±45	287±11	NS
LDL-C (mg/dL)	228±76	191±44	186±46	NS
Triglycerides (mg/dL)	203 (162-299)	279 (209-340)	238 (143-349)	NS
HDL-C (mg/dL)	54±9	53±9	50±12	NS
Non-HDL-C (mg/dL)	264±70	247±39	236±39	NS
Apo AI (mg/dL)	153±22	155±23	149±28	NS
Apo B (mg/dL)	140±42	144±26	129±22	NS
Glucose (mg/dL)	94±9	94±10	94±10	NS
25(OH)VitD (ng/mL)	14.6 (1-38)	14.1 (1-48)	10.4 (6.6-38.4)	NS

Table 20. Serum metabolic parameters at baseline and after 3 months of drug treatment with either rosuvastatin 40 mg (group R), or rosuvastatin 10 mg plus micronized fenofibrate 200 mg (group RF) and with rosuvastatin 10 mg plus omega-3 fatty acids 2 g (group RN).

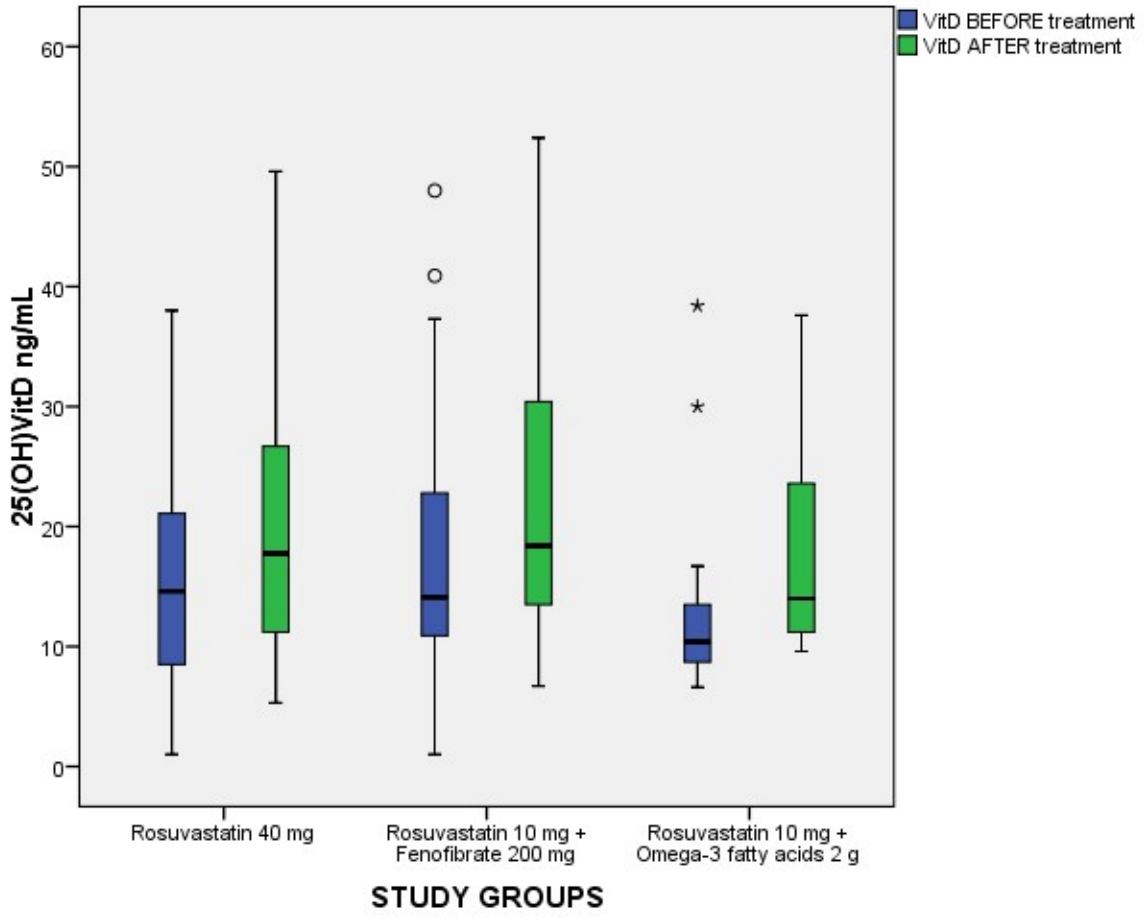
	Baseline	3 months	p vs baseline	% Change
TCHOL (mg/dL)				
Group R	318±75	176±38	0.000	-44%†
Group RF	300±45	197±41	0.000	-33%
Group RN	287±11	196±39	0.000	-31%
Triglycerides (mg/dL)				
Group R	203 (162-299)	137 (62-309)	0.009	-30%
Group RF	279 (209-340)	131 (67-244)	0.000	-54%*
Group RN	238 (143-349)	169 (106-327)	0.004	-26%
HDL-C (mg/dL)				
Group R	54±9	55±8	NS	+1.0%
Group RF	53±9	57±12	0.020	+7.7%*
Group RN	50±12	52±12	NS	+4.8%
LDL-C (mg/dL)				
Group R	228±76	97±38	0.000	-58%†
Group RF	191±44	122±47	0.000	-44%
Group RN	186±46	108±33	0.000	-41%
Non-HDL-C (mg/dL)				
Group R	264±70	125±34	0.000	-52%†
Group RF	247±39	141±39	0.000	-42%
Group RN	236±39	144±34	0.000	-39%
Apo AI (mg/dL)				
Group R	153±22	155±18	NS	+1.0%
Group RF	155±23	162±30	0.04	+4.0%*
Group RN	149±28	152±25	NS	+2.0%
Apo B (mg/dL)				
Group R	140±42	77±22	0.000	-52%†
Group RF	144±26	83±24	0.000	-45%
Group RN	129±22	98±30	0.000	-37%
Glucose (mg/dL)				
Group R	94±9	94±11	NS	+0.5%
Group RF	94±10	93±7	NS	-0.7%
Group RN	94±10	95±9	NS	+1.0%
25(OH)VitD (ng/mL)				
Group R	14.6 (1.0-38.0)	17.8 (5.3-49.6)	0.000	+53%
Group RF	14.1 (1.0-48.0)	18.4 (6.7-52.4)	0.001	+64%
Group RN	10.4 (6.6-38.4)	14 (9.6-37.6)	0.04	+61%

†p <0.05 from comparisons of R vs RF and RN groups,

*p <0.05 from comparisons of RF vs R and RN groups

After treatment, serum 25(OH)VitD levels were found significantly increased in all study groups (Table 20). Specifically, in the rosuvastatin 40 mg group there was a 53% increase (p=0.000), in the commonly used dose of rosuvastatin (10 mg) plus fenofibrate a 64% increase (p=0.001), and in the commonly used dose of rosuvastatin (10 mg) plus omega-3 fatty acids a 61% rise (p=0.04) in serum 25(OH)VitD concentrations. The observed increases in the 25(OH)VitD levels after treatment did not differ significantly between the 3 groups (Figure 17). Lipid changes are summarised in Table 20.

Figure 17. Serum 25(OH)VitD levels before and 3 months after treatments in the 3 treatment groups: rosuvastatin 40 mg, rosuvastatin 10 mg plus micronized fenofibrate 200 mg and rosuvastatin 10 mg plus omega-3 fatty acids 2 g



ii) The effect of simvastatin 40 mg (n=25) versus the effect of the combination of simvastatin 10 mg with ezetimibe 10 mg (n=25) on 25(OH)VitD serum levels in patients with hypercholesterolemia.

The baseline clinical and laboratory characteristics of study participants are listed in Table 21. No significant differences in baseline parameters were found between the 2 groups. Of note both groups presented with low baseline 25(OH)VitD levels with mean values of 6.8 and 6.7 ng/mL, respectively.

In the simvastatin/ezetimibe 10/10 mg group 25(OH)VitD serum levels increased by 36.7% (from 6.8 to 9.3 ng/mL, p=0.000), while in the simvastatin 40 mg group a 79.1% increase (from 6.7 to 12.0 ng/mL, p=0.008) was noticed (Table 22, Figure 18). The increase in 25(OH)VitD levels was significantly greater in the simvastatin 40 mg group compared with simvastatin/ezetimibe 10/10 mg group (p=0.04). Lipid changes were similar between the 2 groups as summarised in Table 22.

Table 21. Baseline characteristics of study participants who received either simvastatin/ezetimibe 10/10 mg or simvastatin 40 mg.

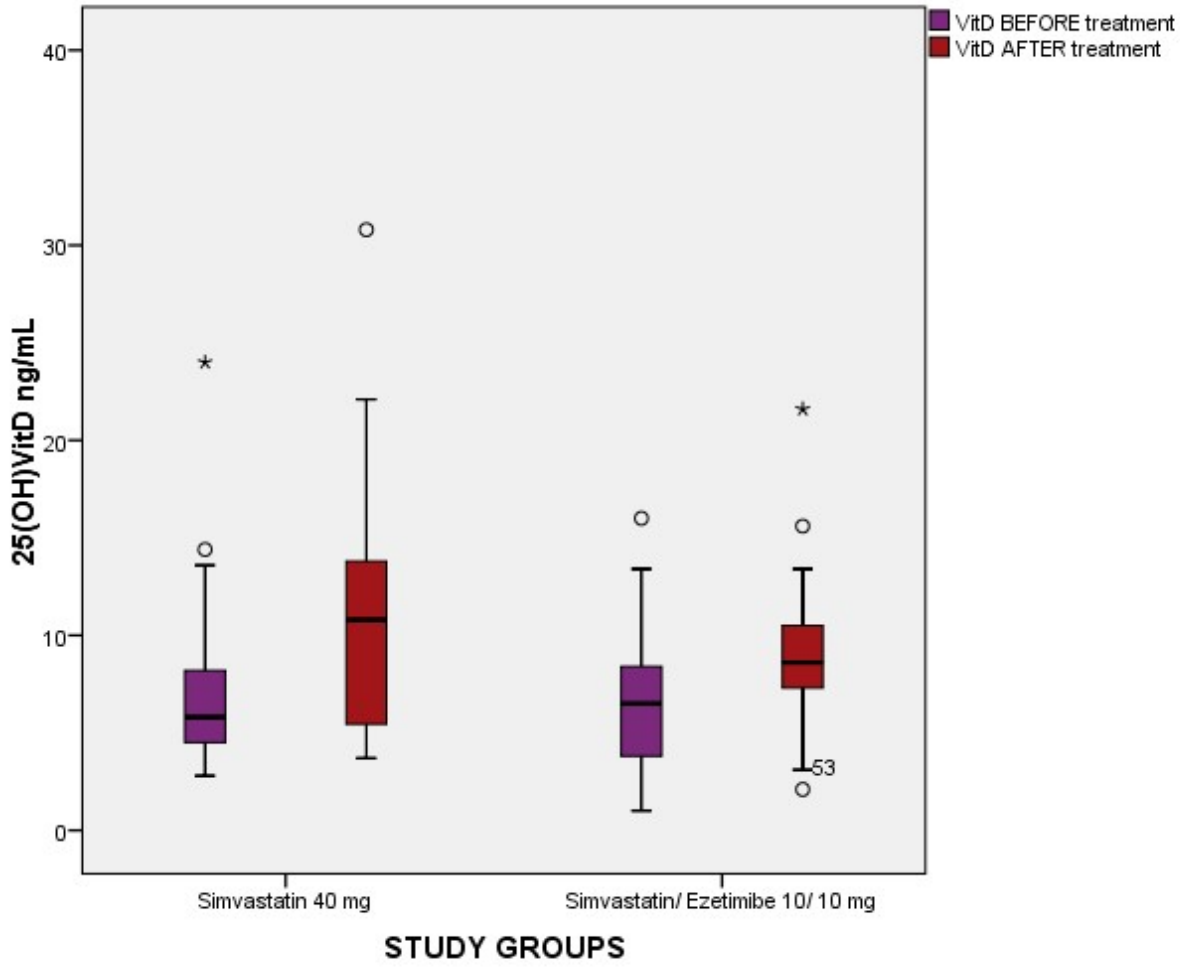
Characteristics	Simvastatin/ezetimibe 10/10mg	Simvastatin 40 mg	p
N (males/females)	25 (12/13)	25 (11/14)	NS
Age (years)	54±12	58±8	NS
Current smokers, %	41	38	NS
Body weight (kg)	81±10	78±6	NS
BMI (kg/m^2)	29±3	28±3	NS
WC (cm)	103±8	102±7	NS
SBP (mm Hg)	123±11	128±12	NS
DBP (mm Hg)	78±7	80±5	NS
TCHOL (mg/dL)	253±53	266±38	NS
LDL-C (mg/dL)	176±48	177±32	NS
Triglycerides (mg/dL)	109 (58-194)	104 (73-210)	NS
HDL-C (mg/dL)	59±11	62±12	NS
Apo AI (mg/dL)	150±24	158±25	NS
Apo B (mg/dL)	108±27	117±23	NS
Glucose (mg/dL)	96±12	99±13	NS
25(OH)VitD (ng/mL)	6.8 (0.2-16)	6.7 (0.5-24)	NS

Table 22. Serum metabolic parameters at baseline and after 3 months of treatment including either simvastatin/ezetimibe 10/10 mg (Simva/eze 10/10 mg) or simvastatin 40 mg (Simva 40 mg).

	Baseline	3 Months	p vs baseline	Change, %
TCHOL (mg/dL)				
Simva/eze 10/10 mg	253±53	169±29	0.000	-33.2
Simva 40 mg	266±38	175±27	0.000	-34.2
Triglycerides (mg/dL)				
Simva/eze 10/10 mg	109 (58-194)	92 (58-166)	0.01	-15.6
Simva 40 mg	104 (73-210)	94 (61-160)	0.01	-9.6
HDL-C (mg/dL)				
Simva/eze 10/10 mg	59±11	60±11	NS	+1.6
Simva 40 mg	62±12	64±12	NS	+1.6
LDL-C (mg/dL)				
Simva/eze 10/10 mg	177±32	92±19	0.000	-48.0
Simva 40 mg	176±48	97±28	0.000	-44.8
ApoAI (mg/dL)				
Simva/eze 10/10 mg	150±24	152±21	NS	+1.3
Simva 40 mg	158±25	164±24	NS	+3.7
ApoB (mg/dL)				
Simva/eze 10/10 mg	117±23	69±13	0.000	-41.0
Simva 40 mg	108±27	66±17	0.000	-38.8
Glucose (mg/dL)				
Simva/eze 10/10 mg	96±12	95±12	NS	-1.0
Simva 40 mg	99±13	105±15	NS	+6.0
25(OH)VitD (ng/mL)				
Simva/eze 10/10 mg	6.8 (1.0-16.0)	9.3 (2.1-21.6)	0.000	+36.7*
Simva 40 mg	6.7 (2.8-24.0)	12 (3.8-30.8)	0.008	+79.1

* p=0.04 difference between the percentage changes of 25(OH)VitD levels in the Simva/eze 10/10 mg versus the Simva 40 mg group

Figure 18. Serum 25(OH)VitD levels before and 3 months after treatment in the 2 treatment groups: simvastatin 40 mg or simvastatin/ezetimibe 10/10 mg



iii) The effect of rosuvastatin 40 mg (n=17) versus the effect of the combination of rosuvastatin 10 mg and fenofibrate 200 mg (n=13) and of the combination of rosuvastatin 10 mg and nicotinic acid/laropiprant 2 g (n=14) on 25(OH)VitD serum levels in patients with mixed dyslipidaemia.

No significant differences in baseline characteristics were noted among the three groups, including initial 25(OH)VitD levels and eGFR (Table 23).

Serum 25(OH)VitD levels did not significantly change in all study groups 3 months after treatment (Table 24). Specifically, in the switch to high-dose rosuvastatin and add-on-statin ER-NA/LRPT groups, there were non-significant decreases in 25(OH)VitD levels (-

4.7% and -14.8%, respectively), while in the add-on-statin fenofibrate group there was a non-significant increase (+13%) (Figure 19). Neither the changes in 25(OH)VitD, nor eGFR follow up levels did differ significantly between groups. Lipid changes are summarised in Table 24.

Table 23. Baseline characteristics of the patients with mixed dyslipidemia not at goal despite treatment with simvastatin 10-40 mg or atorvastatin 10-20 mg or rosuvastatin 5-10 mg, who participated in the study which aimed to investigate whether switching to high-dose rosuvastatin, add-on-statin nicotinic acid or add-on-statin fenofibrate would alter 25(OH)VitD serum levels.

	Switch to high-dose rosuvastatin	Add-on-statin ER-NA/LRPT	Add-on-statin fenofibrate	p
N (males/females)	17 (8/9)	14 (7/7)	13 (7/6)	NS
Age (years)	59±11	61±5	59±12	NS
Current smokers (%)	43	50	46	NS
Body weight (kg)	79±10	81±12	88±14	NS
BMI (kg/m^2)	29±2	29±3	31±4	NS
WC (cm)	98±12	98±7	103±12	NS
SBP (mm Hg)	131±11	130±11	129±12	NS
DBP (mm Hg)	78±6	82±10	80±13	NS
LDL-C (mg/dL)	121±40	115±35	112±32	NS
Triglycerides (mg/dL)	190 (173-210)	213 (190-254)	210 (189-260)	NS
HDL-C (mg/dL)	50±9	47±12	45±11	NS
Non-HDL-C (mg/dL)	157±40	156±37	155±34	NS
Glucose (mg/dL)	94±12	98±20	98±12	NS
eGFR (mL/min)	86±28	90±29	95±29	NS
25(OH)VitD (ng/mL)	16.8 (3.2-37)	12.8 (2.0-54.8)	14.5 (1.0-42)	NS

Table 24. Serum metabolic parameters of the patients with mixed dyslipidemia not achieving the treatment goal despite treatment with simvastatin 10-40 mg or atorvastatin 10-20 mg or rosuvastatin 5-10 mg, who participated in the study which aimed to investigate whether switching to high-dose rosuvastatin, add-on-statin nicotinic acid or add-on-statin fenofibrate would alter 25(OH)VitD serum levels at baseline and after 3 months.

	Baseline	3 months	Change %
Triglycerides (mg/dL)			
Switch to high-dose rosuvastatin	190 (173-210)	152 (140-184)	-20[‡]
Add-on-statin ER-NA/LRPT	213 (190-254)	128 (119-178)	-40[‡,§]
Add-on-statin fenofibrate	210 (189-260)	142 (118-170)	-32[‡,§]
HDL-C, mg/dL (mmol/L)			
Switch to high-dose rosuvastatin	50±9	51±10	+2[†]
Add-on-statin ER-NA/LRPT	47±12	53±11	+13[†,§,#]
Add-on-statin fenofibrate	45±11	48±10	+7[‡,§]
LDL-C (mg/dL)			
Switch to high-dose rosuvastatin	121±40	93±24	-23[‡,#]
Add-on-statin ER-NA/LRPT	115±35	93±34	-19[‡,#]
Add-on-statin fenofibrate	112±32	116±33	+4
Non-HDL-C (mg/dL)			
Switch to high-dose rosuvastatin	157±40	123±23	-22[‡,#]
Add-on-statin ER-NA/LRPT	156±37	117±34	-25[‡,#]
Add-on-statin fenofibrate	155±34	144±36	-7[†]
eGFR (mL/min)			
Switch to high-dose rosuvastatin	86±28	85±26	-0.01
Add-on-statin ER-NA/LRPT	90±29	90±26	0
Add-on-statin fenofibrate	95±29	99±27	+0.04
25(OH)VitD (ng/ml)			
Switch to high-dose rosuvastatin	16.8 (3.2-37)	16.0 (7.9-51.6)	-4.7
Add-on-statin ER-NA/LRPT	12.8 (2.0-54.8)	10.9 (2.4-34)	-14.8
Add-on-statin fenofibrate	14.5 (1.0-42)	16.4 (4.4-30.4)	+13.0

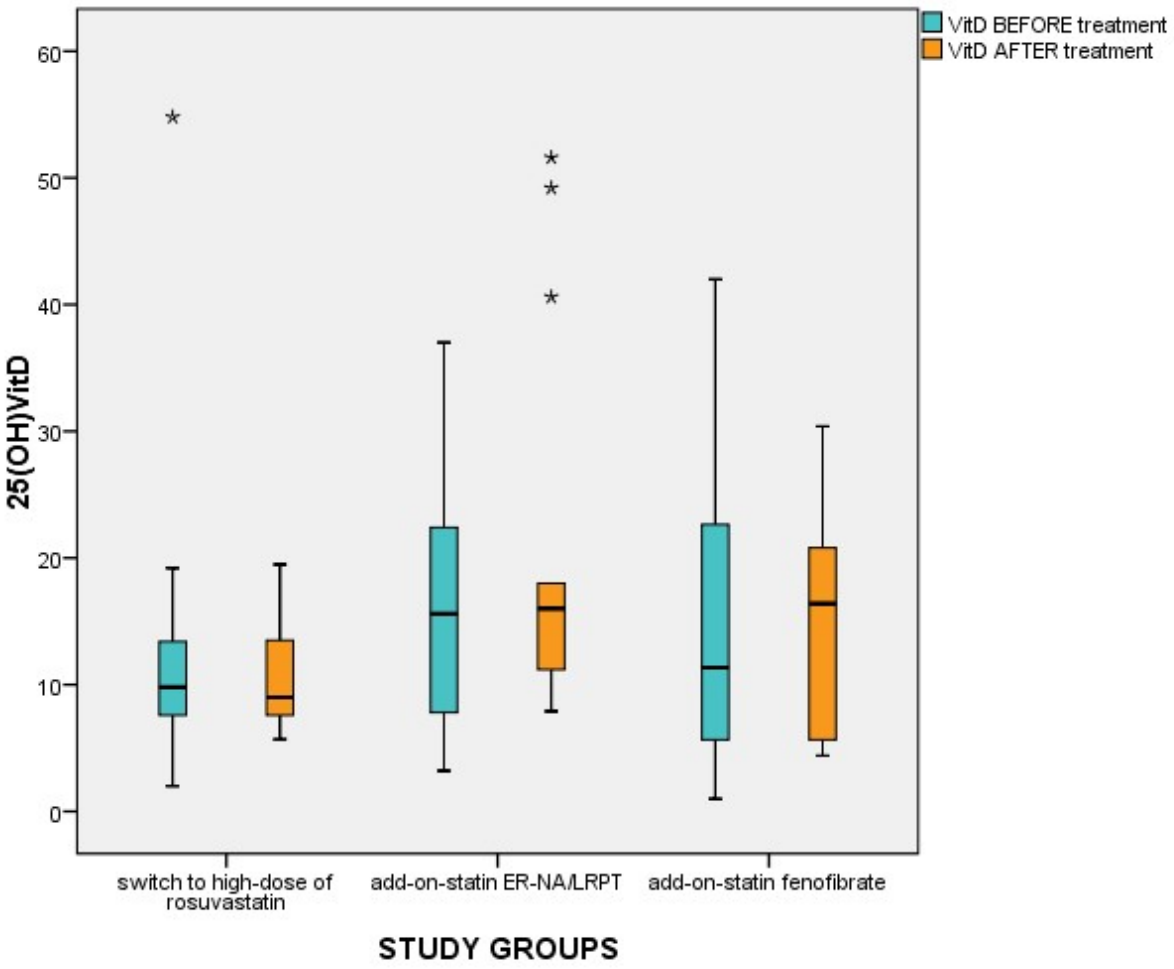
[†]p <0.01 compared to baseline levels

[‡]p <0.001 compared to baseline levels

[§]p <0.01 compared to the switch to rosuvastatin 40 mg group

[#]p <0.01 compared to the add-on-statin fenofibrate group

Figure 19. Serum 25(OH)VitD levels before and 3 months after treatment in the 3 treatment groups: switching to high-dose rosuvastatin, add-on-statin nicotinic acid or add-on-statin fenofibrate.



3.2.2 Assessment of the effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters of adults with MetS and adolescents with obesity.

i) The clinical and laboratory characteristics of **adult** participants in the prospective study (n=50) are shown in Table 25.

No significant differences in baseline characteristics were noted between the 2 groups, except for arylesterase activity which was significantly lower in the No-Suppl vs

Suppl group. There were also no differences in dietary intake between the groups at baseline and after the intervention. A 74% of the participants were VitD deficient at baseline (25(OH)VitD <20 ng/mL).

Three months after intervention, a comparable small weight reduction (1-2 kg) was achieved in both groups (Table 26), implying poor compliance to dietary intervention in both groups since a 500 Kcal/day reduction of energy for 3 months is reported to decrease body weight up to 5-7 kg). In the VitD group, 25(OH)VitD levels increased by 91% (from 16.0 (3.0-35.0) to 30.6 (8.4-67.0) ng/mL, p<0.001), while in the control group a non-significant increase by 30% (from 10.0 (4.0-39.6) to 13.0 (3.5-37.0) ng/mL, p=NS)) was seen (Figure 19). In both groups TCHOL, triglycerides, HDL-C, LDL-C, ApoA1, ApoB, fasting glucose, fasting insulin, HbA1c, HOMA index and diastolic blood pressure did not change significantly. SBP decreased by 3.7% (from 134±14 to 129±13 mmHg, p=0.05) in the VitD group, while it decreased only by 1.5% in the control group (from 132±13 to 130±16 mmHg, p=NS) (Table 26). In the VitD group the increase of 25(OH)VitD levels was negatively correlated with the decrease of SBP (r = -0.398, p = 0.049).

Also no significant changes in serum sdLDL levels, sdLDL proportion, mean LDL size and $LpPLA_2$ activity, as well as in leptin and adiponectin levels and leptin to adiponectin ratio were noted in both groups (Table 26). No differences in the changes of the same parameters were noticed between groups after the intervention.

Plasma ox-LDL levels and serum paraoxonase and arylesterase activities of PON-1 did not significantly change in both groups. Correction for lipid and apolipoprotein levels did not change the results (Table 26). Urine 8-iso-PGF_{2a} levels significantly decreased by 22.7% in the Suppl group [from 48.8 (26.8 to 137.1) to 37.7 (12.3-99.0) ng/mmol creatinine, p = 0.015], whereas a non-significant reduction of 14.4% was observed in the No-Suppl group [from 45.8 (16.6 to 99.3) to 39.2 (13.3-120.1) ng/mmol creatinine, p=NS]. However, the reduction in 8-iso-PGF_{2a} levels did not differ significantly between the 2 groups (Table 26).

Table 25. Baseline clinical and laboratory characteristics of adult participants

	VitD Suppl Group	Non-Suppl Group	P
N	25	25	NS
Age (years)	52±9	51±12	NS
Sex (m/f)	15/10	11/14	NS
Smoke (yes/no)	4/21	6/18	NS
Weight (kg)	89±16	89±13	NS
BMI (kg/m^2)	31.0±5.0	33.4±6.0	NS
Waist circumference (cm)	107±13	111±10	NS
SBP (mmHg)	134±14	132±13	NS
DBP (mmHg)	85±6	85±9	NS
TCHOL (mg/dL)	219±36	231±34	NS
HDL-C (mg/dL)	48±10	50±9	NS
LDL-C (mg/dL)	140±35	147±26	NS
Triglycerides (mg/dL)	150 (56-336)	146 (84-339)	NS
Apo A1 (mg/dL)	136±26	143±13	NS
Apo B (mg/dL)	92±25	107±16	NS
Fasting Glucose (mg/dL)	103±15	97±11	NS
Fasting Insulin (mg/dL)	10.5 (5.9-19.7)	9.2 (2-19.8)	NS
HOMA index	2.5 (0.4-6.6)	2.6 (1.5-4.6)	NS
HbA$_1$c (%)	6.2±0.8	6.0±0.5	NS
25(OH)VitD (ng/mL)	16 (3-35)	10 (4-40)	NS
PTH (pg/mL)	56±27	58±20	NS
MetS criteria (number of components)	3.4±2.0	3.0±2.0	NS
sdLDL-cholesterol (mg/dL)	7.0 (0.0-22.0)	9.0 (0.0-40.0)	NS
sdLDL proportion (%)	3.8±2.8	5.7±5.2	NS
Mean LDL size (nm)	266.5±3.9	264.8±6.3	NS
LpPLA$_2$ activity (nmol/mL/min)	57.4±13.3	56.4±15	NS
Leptin (ng/mL)	17.9 (3.9-93.7)	11.2 (3.0-106.3)	NS
Adiponectin (µg/mL)	8.1±3.3	8.2±3.6	NS
Leptin : Adiponectin Ratio	2.4 (0.4-75)	1.8 (0.3-76.2)	NS
Ox-LDL (U/L)	67.2±16.9	70.3±15.2	NS
Paraoxonase (U/L)	82.2 (16.1-207.4)	80.6 (19.5-287.4)	NS
Arylesterase (U/mL)	97.7±22.7	79.3±26.0	**0.013**
Urine 8-epi PGF$_{2a}$ (ng/mmol creatinine)	45.8 (16.6-99.3)	48.8 (26.8-137.1)	NS
Ox-LDL/LDL (U/mg)	0.060±0.008	0.050±0.010	NS
Ox-LDL/ApoB (U/mg)	0.070±0.008	0.080±0.040	NS
Paraoxonase/HDL (U/mg)	0.17±0.12	0.19±0.15	NS
Paraoxonase/ApoA1 (U/mg)	0.06±0.04	0.08±0.07	NS

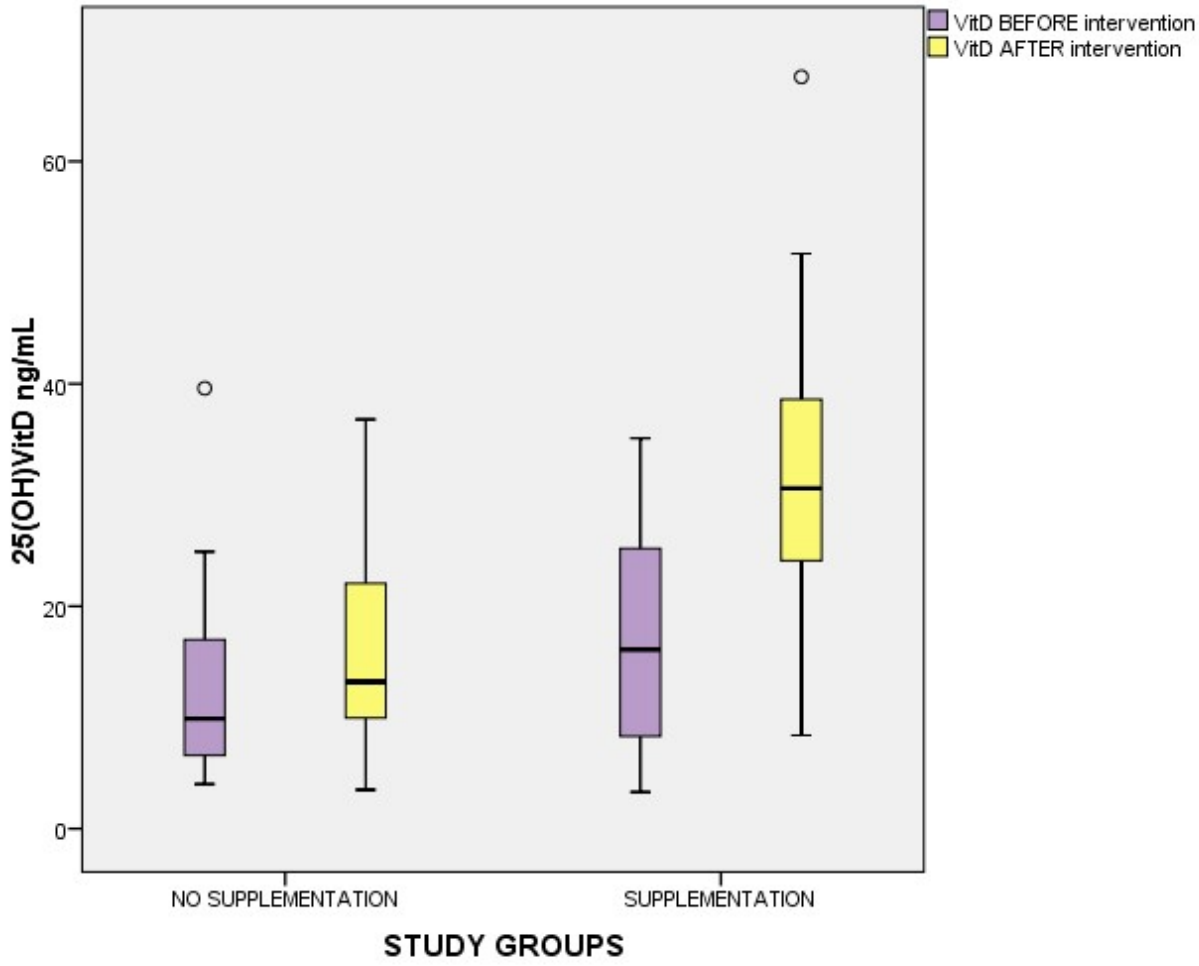
Table 26. Clinical and laboratory characteristics of adult participants at baseline and 3 months after intervention.

	Baseline	3 months	Change (%)	p* vs Baseline	p* change between groups
Weight (kg)					
VitD Suppl Group	89±16	88±17	-1.1	NS	NS
Non-Suppl Group	89±13	87±12	-2.2	**0.01***	
BMI (kg/m^2)					
VitD Suppl Group	31.0±5	30±5	-3.2	NS	NS
Non-Suppl Group	33.4±6	32±5	-4.1	**0.008***	
Waist circumference (cm)					
VitD Suppl Group	107±13	106±13	-0.9	NS	NS
Non-Suppl Group	111±10	107±9	-3.6	**0.002***	
SBP (mmHg)					
VitD Suppl Group	134±14	129±13	-3.7	NS	NS
Non-Suppl Group	132±13	130±16	-1.5	NS	
DBP (mmHg)					
VitD Suppl Group	85±6	83±6	-2.3	NS	NS
Non-Suppl Group	85±9	82±10	-3.5	NS	
TCHOL (mg/dL)					
VitD Suppl Group	219±36	224±37	+2.3	NS	NS
Non-Suppl Group	231±34	232±42	+0.4	NS	
HDL-C (mg/dL)					
VitD Suppl Group	48±10	49±9	+2	NS	NS
Non-Suppl Group	50±9	49±10	-2	NS	
LDL-C (mg/dL)					
VitD Suppl Group	140±35	145±34	+3.5	NS	NS
Non-Suppl Group	147±26	152±37	+3.4	NS	
Triglycerides (mg/dL)					
VitD Suppl Group	150 (56-336)	136 (46-261)	-9.3	NS	NS
Non-Suppl Group	146 (84-339)	131 (73-307)	-10.3	NS	
Fasting Glucose (mg/dL)					
VitD Suppl Group	103±15	102±23	-0.9	NS	NS
Non-Suppl Group	97±11	96±14	-1.0	NS	
Fasting Insulin (µU/mL)					
VitD Suppl Group	10.5 (5.9-19.7)	9.3 (3.1-27.9)	-11.4	NS	NS
Non-Suppl Group	9.2 (2-19.8)	8.4 (4.6-15.9)	-8.6	NS	
HOMA index					
VitD Suppl Group	2.5 (0.4-6.6)	2.3 (0.7-11.5)	-8	NS	NS
Non-Suppl Group	2.6 (1.5-4.6)	1.8 (1.0-4.5)	-3	NS	
HbA1c (%)					
VitD Suppl Group	6.2±0.8	6.2±0.7	0	NS	NS
Non-Suppl Group	6.0±0.5	5.6±0.5	-6.6	NS	
25(OH)VitD (ng/mL)					
VitD Suppl Group	16.0 (3.0-35.0)	30.6 (8.4-67.0)	+91	**0.000***	
Non-Suppl Group	10.0 (4.0-39.6)	13.0 (3.5-37.0)	+30	NS	**0.007***
PTH (pg/mL)					
VitD Suppl Group	56±27	51±19	-9	NS	NS

Non-Suppl Group	58±20	48±19	-17	NS	
sdLDL-cholesterol (mg/dL)					
VitD Suppl Group	9.0 (0.0-40)	4.0 (0.0-46)	-55.5	NS	NS
Non-Suppl Group	7.0 (0.0-22)	5.0 (2.0-25)	-28.6	NS	
sdLDL proportion (%)					
VitD Suppl Group	5.7±5.2	4.5±4.4	-21.0	NS	NS
Non-Suppl Group	3.8±2.8	3.3±2.3	-13.2	NS	
Mean LDL size (nm)					
VitD Suppl Group	264.8±6.3	266.6±5.2	+0.7	NS	NS
Non-Suppl Group	266.5±3.9	267.0±3.5	+0.2	NS	
$LpPLA_2$ activity (nmol/mL/min)					
VitD Suppl Group	56.4±15.0	55.7±13.5	-1.2	NS	NS
Non-Suppl Group	57.4±13.3	52.7±12.4	-8.1	NS	
Leptin (ng/mL)					
VitD Suppl Group	11.2 (3.0-106.3)	10.3 (2.8-43.8)	-8.0	NS	NS
Non-Suppl Group	17.9 (39.0-93.7)	14.6 (3.0-66.2)	-18.4	NS	
Adiponectin (µg/mL)					
VitD Suppl Group	8.2±3.6	8.1±3.9	-1.2	NS	NS
Non-Suppl Group	8.1±3.3	8.3±3.1	+2.4	NS	
Leptin : Adiponectin Ratio					
VitD Suppl Group	1.8 (0.3-76.2)	1.4 (0.3-52.6)	-22.2	NS	NS
Non-Suppl Group	2.4 (0.4-75)	1.7 (0.4-76.2)	-29.2	NS	
Urine 8-epi PGF2a (ng/mmol creatinine)					
VitD Suppl Group	48.8 (26.8-137.1)	37.7(12.3-99)	-22.7	0.015	NS
Non-Suppl Group	45.8 (16.6-99.3)	39.2 (13.3-120.1)	-14.4	NS	
Ox-LDL (U/L)					
VitD Suppl Group	70.3±15.2	75.9±21.2	+7.9	NS	NS
Non-Suppl Group	67.2±16.9	67.3±19.3	+0.1	NS	
Paraoxonase (U/L)					
VitD Suppl Group	80.6 (19.5-287.4)	97.2 (21.0-236.0)	+20.6	NS	NS
Non-Suppl Group	82.2 (16.1-207.4)	95.7 (25.0-202.5)	+16.4	NS	
Arylesterase (U/mL)					
VitD Suppl Group	79.3±26	81.2±22.7	+2.4	NS	NS
Non-Suppl Group	97.7±22.7	91.7±22.8	-6.3	NS	
OxLDL/LDL (U/mg)					
VitD Suppl Group	0.05±0.01	0.05±0.01	0.0	NS	NS
Non-Suppl Group	0.06±0.008	0.06±0.02	0.0	NS	
OxLDL/ApoB (U/mg)					
VitD Suppl Group	0.08±0.04	0.07±0.01	-12.5	NS	NS
Non-Suppl Group	0.07±0.008	0.08±0.02	+14.3	NS	
Paraoxonase/HDL (U/mg)					
VitD Suppl Group	0.19±0.15	0.19±0.15	0.0	NS	NS
Non-Suppl Group	0.17±0.12	0.19±0.12	+11.7	NS	
Paraoxonase/ApoA1 (U/mg)					
VitD Suppl Group	0.08±0.07	0.07±0.05	-12.5	NS	NS
Non-Suppl Group	0.06±0.04	0.07±0.04	+16.6	NS	

* p was considered significant if it was <0.01

Figure 20. Serum 25(OH)VitD levels at baseline and 3 months after treatment in adult participants in the prospective study



ii) Table 27 shows the clinical characteristics and laboratory findings at baseline and after intervention in **adolescents** with obesity and VitD insufficiency/deficiency (n=15) who were given VitD supplementation along with dietary instructions. Over half of them (9 out of 15, 60%) were VitD deficient (25(OH)VitD) <20 ng/mL). Three months later, 25(OH)VitD increased significantly by 88.4% [from 17.3 (12.5-27.8) to 32.6 (14.3-68.0) ng/mL, p=0.005] (Table 27). Interestingly, at the same time significant reductions were seen in HbA_1c (p=0.03) and leptin levels (p=0.03). On the contrary, LDL-C levels significantly increased (p=0.022). Other clinical and laboratory metabolic parameters (BMI, WC, BP, TCHOL, HDL-C, triglycerides, glucose, insulin, HOMA index, PTH) along with oxidative stress markers (ox-LDL, paraoxonase, arylesterase and urine isoprostanes) remained unchanged (Table 27).

Table 27. Metabolic parameters of adolescents with obesity and VitD insufficiency/deficiency at baseline and 3 months after intervention

	Baseline	After 3 Months	p vs baseline	Change, %
N (males/females)	15 (10/5)			
Age (years)	15.4±1.8			
Smoking, (yes/no)	5/10	5/10		
Weight (kg)	97.6±16.9	96.4±19.5	NS	-1.3
BMI (kg/m^2)	35.0±7.9	34.1±8.4	NS	-2.6
WC (cm)	115.6±1.2	114.6±1.5	NS	-0.8
SBP (mm Hg)	134±11	129±17	0.043	-3.9
DBP (mm Hg)	75±8	73±10	NS	-3.0
TCHOL (mg/dL)	154.8±10.9	163.4±15.5	NS	+5.5
HDL-C (mg/dL)	40.0±4.7	40.8±5.0	NS	+2.0
LDL-C (mg/dL)	85.4±9.5	92.1±15.8	**0.022***	+7.8
Triglycerides (mg/dL)	83.0 (65.0-208.0)	76.0 (56.0-188.0)	NS	-8.4
Fasting glucose (mg/dL)	88.6±8.9	89.6±7.7	NS	+1.1
Fasting insulin (μU/mL)	15.4 (7.1-18.7)	13.9 (7.9-20.0)	NS	-9.7
HOMA index	3.7 (1.3-4.4)	2.9 (1.8-4.9)	NS	-21.6
HbA$_1$c (%)	5.8±0.2	5.5±0.1	**0.03***	-5.2
Leptin (ng/mL)	19.7 (7.8-45.5)	15.1 (4.3-37.3)	**0.03***	-23.3
25(OH)VitD (ng/mL)	17.3 (12.5-27.8)	32.6 (14.3-68.0)	**0.005***	+88.4
iPTH (pg/mL)	32.0±16.6	37.5±13.3	NS	+17.2
Ox-LDL (U/L)	56.5±12.4	57.3±14.6	NS	+1.4
Paraoxonase (U/L)	59.8 (47.6-151.4)	61.5 (51.5-161.3)	NS	+2.8
Arylesterase (U/mL)	70.5±11.6	66.0±9.5	NS	-6.4
Urine 8-epi PGF2α (ng/mmol creatinine)	41.5 (23.6-117.4)	30.0 (22.0-41.5)	NS	-27.7

* p was considered significant if it was at ≤ 0.03

CHAPTER IV.

DISCUSSION

4.1 Discussion for the Cross-sectional study

Serum 25(OH)VitD levels in adults with MetS and adolescents with obesity and their relationship with CVD risk factors

The **adults** with MetS were found to have significantly lower 25(OH)VitD levels compared to controls, which were inversely associated with triglycerides and sdLDL-C levels. However, in the multivariate regression analysis, the sdLDL-C levels were found to be affected only by triglycerides and not by 25(OH)VitD concentration. There was no association of 25(OH)VitD with waist circumference, blood pressure, HDL-C and fasting glucose (the other diagnostic criteria for MetS) as well as emerging CVD risk factors like $LpPLA_2$ and hsCRP.

Considerable research has been made on associations between VitD levels and the prevalence of the MetS, in support of an inverse relationship between serum 25(OH)VitD and MetS. The large NHANES III and NHANES 2003-2004 have shown a significant inverse association between serum 25(OH)VitD concentration and MetS as a whole, as well as with each one of its components [453, 454]. Also two more recent MAs claimed a significant association between high 25(OH)VitD levels and reduced MetS prevalence [382, 383]. Several epidemiological studies have also shown an association between low 25(OH)VitD serum levels and MetS and/or its components [92, 319, 454-456], while others did not [457-461]. Thus a relationship may exist between 25(OH)VitD levels and MetS but cannot be alleged as causation. A recently published study in diabetic patients with MetS showed a high prevalence of VitD deficiency and an inverse correlation with glycemic control and CVD risk factors, except for HDL-C, insulin resistance and obesity. Also SBP was the only factor which could be predicted from VitD concentrations [462]. Moreover, a recent study provided some evidence that the active VitD metabolite, $1,25(OH)_2$VitD, acting like a potent hormone that binds to VDRs and regulates transcription of several genes, is also associated with MetS and its components (i.e. lower risk for high triglycerides and low HDL-C) [463]. The same study showed inverse associations between 25(OH)VitD and MetS, triglycerides and waist circumference (WC) [463].

Our results are in agreement with previous findings showing that subjects with MetS have significantly lower 25(OH)VitD levels compared with non-MetS. A possible explanation for this observation could be the sequestration of the fat soluble VitD in the adipose tissue [365], which is in abundance in MetS subjects, making the stores less available to become biologically activated [155]. Of note, VitD deficiency was reported more prevalent in morbidly obese patients with MetS compared with those obese without MetS in one study [464]. However, we cannot exclude the possibility that obesity and associated co-morbid conditions could reduce outdoor physical activity and sun exposure, and subsequently lead to VitD deficiency. On the other hand, VitD deficiency could have an indirect effect on the development of obesity, which is a basic characteristic of MetS.

PTH which is reported elevated in VitD deficiency increases the cytosolic calcium level in isolated adipocytes [465], thus impeding the catecholamine-induced lipolysis [466] and promoting the expression of fatty acid synthetase [334]. There is also evidence that apart from low VitD, elevated PTH levels could be associated with glucose intolerance and insulin resistance [251, 322, 467]. Reis et al showed that MetS was positively related with PTH concentration among older men but not women [454, 459]. However Lee et al did not find any relationship between MetS and PTH levels in men and no evidence of an age interaction ([320]. Similarly in our study, PTH levels were in the normal range in the MetS and not significantly different from the non-MetS subjects.

Dyslipidemia (with increased triglycerides and low HDL-C levels) is a hallmark of MetS and may substantially contribute to the increased CVD risk observed in this population. Our study confirmed an inverse relationship between 25(OH)VitD and triglycerides, but we could find no significant association between 25(OH)VitD and HDL-C. Cross-sectional analyses report an association between optimal VitD status and a favorable lipid profile, but the results are not consistent. A large cross-sectional analysis of 108,711 subjects showed that the “optimal” 25(OH)VitD group relative to the “deficient” group displayed lower TCHOL, lower LDL-C, higher HDL-C and lower triglycerides [316]. Another large study (n=15,088), based on NHANES III, also found that mean 25(OH)VitD levels were lower in subjects with hypertriglyceridemia [100]. The association between serum 25(OH)VitD and lipids has been analyzed also in other studies, but with inconsistent results. Jorde et al. (n=10,105) found positive associations between serum 25(OH)VitD and serum TCHOL, HDL-C and LDL-C, and negative associations

between serum 25(OH)VitD and both LDL-C/HDL-C ratio and triglycerides [317]. The same researchers also reviewed 22 other cross-sectional studies and showed that serum 25(OH)VitD was positively associated with HDL-C, resulting in a favorable LDL-C (or TCHOL) to HDL-C ratio, and negatively associated with triglycerides [318]. Hypponen et al. (n= 6,810) [319] and Lee et al. (n=3,069) [320] found a negative association between serum 25(OH)VitD and triglycerides, but the association between serum 25(OH)VitD and HDL-C was not significant after adjustment for confounders, which is in the same line with our results. Also Chiu et al (2004) reported a negative correlation of 25(OH)VitD concentration with TCHOL and LDL-C but no relation with HDL-C [322]. Others again found no association of 25(OH)VitD with HDL-C but also with triglycerides and negative association with LDL-C [251, 321].

Of interest is the inverse relationship we found between 25(OH)VitD serum levels and sdLDL-C but not with LDL size on the univariate analysis. However multivariate regression analysis showed that sdLDL-C levels were affected only by serum triglycerides and not by 25(OH)VitD levels which is in agreement with others [327]. Also previous studies have proposed that the most important single determinant of LDL particle distribution and size is the pool of triglyceride-rich lipoproteins [443]. In general, the higher the triglyceride levels, the smaller the LDL size [327]. Furthermore there is considerable evidence that sdLDL-C contribute to the pathogenesis of atherosclerosis and accelerate its progression [327].

We also searched for a possible relationship between 25(OH)VitD and $LpPLA_2$ as well as hsCRP, which are considered as powerful predictors of CVD [468]. We could find no association between 25(OH)VitD and $LpPLA_2$ activity or hsCRP.

Observational data strongly associate low 25(OH)VitD levels with an increase in blood pressure and higher risk of hypertension [197-199]. An analysis of NHANES III 1988–1994 of 12,644 participants aged >20 years showed an inverse association between VitD levels and blood pressure [200]. Similar results were obtained from analysis of NHANES 2003–2006 of 7228 participants [201]. However this was not the case in the present study.

Several observational studies have also shown lower 25(OH)VitD levels in patients with T2DM compared with the general population, as well as an inverse association between 25(OH)VitD levels and fasting plasma glucose, impaired glucose tolerance, and

HbA_1c levels [100, 245-250]. In this analysis though we found no association of 25(OH)VitD levels with fasting glucose in MetS patients.

With regard to **adolescents** in this cross-sectional study we observed that VitD deficiency was more prevalent among those with obesity (73.9% vs 17.6% in normal weight controls). Adolescents with obesity had significantly lower 25(OH)VitD levels compared with normal weight controls. In adolescents with obesity, 25(OH)VitD was inversely associated with leptin even after adjustment for BMI. On the contrary, 25(OH)VitD was not related with other parameters, such as BMI, BP, lipids, glucose, insulin, HOMA index, adiponectin, leptin/adiponectin ratio and visfatin levels.

Worldwide studies report high incidences of suboptimal VitD status in obese youth (70%) [351], in agreement with our findings, which have been attributed to reasons similar to those described in adults.

Recent research indicated that 25(OH)VitD may interfere in the regulation of the adipoinsular axis as well. Some studies in children/adolescents with obesity have found a positive correlation between 25(OH)VitD levels and adiponectin compared with controls [312, 313], but this was not confirmed in the present study, since the adiponectin levels did not differ between the groups despite their differences in their VitD status. Others showed an inverse correlation of 25(OH)VitD levels with the leptin/adiponectin ratio [309]. However there are no reports to our knowledge of an association between 25(OH)VitD and leptin levels alone in children and adolescents. Interestingly, our results showed that 25(OH)VitD levels were inversely related to leptin levels (but not to leptin/adiponectin ratio) in adolescents with obesity. On the other hand, no differences were seen in the visfatin levels between obese and normal weight adolescents in the present study and no relationship with 25(OH)VitD, while we found no relevant data in the literature.

Accumulating evidence has indicated that serum 25(OH)VitD levels may be negatively associated with blood pressure in most [78, 218, 219], but not all [220] studies in youth. Interestingly, a recent analysis from NHANES 2007-2010 (n=2908, aged 8-18 years) showed that 25(OH)VitD was not associated with SBP when adjusting for BMI [221]. In our study also no correlation was detected between 25(OH)VitD levels and SBP or DBP in the adolescents with obesity.

Studies regarding the relationship between VitD status and lipid profile in children/adolescents with obesity have also reported inconsistent results, with some being positive [229], and others not [220, 345]. Even in studies investigating correlations between VitD and lipids, there is great disparity. Hence, 25(OH)VitD has been reported to be inversely related to TCHOL [350] and triglycerides [352], negatively [350] or positively related to LDL-C [269], and positively related to HDL-C levels [78, 228, 351]. In the present study we found no relationship between 25(OH)VitD levels and lipids.

Moreover, many cross-sectional studies in young ages showed that 25(OH)VitD levels correlated with fasting glucose [191, 192], fasting insulin [275, 276] and/or HOMA-IR [193, 220, 275], while some did not [274, 405]. Similarly, in the present study 25(OH)VitD was not found to be associated with any carbohydrate metabolism marker.

These inconsistences may be attributed to the relatively small number of participants which does not allow us to generalize these results. Another main limitation is also that a causal relationship between 25(OH)VitD and emerging risk factors in subjects with MetS could not be assessed because of the cross-sectional design of this part of the study. In the prospective/interventional part of our study we tried to examine more precisely some of these associations, as analysed below.

4.2 Discussion for the Prospective study

4.2.1 Possible impact on 25(OH)VitD serum levels from the use of lipid-lowering medication in patients with dyslipidaemia.

i) In this analyses we showed, for the first time to our knowledge, that high-dose rosuvastatin monotherapy 40 mg as well as usual-dose rosuvastatin 10 mg plus micronized fenofibrate 200 mg and usual-dose rosuvastatin 10 mg plus omega-3 fatty acids 2 g were all associated with significant and to a similar degree increases in serum 25(OH)VitD levels.

Grimes first proposed that since the unexpected and unexplained clinical benefits of statins have also been shown to be properties of 25(OH)VitD, and therefore mimic many of the actions of 25(OH)VitD, they may be considered as VitD analogues [469].

Several statins, such as lovastatin [412], simvastatin [413], atorvastatin [414], and especially rosuvastatin [410, 470] appeared to increase 25(OH)VitD serum levels contrary to the initial concern that statins would impair the formation of steroids dependent on cholesterol synthetic pathway, including VitD synthesis [416]. Several potential mechanisms have been proposed to explain the observed increase in 25(OH)VitD concentrations after statin therapy, as analysed in the introduction. On the contrary, more recent studies found no effect of statin use on 25(OH)VitD concentration [417, 418] and so did a systematic review and MA [419]. Another MA was inconclusive, since across RCTs, treatment with statins was associated with a significant increase in serum VitD concentrations, but across studies of non-RCT design, treatment with statins was associated with a decrease in VitD concentrations [420].

These contradictory findings do not allow us to reach general conclusions, and may be attributed to different populations studied, to various statin types studied (that have different metabolism, potency and bioavailability), the different doses, different baseline 25(OH)VitD levels and follow-up intervals. Moreover, other limitations of the existing studies are their small sample size, research design and subject traits (gender, ethnicity, age, etc).

In our study, the high-dose rosuvastatin monotherapy was associated with a 53% increase in 25(OH)VitD levels after 3 months of treatment. Previous studies of Yavuz et al [410] and Ertugrul et al [470] have also examined the effect of rosuvastatin at 10 and 20 mg daily on 25(OH)VitD levels after 8 weeks of treatment, and found significant increases in 25(OH)VitD concentrations by 159% and 198%, respectively, which were even higher than ours. On the contrary, Anagnostis et al reported no effect of rosuvastatin 10 mg daily on 25(OH)VitD levels after 12 weeks of treatment of patients with dyslipidemia [424]. The reasons for this difference are mainly unknown. Differences in baseline 25(OH)VitD concentration, study population and the different doses used as well as study duration may account for this inconsistency. Nevertheless, this increase in 25(OH)VitD levels may represent a novel pleiotropic effect of statins [471].

We also found that combinations of the usual-dose rosuvastatin with either fenofibrate 200 mg or with omega-3 fatty acids 2 g were associated with significant increases in 25(OH)VitD levels of 64% and 61%, respectively. These increases were of similar degree to the high-dose rosuvastatin monotherapy. Based on our study design we

cannot conclude whether fenofibrate or omega-3 fatty acids have synergistic effects in increasing 25(OH)VitD concentrations or if this increase is only associated with rosuvastatin, independently of dose. To our knowledge, there are no published data concerning possible effects of fibrates or omega-3 fatty acids on 25(OH)VitD serum levels.

However, there are some limitations in the design of this cross-sectional study. We included no group receiving monotherapy with fenofibrate or omega-3 fatty acids, as statin use must be a component of any lipid-lowering treatment. Subsequently, we cannot distinguish if fenofibrate or omega-3 fatty acids alone might increase 25(OH)VitD levels, or if it is the rosuvastatin per se independently of dosage (i.e. usual dose (10mg) and maximum dose (40 mg)) that leads to similar increases in 25(OH)VitD levels. Additional limitations include the open-label design and the relatively short period of follow-up (3 months).

On the other hand we tested the efficacy of treatments used in every-day clinical practise in the management of combined dyslipidaemia, on altering 25(OH)VitD levels, which may be clinically important, since low levels of 25(OH)VitD have been recognised as an independent CVD risk factor [91]. Additionally, this was an adequately powered study, with all comparisons being adjusted for baseline levels and endpoints being blindly assessed.

ii) Our study also showed that simvastatin 40 mg was associated with a more than double increase in 25(OH)VitD levels (79.1%) compared with simvastatin/ezetimibe 10/10 mg (36.7%) in patients with primary hypercholesterolemia while the lowering effect in TCHOL, LDL-C and triglycerides was similar in both groups.

Data from other studies are inconclusive for simvastatin. A small study showed that treatment with 10 and 20 mg raised plasma levels of 25(OH)VitD and 1,25$(OH)_2$VitD (the active metabolite) in a dose-dependent ratio [413]. On the contrary, other studies found no effect of simvastatin on 25(OH)VitD [421-423]. An RCT examining patients with dyslipidemia, found no effect of simvastatin 40 mg treatment compared with placebo on 25(OH)VitD levels after one month [421]. Also a recent RCT failed to show any effect of simvastatin 40 mg treatment compared with placebo for one year on 25(OH)VitD levels in healthy postmenopausal women with osteopenia but without known hyperlipidemia [422]. An explanation for the controversies given by the authors was that the observed increase of

25(OH)VitD levels after statin treatment found in previous uncontrolled studies might be due to unmeasured changes in indices affecting 25(OH)VitD levels. These include the coincidence of lifestyle changes in relation to the administration of statin drugs (i.e. increased exercise and sun exposure, consumption of food items rich in VitD or supplements) [422]. However in the present study, treatment with simvastatin 40 mg for 3 months resulted in a 79.1% increase of serum 25(OH)VitD levels. Of note, most of our patients were VitD deficient at baseline. The reasons are unknown but may be related to limited sun exposure during the autumn and winter seasons that they were examined. On the contrary, in Rejnmark's et al study only 3 patients were VitD deficient (25(OH)VitD <10 ng/mL) [422]. It can be assumed that the lower the 25(OH)VitD baseline levels, the greater the observed increase following statin treatment.

The more than double 25(OH)VitD increase with simvastatin 40 mg compared with simvastatin/ezetimibe 10/10 mg found in this study could be attributed either to a dose-dependent effect of simvastatin on raising 25(OH)VitD or to an amelioration of VitD intestinal absorption by ezetimibe or both. It has not been clarified if there is a dose-dependent effect of statins on 25(OH)VitD levels, or what effect could ezetimibe monotherapy have on serum 25(OH)VitD levels in humans.

The lipid lowering effect of ezetimibe is mediated through a specific inhibition of Niemann-Pick C1 Like 1 (NPC1L1) cholesterol transporter, which recently was shown to be moderately involved in 25(OH)VitD intestinal absorption as well [472]. Notably, the contribution of intestinal absorption to serum VitD levels, when no supplements are taken, is relatively small, since 80-90% of VitD derives from endogenous production in the skin [473]. Therefore, it can be assumed that ezetimibe's effect on serum 25(OH)VitD levels may be of limited extent.

The increase in 25(OH)VitD levels may represent a novel pleiotropic effect of statins, as discussed earlier. Ezetimibe has been found to either enhance or abrogate the pleiotropic effects of statins [474], but its effect on 25(OH)VitD levels is largely unstudied.

There are certain limitations in this study too. We included no group receiving monotherapy with ezetimibe, as statins are first-line lipid-lowering drugs. Also, we did not include a 10 mg simvastatin group as current guidelines suggest a -40% decrease in LDL-C, which can only be achieved with a dose of 40 mg simvastatin. Additional limitations include the open-label design and the relatively short period of follow-up (3 months). Even

though the number of patients in each treatment group was small it was an adequately powered study.

Moreover, this was a clinically relevant study, since we tested the efficacy of equivalent treatments in terms of lowering LDL-C levels and which are used in everyday clinical practice on the 25(OH)VitD levels. Additionally, all comparisons were adjusted for baseline levels and end points were blindly assessed.

iii) Furthermore, in this study, we showed for the first time to our knowledge, that neither the switch to high-dose rosuvastatin 40 mg, nor add-on-statin (10 mg) ER-NA/LRPT 2 g or fenofibrate 200 mg was associated with significant changes in 25(OH)VitD levels in patients with mixed dyslipidemia and not at goal while on treatment with a conventional statin dose.

In contrast to our previous work mentioned in section (i) in the present analysis, the switch to the highest dose of rosuvastatin was not associated with any change in 25(OH)VitD levels probably due to the different population who also had not responded to previous treatment for lowering lipid levels. Also the positive effect of rosuvastatin on 25(OH)VitD levels seems to be dose-independent.

Hence the initial treatment of these patients with either simvastatin 10-40 mg or atorvastatin 10-20 mg or rosuvastatin 5-10 mg may have already affected VitD metabolism so that the switch to the maximum dose of rosuvastatin caused no further change despite the low 25(OH)VitD levels. A suggestion that could be made is that since they do not respond to the lipid lowering drugs, in a similar way there is no response for VitD metabolism. However, data from direct comparisons of several statins at various dosages in such populations are lacking and safe conclusions cannot be reached.

Neither the addition of fenofibrate (200 mg) to a standard statin dose was associated with significant changes in 25(OH)VitD levels. Taking into consideration our previous findings, i.e. that the combined treatment with rosuvastatin 10 mg plus fenofibrate 200 mg led to a 64% increase ($p=0.001$) in 25(OH)VitD levels one may speculate that fenofibrate has limited effect on 25(OH)VitD levels and that the observed increase could be attributed to rosuvastatin alone. However, only studies with fenofibrate monotherapy could answer this question.

Furthermore, the addition of ER-NA/LRPT to a standard statin dose did not significantly affect 25(OH)VitD levels. There are no other data about the effect of ER-NA/LRPT on 25(OH)VitD concentration in the literature. Of note, ER-NA/LRPT has been withdrawn from the market due to a plethora of serious nonfatal side effects [431].

Notably, both add-on-statin ER-NA/LRPT [475, 476] and fenofibrate studies [477] did not show any clear additional clinical profit compared with statin monotherapy as well as with respect to VitD levels as shown in the present study.

A major limitation of this study is that we included no group receiving monotherapy with ER-NA/LRPT or fenofibrate, as statin use must be the basis of any lipid-lowering treatment. Also, study participants did not have identical baseline 25(OH)VitD levels, but they were taken into account as covariates in statistical analysis. Last, the number of patients analysed was rather small.

On the other hand, this is a clinically relevant study too, conferring novel results on the effect of switching to the highest dose of rosuvastatin from a standard statin dose or adding-on-statin nicotinic acid or fenofibrate on 25(OH)VitD levels.

Overall, our results showed that rosuvastatin and simvastatin treatment were associated with increases in serum 25(OH)VitD levels. Other lipid-lowering drugs including ezetimibe, fenofibrate, omega-3 fatty acids and nicotinic acid seemed to have minimal if any effect on 25(OH)VitD serum concentration of our patients, but their exact effect could not be clarified unequivocally due to study design limitations.

This is a novel field we tried to investigate with the present research that merits further investigation with suitably designed studies to clarify possible benefits in cardiovascular and overall health. Raising 25(OH)VitD serum levels through other ways (for example through sun exposure, prescription of supplements or even by lipid-lowering treatment) could be clinically important, since VitD deficiency has emerged as a cardiovascular risk factor [91]. Whether optimizing 25(OH)VitD serum status can prevent or ameliorate various chronic diseases is however currently under debate.

4.2.2 Effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters of adults with MetS and adolescents with obesity.

i) In this study we showed that VitD supplementation (2000 IU/day) plus dietary intervention in **adults** with MetS was not associated with any significant change in various CVD risk factors compared with dietary intervention alone despite the significant increases in their 25(OH)VitD levels. Of note is that our MetS population was 74% VitD deficient at baseline (25(OH)VitD ~13 ng/mL and after VitD supplementation reached normal levels, while the no-supplemented did not.

Several epidemiological studies have indicated an association between low 25(OH)VitD serum levels and MetS and/or its components [92, 319, 454-456], while others did not confirm these associations [457-461].

However the "VitD-CVD hypothesis" in MetS subjects has not been confirmed by reversal of CVD risk factors through VitD supplementation in a number of studies. In our study the 91% increase in 25(OH)VitD serum levels was not associated with changes in lipids, carbohydrate metabolism parameters and DBP, but with only a 3.7% decrease in SBP which was not significant and did not differ from the non-suppl group. Also no large RCT has been carried out with onset of MetS/diabetes as the primary outcome [92]. A small RCT that included 126 individuals with MetS and VitD deficiency and treated them with either 700 IU/day of VitD or placebo for 1-year, also found no improvement in the MetS risk factors despite the significant increase in serum VitD levels in the treated group [388]. On the contrary, a recently published study in 160 postmenopausal women with VitD deficiency found that supplementation with 1000 IU VitD for 9 months was associated with a reduction in the MetS risk profile as well as hypertriglyceridemia and hyperglycemia [389]. Another study with 80 MetS subjects randomized to receive 50,000 IU VitD/week for 16 weeks found a significant change only in triglycerides but in no other metabolic or anthropometric parameters [478]. In conclusion, to date it is still unclear whether VitD replacement may translate to substantial health benefits in subjects with MetS.

Some mechanisms for the possible anti-hypertensive effect of VitD have been suggested and were extensively analyzed in the introduction. However, data from interventional studies and especially from RCTs investigating the effect of VitD

supplementation on blood pressure are conflicting. A MA of 4 RCTs found a reduction of SBP by -2.44 mm Hg, but no effect on DBP [213], which are similar to our findings. Another MA by Witham et al of 11 RCTs showed that administration of VitD and ultraviolet A and B radiation was associated with a non-significant SBP reduction by -3.5 mm Hg and a significant DBP reduction by -3.1 mm Hg [214]. On the other hand, no beneficial effects of VitD supplementation were reported in other recent MAs of RCTs on SBP or DBP [93, 167, 172]. Of note however is that some studies included patients with mild hypertension, others pregnant women or healthy normotensive persons. In summary so far, the majority of evidence from RCTs is not supportive of VitD treatment for improving BP [215]. As Tamez et al suggest, larger RCTs targeting hypertensive patients with profound VitD deficiency are needed [217].

Moreover, in this study we found no significant changes in carbohydrate metabolism indexes (fasting glucose, HbA1c, HOMA index) after VitD supplementation in patients with MetS. Several direct and indirect mechanisms have been proposed to explain the alleged association as discussed in the introduction. Overall, however, interventional studies have not proved a beneficial effect of VitD supplementation on optimizing glucose metabolism parameters [253], in line with our findings. Some but not all studies have shown that the potential benefits of VitD supplementation could be more prominent among pre-diabetic individuals [256, 479]. Overall, current literature does not support the use of VitD supplements for the prevention and/or treatment of diabetes.

In our study VitD supplementation did not have any effect on serum lipids and apolipoproteins either. Several cross-sectional studies have demonstrated an inverse relationship between VitD deficiency and an atherogenic lipid profile and investigators speculated that 25(OH)VitD could affect lipid metabolism either directly or indirectly, as analyzed earlier. Yet, interventional studies with VitD supplementation have led to conflicting results, with most showing that VitD supplementation might not be translated into clinically meaningful changes in lipid concentrations [316, 338]. A MA of 19 RCTs found no beneficial effect of VitD supplementation on lipid profile parameters [341], similarly to our findings.

We noticed that PTH levels did not significantly change in both groups of our study participants. This finding is consistent with current literature which questions the utility of

PTH measurements for identification of optimal VitD levels and regards that serum 25(OH)VitD and PTH relationship is inconsistent [480].

In this study we showed that VitD supplementation in the dose used plus dietary instructions was not associated with any meaningful improvement of several emerging CVD risk factors in patients with MetS as compared with dietary instructions alone.

MetS is a constellation of known CVD risk factors (abdominal obesity, atherogenic dyslipidemia, disturbed carbohydrate metabolism and elevated blood pressure), and of others like abnormal body fat distribution, endothelial dysfunction, proinflammatory and prothrombotic states that could be included as various "emerging" CVD risk factors [481]. In particular, patients with MetS have increased atherogenic sdLDL-C levels [326, 327], and elevated Lp-PLA_2 activity [328], both of which have been associated with increased CVD risk [482, 483]. Also, MetS subjects have been found with increased leptin and decreased adiponectin serum levels [282]. These features are associated with a chronic low-grade inflammatory state.

The possible relationship between 25(OH)VitD and emerging CVD risk factors is also controversial. In the cross-sectional part of the study we showed that in MetS patients, the low 25(OH)VitD levels were associated with increased sdLDL-C levels [331], as has also been reported by another recent study [484]. However, 25(OH)VitD was not related either to mean LDL size or to $LpPLA_2$ activity [331]. Also in the interventional study, the treatment with VitD (2000 IU/day for 3 months) did not lead to any improvement of sdLDL-C levels, mean LDL size or $LpPLA_2$ activity. On the contrary, a small recent study in patients with obstructive sleep apnoea and increased body mass index (BMI=30.4 kg/m^2) who received 4000 IU/day VitD or placebo per os for 15 weeks showed significant decreases in both LDL-C and $LpPLA_2$ [332]. The mechanism through which VitD could have an impact on the lipid profile is not clear, but some assumptions that have been made were presented in the introduction. However adequate data on this matter are still missing.

Also our results showed that patients irrespective of VitD supplementation or not had no significant changes in serum leptin and adiponectin levels and leptin to adiponectin ratio after 3 months of dietary intervention. In support of our findings comes a recent systematic review and MA which reported that the inverse association between 25(OH)VitD and serum leptin levels found in most observational studies, was not confirmed in interventional ones [291]. Indeed interventional studies have shown

conflicting results. Some showed that VitD supplementation led to increases in serum leptin levels [297, 299] and one showed that it decreased leptin concentration [300]. A MA of RCTs concluded that VitD supplementation did not affect leptin levels [301] and so did a secondary analysis of the D-Health trial [302], similar to our results.

Regarding the association of 25(OH)VitD with adiponectin levels, previous studies have given equivocal results. Most interventional studies generally concluded of no effect of VitD supplementation on adiponectin levels [301, 302, 305], while one showed an increase in adiponectin levels [300] and another only a marginal raise [306]. In the present study we found no effect of VitD supplementation on adiponectin concentration after treatment.

Only few studies have investigated the relationship between 25(OH)VitD levels and the leptin to adiponectin ratio. Interventional studies reported that VitD supplementation reduced the leptin to adiponectin ratio [201, 273, 306], but this was not the case in the present study. The inconsistencies between the different studies may be due to the fact that adipokine levels are affected by genetic factors, degree of tissue adiposity, maturity of adipocytes, age at diagnosis and severity of associated conditions [307].

In addition we found that VitD supplementation plus dietary intervention did not meaningfully alter several oxidative stress markers compared with dietary intervention alone in patients with MetS. On the other hand, one in vitro study showed that addition of VitD to cultured human umbilical vein endothelial cells undergoing oxidative stress prevented cell death by inhibiting superoxide anion generation [125].

MetS has been associated with increased oxidative stress, which plays a crucial role in the formation, progression and rupture of atherosclerotic plaques [127]. VitD deficiency has been associated with increased oxidative stress in obese individuals, patients with chronic diseases and the elderly [129].

There is little data on the association between VitD and the oxidative stress markers assessed in the present study (ox-LDL, PON-1 activities, and urinary 8-isoprostanes). In particular, a recent study found that serum ox-LDL levels were significantly higher in type 2 diabetic patients with hypovitaminosis D compared to those with normal VitD status [134]. On the other hand, a study in VitD deficient but otherwise healthy persons and VitD sufficient controls showed that ox-LDL and LDL levels did not differ between groups and there was no association between VitD and ox-LDL levels [124]. In the same study,

treatment with 50,000 IU VitD/week per os for 8 weeks did not result in any changes in the ox-LDL levels [124], similar to our study with the MetS subjects.

Regarding PON-1 activity, a previous study showed that supplementing clinically healthy but VitD deficient people with intra-muscular (IM) 300,000 IU VitD monthly for 3 months was not associated with changes in serum paraoxonase activity compared with VitD sufficient controls [135]. These findings are in line with ours, since we found no effect of VitD supplementation on PON-1 activity levels in MetS patients, and no alterations in ARYL activity either.

Observational studies have also given conflicting results about the relationship between VitD and isoprostane levels. An analysis of the Framingham Offspring Study showed that plasma 25(OH)VitD concentration was inversely associated with urinary isoprostanes [136]. Similar results were obtained in a study in type 2 diabetic patients with hypovitaminosis D [134]. Another study though did not support an association of 25(OH)VitD levels with plasma isoprostanes in African-Americans [117]. Of note, data from interventional studies are scarce. In particular, one study in type 2 diabetic patients showed that treatment with 5000 IU VitD/day per os for 12 weeks versus placebo was not associated with improvement in plasma 8-isoprostanes [137]. In the present analysis in MetS subjects, urinary 8-isoprostanes (8-iso-PGF_{2a}) decreased by 22.7% (p=0.015) in the VitD Suppl group and by 14.4% (p=NS) in the Non-Suppl group. However the reduction in 8-iso-PGF_{2a} urine levels between the 2 groups did not differ significantly.

In order to explain our null findings we should take into consideration the following parameters. Compared with clinically healthy controls participating in former studies of our investigating team group, MetS patients had increased ox-LDL levels (68.8 vs 45 U/L in controls (n=50, M/F: 23/27, age 54±11 years, BMI 25±3 Kg/m^2)) [485], paraoxonase activity (80.3 vs 77.4 U/L in controls (n=30, M/F: 16/14, age 33±9 years, BMI 24±3 Kg/m^2)) [486], arylesterase activity (88.1 vs 66.6 U/mL in controls) [486] and urine 8-iso-PGF_{2a} levels (48.0 vs 5.5 ng/mmol creatinine in controls)) [486], indicating high oxidative stress levels at baseline. It is probable that intervention with either dietary instructions alone or in combination with 2000 IU/day VitD for 3 months was not enough to reduce considerably these increased oxidative stress markers. Indeed, subjects lost only 1-2 Kg and a higher dose of VitD and/or of longer duration may have been required. Moreover, the selected markers may not have been sensitive enough to evaluate changes in oxidative

stress status. This prospective part of the study has also some limitations. It was a pilot study with a small number of participants. Therefore, safe conclusions cannot be reached. Another limitation is that supplementation dose (2000 IU/day) and duration (3 months) may be inadequate to treat VitD deficiency given that subjects had very low 25(OH)VitD levels at baseline. According to previous suggestions, concentrations of at least 35-60 ng/mL would be necessary to induce an effect [487], while in our treatment group VitD levels only reached on average 30.6 ng/mL. Moreover, the actual energy and nutrient intake as well as sunlight exposure of each participant were not precisely calculated. Also, we were unable to directly compare our findings with healthy matched controls, as such a group was not included in the original study.

According to some studies, supplementation has generally been associated with improvement of oxidative stress and inflammation [125], though not consistently. Conflicting data among studies have been attributed to differences in a) studied populations, such as comorbidities and number of participants, b) baseline VitD status as well as dose, route and duration of treatment, c) study design and d) measured oxidative stress markers.

Overall, although results from observational epidemiological studies in adults were encouraging, RCTs with VitD supplementation have not so far given unequivocal results for beneficial cardiovascular effect protection [166-175]. Most MAs showed neither beneficial nor harmful effects on estimates in response to the interventions on risk of CVDs, in terms of any CV events, myocardial infarctions (MI), stroke/cerebrovascular disease, or mortality from CVDs [96]. However, most published trials have either had a relatively small sample size or did not include CVD outcomes as a pre-specified outcome. Only the recently published study by Scragg et al. had CVD as primary outcome. This study showed that oral high-dose VitD supplementation (initial dose of 200,000 IU followed a month later by 100,000 IU monthly or placebo) for a median of 3.3 years did not prevent CVD events. Other researchers have suggested that monthly dosage may be less effective than daily or weekly in CVD protection [176]. Results from specifically designed large-scale VitD supplementation trials of longer duration have been published and give more clear results. The VITamin D and OmegA-3 TriaL (VITAL) study, a placebo-controlled, double-blind 2×2 factorial trial of over 25,875 multi-ethnic participants randomized to 2,000 IU/day of vitamin D3 and omega-3 fatty acid supplements for 5 years

has showed that VitD supplementation did not result in a lower incidence of cardiovascular events (including myocardial infarction, stroke, or death from cardiovascular causes) than placebo [181]. Also the Vitamin D Assessment (ViDA) study, a randomised, double-blind, placebo-controlled trial, to evaluate the efficacy of monthly VitD supplementation (100,000 IU or placebo) showed no beneficial effect of VitD supplementation on incidence of cardiovascular disease as well as falls, non-vertebral fractures and all cancer types [182]. The EVITA study that examined the effect of VitD supplementation (4000 IU VitD daily versus placebo) on all-cause mortality in 400 heart failure patients (HF) with 25(OH)VitD levels <30 ng/mL for 3 years showed that daily VitD supplementation did not reduce mortality in patients with advanced HF but was associated with a greater need for mechanical circulatory support implants. These data indicate caution regarding long-term supplementation with moderately high VitD doses [165]. The D-Health Trial, a placebo-controlled trial with 21,315 participants randomized to 60,000 IU/month of vitamin D3 for 5 years with primary outcome all-cause mortality and secondary outcomes total cancer incidence and colorectal cancer incidence is still underway [183].

ii) Furthermore in this study we examined the effect of VitD supplementation (2000 IU/day pos for 3 months) in **adolescents** with obesity and VitD insufficiency/deficiency. At follow-up we found that VitD supplementation effectively increased 25(OH)VitD levels and was associated with marginal decreases in HbA_1c and leptin as well as an increase in LDL-C levels. Other clinical and laboratory metabolic parameters (BMI, WC, DBP, TCHOL, HDL-C, triglycerides, glucose, insulin, HOMA index, PTH) along with oxidative stress markers (ox-LDL, PON-1, ARYL and urine isoprostanes) remained unchanged.

The recently published ODIN Project (food-based solutions for optimal vitamin D nutrition and health through the life cycle) where adolescents were assigned to receive either placebo or VitD (400 or 800 IU/day) for 20 weeks, reported no effect on SBP or DBP [225]. In our study, after VitD supplementation we found a small but not significant reduction in SBP by -3.9% and DBP also did not change significantly. In the adults also as reported earlier, a similar decrease (-3.7%) in SBP was found after supplementation, which again did not differ statistically from its basal value or from that of the Non-Suppl group at 3 months.

Data from interventional trials are also sparse and inconclusive regarding the effect of VitD supplementation on lipids. In one study supplementing adolescents with obesity with 2000 IU VitD/day caused a slight increment of only 6 ng/mL in 25(OH)VitD concentrations and no change in the lipid profile after 12 weeks [355]. Similarly, the ODIN Project, found no effect on lipid profile in the fully adjusted analysis in mainly healthy normal weight adolescents [225]. In the present study we found an increase of 7.8% in the LDL-C levels after VitD supplementation, while other lipids remained unchanged. To our knowledge, this finding has not been previously reported in adolescents. However, a MA of RCTs in adults also observed that VitD supplementation significantly increased LDL-C levels [340]. This effect seemed to be more evident in the subjects with obesity and especially in those with relatively shorter durations of intervention, while there was no such effect in the normal weight subjects. The authors proposed that confounders such as obesity with its side effects must be implicated leading to this finding. Obesity is generally accompanied by dyslipidemia with increased triglycerides and LDL-C levels and decreased HDL-C levels as well as a preferential uptake of VitD by adipose tissue. In addition some interventional studies give vitamin D2 that is less bioactive than vitamin D3 [340]. Whether VitD supplementation would improve insulin sensitivity and glucose metabolism in childhood and adolescence is also debatable. Some RCTs showed beneficial effects on insulin sensitivity [273, 355], while others did not [274, 276, 277]. In this study, adolescents with obesity and VitD insufficiency/deficiency and insulin resistance (median HOMA-IR 3.7) who received VitD, had a 5.2% reduction in HbA_{1c} levels, but no changes in fasting glucose, fasting insulin and HOMA-IR for the time studied. On the contrary, HbA_{1c} levels were not affected by VitD supplementation in adolescents with obesity in another study [273]. Differences in the age groups studied, severity of weight excess and degree of glucose tolerance, as well as doses and duration of VitD supplementation may be responsible for the observed discrepancies.

In the cross-sectional part of this study we found that in adolescents with obesity, 25(OH)VitD was inversely associated with leptin even after adjustment for BMI. In accordance to those findings, in the interventional part, correction of hypovitaminosis D led to a significant reduction (23.3%) of leptin levels. Our results agree with the other few studies. Rambhojan et al (2016) also reported an inverse correlation between 25(OH)VitD and leptin levels in clinically healthy adolescents [309]. In addition they showed an inverse

correlation of 25(OH)VitD levels with the leptin/adiponectin ratio and in the obese ones after a year's life style intervention program they observed a rise in 25(OH)VitD and decrease in the ratio [309]. Also, an RCT found serum leptin/adiponectin ratio to be significantly lower in the VitD supplemented group compared with the placebo [273]. More upcoming research with focus in this association, will help reaching safer conclusions.

With regard to oxidative stress, it has been reported that VitD deficiency may be implicated in this process even in the obese youth and that VitD supplementation may have favorable effect on their antioxidant system [145]. In our study however, treatment of adolescents with obesity with VitD p.os. plus dietary intervention was not associated with any changes in ox-LDL, paraoxonase and arylesterase activities (i.e. a PON-1 activity was more closely related to PON-1 mass) and urinary isoprostanes. Again, there is insufficient data for this age group.

This part of our study had some limitations as well. The number of participants in the prospective study was small, with just over half having VitD deficiency (the rest had insufficiency), while a placebo arm was not included. Also, the supplementation dose (2000 IU/day) and duration (3 months) may have been inadequate to show a significant change in various metabolic markers. Additionally, the actual energy and nutrient intake of each participant were not precisely calculated. However, this was a clinically relevant study, since we evaluated the relationship of 25(OH)VitD with novel parameters considered as primary CVD risk factors (such as ox-LDL, paraoxonase, arylesterase, urine isoprostanes and leptin). They have been rarely, if not at all, explored before, especially in the adolescent population.

However overall, the possible beneficial effect of VitD on CVD risk factors and events still remains unclear based on both ours and current literature data. Although the majority of observational studies have reported low 25(OH)VitD levels to be associated with an increased risk of adverse health outcomes including CVD, most published RCTs have failed like ours to document a beneficial effect of VitD supplementation. RCTs' findings question the inverse association between 25(OH)VitD levels and CVD. Besides some researchers suggest that it could be the result of disease process causing low 25(OH)VitD levels rather than low 25(OH)VitD levels causing the disease i.e., low 25(OH)VitD levels may only be a marker of ill health [96]. As analysed earlier obesity per

se is regarded as a causal risk factor for VitD deficiency and this in turn could contribute to the adverse health effects associated with obesity [353].

Moreover, we found conflicting data among trials examining the same alleged relationships. This may be attributed to differences in a) study populations and number of participants, b) baseline VitD status, c) dose, route and duration of treatment, and d) study design. More analytically, only a few studies have actually investigated effects in populations with low 25(OH)VitD levels and the sample size in most RCTs was relatively small [96]. In addition, even the definition of what is an "optimal" serum 25(OH)VitD concentration is controversial. Additionally, it has been documented that the use of different methods for measuring 25(OH)VitD levels may cause huge variations in the levels measured and thereby affecting the classification of VitD status [488]. Nevertheless, some investigators suggested serum 25(OH)VitD concentrations >32 ng/ml as necessary for lipid and cardiovascular health [489] and others serum 25(OH)VitD levels ≥34,4 ng/mL as optimal for reductions in blood pressure, markers of arterial stiffness, and reductions in hs-CRP [10]. These serum 25(OH)VitD status was achieved with VitD supplemental doses ≥4,000 IU/d, the current tolerable upper level of intake [10]. Of note however is that overweight and obese individuals require two to three times the amount of VitD to increase their serum 25(OH)VitD concentrations to the same extent as those with a normal BMI [490]. It may also be questioned whether effects of supplementation with VitD are equal to endogenous synthesis especially for VitD2. The duration of supplementation is an important factor in assessments of VitD status too and in many cases it has been relatively short [96]. With a half-life of about 2 months, to achieve and maintain a steady serum 25(OH)VitD concentration requires a follow-up period of 3 months and more. Finally, it has been highlighted that most of the available trials were not designed to study non-skeletal outcomes of VitD supplementation as a primary end-point. For all these reasons we see discrepancies between the results coming from observational and randomized trials and a causal relationship between low 25(OH)VitD levels and CVD cannot be supported unequivocally at present. Therefore, further adequately powered RCTs designed to test the effect of VitD supplementation on CVD risk factors and events as a primary outcome, only in individuals with VitD deficiency and at appropriate doses are still needed to be carried out, as well as long-term cohort studies using standardized methods for serial measurement of 25(OH)VitD levels.

CHAPTER V.

CONCLUDING REMARKS

Overall, the findings of this study are summarized below:

1) Adults with MetS had significantly lower 25(OH)VitD levels compared with those without MetS.

VitD deficiency was also more prevalent among Greek **adolescents** with obesity (73.9% vs 17.6% in normal weight controls).

2) In **adults** 25(OH)VitD levels were significantly and inversely associated with triglycerides and sdLDL-C levels in patients with MetS. However, in multivariate regression analysis, sdLDL-C levels were found to be affected only by triglycerides and not by 25(OH)VitD concentration. There was no association of 25(OH)VitD with waist circumference, blood pressure, HDL-C and fasting glucose (the other diagnostic criteria for MetS) as well as $LpPLA_2$ and hsCRP levels.

In **adolescents** with obesity, 25(OH)VitD was inversely associated with leptin even after adjustment for BMI. On the contrary, 25(OH)VitD was not related with other parameters, such as BMI, blood pressure, lipids, glucose, insulin, HOMA index, adiponectin, leptin/adiponectin ratio and visfatin levels.

3) VitD supplementation (2000 IU/day p.os for 3 months) plus dietary intervention in **adults** with MetS was not associated with any significant change in classic CVD risk factors compared with dietary intervention alone despite the significant rise in the 25(OH)VitD levels in the first. In both groups (VitD Suppl vs Non-Suppl) triglycerides, HDL-C, LDL-C, fasting glucose, HbA_1c, HOMA index and DBP did not significantly change and even SBP that decreased by 3.7% in the VitD Suppl group versus 1.5% in the Non-Suppl group did not differ significantly between groups. But in the VitD Suppl group the serum 25(OH)VitD increase was inversely correlated with SBP decrease ($r = -0.398$, $p = 0.049$) which is of clinical importance.

However, treatment with VitD was not associated with any changes in emerging CVD risk factors. Patients in both groups had no significant changes in sdLDL-C levels, mean LDL size or $LpPLA_2$ activity, serum leptin and adiponectin levels and leptin to adiponectin ratio after VitD supplementation. No differences in the changes of the same parameters were noticed between groups.

Moreover, VitD administration plus dietary intervention in the same patients was not associated with meaningful reductions in oxidative stress markers compared with dietary

intervention alone. Ox-LDL, PON-1 and ARYL did not change significantly at follow-up in both groups, except for urine 8-iso-PGF_{2a} levels that decreased by 22.7% in the VitD Suppl group (p = 0.015) versus 14.4% in Non-Suppl group (p=NS). But again no difference was noted in the reduction of the 8-iso-PGF_{2a} levels between the 2 groups.

4) VitD supplementation (2000 IU/day p.os for 3 months) in **adolescents** with obesity and VitD insufficiency/deficiency effectively increased their 25(OH)VitD levels and was associated with marginal decreases in HbA_1c and leptin as well as an increase in LDL-C levels. Other clinical and laboratory metabolic parameters (BMI, waist circumference, BP, TCHOL, HDL-C, triglycerides, glucose, insulin, HOMA index, PTH) along with oxidative stress markers (ox-LDL, PON-1, ARYL and urine 8-iso-PGF_{2a}) remained unchanged.

5) High-dose rosuvastatin monotherapy as well as usual-dose rosuvastatin plus micronized fenofibrate and usual-dose rosuvastatin plus omega-3 fatty acids resulted to significant and similar increases in serum 25(OH)VitD levels in patients with mixed dislipidemia.

Also patients with primary hypercholesterolemia while achieving similar lower levels of LDL-C with 40 mg simvastatin alone or with 10/10 mg simvastatin/ezetimibe, they had more than double increase in their 25(OH)VitD levels with simvastatin monotherapy.

On the contrary neither the switch to high-dose rosuvastatin, nor add-on-statin ER-NA/LRPT or fenofibrate was found to cause any significant changes in 25(OH)VitD levels in patients with mixed dyslipidemia and not at goal while on treatment with a conventional statin dose.

Overall according to our results unlike rosuvastatin and simvastatin other lipid-lowering drugs like ezetimibe, fenofibrate, omega-3 fatty acids and nicotinic acid seem to have minimal if any effect on 25(OH)VitD serum concentrations in patients with dyslipidemia.

SUMMURY

INTRODUCTION

In the last 20 years VitD deficiency (25 (OH) VitD <20 ng/mL) is returning as a new public health problem. Low VitD status has been associated with many chronic skeletal and non-skeletal diseases beginning in childhood/adolescence which manifest later in adulthood. These include cardiovascular diseases (CVD), metabolic syndrome (MetS), obesity, hypertension, dyslipidemia, diabetes and others such as malignancies, infections, neuropsychiatric and autoimmune diseases. However, the effect of VitD deficiency on the corresponding cardiovascular risk factors in children and adolescents has been studied much less than in adults and with conflicting results.

In addition, the main element of the prevention and treatment of CVD in adults is the drug treatment of dyslipidemia. According to some studies, statins may affect serum VitD levels as a new pleiotropic effect, while there are insufficient data on the effect of other hypolipidemic drugs in VitD metabolism.

Finding and correcting modifiable cardiovascular risk factors (including VitD deficiency/insufficiency) as soon as possible is an important step in CVD prevention. However, so far VitD substitution studies in children/adolescents and large randomized clinical trials in adults with CVD as primary endpoint have resulted in conflicting results in terms of the clinical benefit of its administration.

AIMS & METHODS:

The present study consists of 2 parts: a cross-sectional and a prospective.

Aims & Methods of cross-sectional study:

Determination of serum 25(OH)VitD levels in Greek patients, a) adults MetS (N=52) and b) adolescents with obesity (N=69) as well as corresponding to age and sex controls (58 adults, 34 adolescents). Furthermore, investigation of the possible correlation of VitD levels with the diagnostic criteria of MetS and other biochemical parameters observed within it.

Aims & Methods of prospective study:

A) Assessment of the effect of hypolipidemic drugs on 25(OH)VitD serum levels in adult patients with dyslipidemia, 3 months after the start of treatment, based on the following 3 treatment protocols:

i) Effect of rosuvastatin 40 mg (N=22) against the combination of rosuvastatin 10 mg plus phenofibrate 200 mg (N=21) or the combination of rosuvastatin 10 mg plus omega-3 fatty acids 2 g (N=17) on 25(OH)VitD serum levels, in patients with mixed dyslipidemia.

ii) Effect of simvastatin 40 mg (N=25) against the combination of simvastatin 10 mg plus ezetimibe 10 mg (N=25) on 25(OH)VitD serum levels in patients with primary hypercholesterolemia.

iii) Effect of rosuvastatin 40 mg (N=17) against the combination of rosuvastatin 10 mg plus phenofibrate 200 mg (N=14) or the combination of rosuvastatin 10 mg plus nicotinic acid/laropiprant 2 g (N=13) on 25(OH)VitD serum levels in patients with mixed dyslipidemia, who had not achieved the therapeutic goals while already receiving a conventional statin dose (simvastatin 10-40 mg or atorvastatin 10-20 mg or rosuvastatin 5-10 mg).

B) Assessment of the effect of cholecalciferol (VitD3) administration (2000 IU/day) on metabolic parameters in adults with MetS and adolescents with obesity.

i) Adults with MetS were randomized based on gender and age to apply either only healthy-dietary guidelines (N=25), or to receive 2000 IU VitD/day pos along with healthy-dietary guidelines (N=25) and were evaluated clinically and laboratory in baseline and 3 months after the intervention.

ii) Adolescents with obesity (BMI=35.0±7.9) and VitD deficiency (25(OH)VitD <20 ng/mL) (N=15) received 2000 IU VitD/day pos along with healthy-dietary guidelines and were re-assessed 3 months later.

The primary endpoints were changes in MetS parameters 3 months after the start of treatment, including: waist circumference, blood pressure (systolic and diastolic), fasting triglycerides, HDL-C and fasting glucose levels.

Secondary endpoints include changes in: serum 25(OH)VitD and PTH levels, glucose metabolism homeostasis (HOMA index: fasting insulin x fasting

glucose/405), glycosylated hemoglobin (HbA1c), serum LDL-C levels, LDL-C subclasses (mean LDL-C particle size, small dence-LDL-C levels), high sensitivity C reacting protein (hsCRP) levels, Lp-PLA_2 activity (lipoprotein-associated phopspholipase A_2), paraoxonase-1 (PON1) and arylesterase activity (ARYL), oxidative stress markers [urine 8-isoprostanes (8-iso-PGF_2a) and serum oxidized LDL (oxLDL) levels], serum adipokine levels (leptin, adiponectin, visfatine).

RESULTS

Results of the cross-sectional study:

a) **Adults with MetS** had significantly lower serum 25(OH)VitD levels than controls (11.8 (0.6-48.3) ng/mL vs 17.2 (4.8-62.4) ng/mL, p=0.027). Overall, 91.3% of study participants had VitD insufficiency (25(OH)VitD <30 ng/mL), of which 65% had VitD deficiency (25(OH)VitD) <20 ng/mL). In addition, the incidence of VitD deficiency tended to be higher in patients with MetS (65.3%) than in controls (59.2%).

In adults with MetS, univariate regression analysis showed that serum 25(OH)VitD levels correlated negatively with triglycerides (r = -0.416, p = 0.003), but not with other diagnostic criteria of MetS. In addition, 25(OH)VitD had a negative correlation with sdLDL-C (p = 0.03) and PTH levels (r = -0.376, p = 0.04), but not with LDL size, Lp-PLA_2 activity and hsCRP. However, stepwise multivariate linear regression analysis showed that sdLDL-C levels were significantly affected only by triglyceride levels and not by 25(OH)VitD levels.

b) **Adolescents with obesity** had lower serum 25(OH)VitD levels than normal-weight controls [12.0 (3.0-36.0) versus 34.0 (10.0-69.0) ng/mL respectively, p = 0.000]. Overall, 74.7% of participants had VitD insufficiency [25(OH)VitD <30 ng/mL], of which 52.4% had VitD deficiency [25(OH)VitD) <20 ng/mL]. Also, the incidence of VitD deficiency was higher among obese (73.9%) than normal-weight adolescents (17.6%) (p = 0.001).

In obese adolescents, serum 25(OH)VitD levels were found to be negatively correlated with serum leptin levels (r = -0.280, p = 0.037), and this relationship did not appear to be affected by BMI (r = -0.340, p = 0.009).

Results of the prospective study:

A.i) Three months after **drug treatment** of adults with mixed dyslipidemia, serum levels of 25(OH)VitD increased significantly in all 3 treatment groups: in the group receiving rosuvastatin 40 mg they increased by 53% (p = 0.000), in group receiving rosuvastatin 10 mg plus phenofibrate by 64% (p = 0.001), and in the group receiving rosuvastatin 10 mg plus omega-3 fatty acids by 61% (p = 0.04). Increases in serum 25(OH)VitD levels were comparable in all 3 treatment groups.

A.ii) Three months after drug treatment of adults with primary hypercholesterolemia, serum 25(OH)VitD levels increased significantly in both groups: in the recipients of simvastatin/ezetimibe 10/10 mg they increased by 36.7%, while in the group receiving simvastatin 40 mg by 79.1%. The increase in 25(OH)VitD levels was significantly higher in the simibastatin 40 mg group compared to that in simvastatin/ezetimibe 10/10 mg group (p = 0.04).

A.iii) Three months after the modification of medication in adults with mixed dyslipidemia who had not achieved the therapeutic goals with a conventional statin dose, serum 25(OH)VitD levels did not change significantly in all 3 treatment groups: in both groups receiving rosuvastatin 40 mg and rosuvastatin 10 mg plus nicotinic acid/laropiprant, a tendency to decrease was observed in 25(OH)VitD serum levels (-4.7% and -14.8%, respectively), which was not statistically significant. In the group that received rosuvastatin 10 mg plus phenofibrate an increase in 25(OH)VitD serum levels (+13%) was found, which was also statistically non-significant. The above changes in serum 25(OH)VitD levels did not differ statistically significantly between the 3 groups.

B.i) In **adults with MetS** who received pos VitD supplementation, 25(OH)VitD levels increased by 91% (from 16.0 (3.0-35.0) to 30.6 (8.4-67.0) ng/mL, p <0.001), while those who did not receive VitD showed a statistically non-significant increase of 30% (from 10.0 (4.0-39.6) to 13.0 (3.5-37.0) ng/mL, p = NS). The levels of TCHOL, triglycerides, HDL-C, LDL-C, ApoA1, ApoB, fasting glucose and insulin, HbA1c, HOMA index and diastolic BP did not change significantly in both groups. In the group that received VitD, systolic BP decreased by 3.7% (from 134±14 to 129±13 mmHg, p = 0.05), while in the group that did not receive VitD it decreased by only

1.5% (from 132±13 to 130±16 mmHg, p=NS). In the group that received VitD, the increase in 25(OH)VitD was associated with a decrease in systolic BP (r = -0.398, p = 0.049). However, no significant changes were observed in sdLDL-C, sdLDL proportion, mean LDL size and $LpPLA_2$ activity levels, as well as serum leptin and adiponectin levels and leptin/adiponectin ratio in both groups. Also, serum levels of ox-LDL-C and paraoxonase and arylesterase activities of PON-1 did not change significantly in either group. In contrast, the levels of urine isoprostane (urine 8-iso-PGF_2a) decreased significantly by 22.7% in the group receiving pos VitD [from 48.8 (26.8 to 137.1) to 37.7 (12.3-99.0) ng/mmol creatinine, p=0.015], while a downward trend of 14.4% was found in the group that did not receive VitD, but it was not statistically significant [from 45.8 (16.6 to 99.3) to 39.2 (13.3-120.1) ng/mmol creatinine, p=NS]. However, the reduction in 8-iso-PGF_2a urine levels did not differ statistically significantly between the 2 groups.

B.ii) In **adolescents with obesity and VitD insufficiency** who received pos VitD, 25(OH)VitD levels increased significantly by 88.4% 3 months later [from 17.3 (12.5-27.8) to 32.6 (14.3-68.0) ng/mL, p=0.005]. At the same time, significant reductions were found in HbA1c (p=0.03) and leptin levels (p=0.03), while LDL-C levels increased (p=0.022). The other clinical and laboratory parameters examined (BMI, waist circumference, BP, TCHOL, HDL-C, triglycerides, glucose, insulin, HOMA index, PTH) and oxidative stress markers (ox-LDL, paraoxonase, arylesterase and urine isoprostanes) did not show significant alterations.

CONCLUSIONS

1) Adults with MetS had significantly lower 25(OH)VitD levels compared with those without MetS.

VitD deficiency was also more prevalent among Greek **adolescents** with obesity (73.9% vs 17.6% in normal weight controls).

2) In **adults** 25(OH)VitD levels were significantly and inversely associated with triglycerides and sdLDL-C levels in patients with MetS. However, in multivariate regression analysis, sdLDL-C levels were found to be affected only by triglycerides and not by 25(OH)VitD concentration. There was no association of 25(OH)VitD with waist

circumference, blood pressure, HDL-C and fasting glucose (the other diagnostic criteria for MetS) as well as $LpPLA_2$ and hsCRP levels.

In **adolescents** with obesity, 25(OH)VitD was inversely associated with leptin even after adjustment for BMI. On the contrary, 25(OH)VitD was not related with other parameters, such as BMI, blood pressure, lipids, glucose, insulin, HOMA index, adiponectin, leptin/adiponectin ratio and visfatin levels.

3) VitD supplementation (2000 IU/day p.os for 3 months) plus dietary intervention in **adults** with MetS was not associated with any significant change in classic CVD risk factors compared with dietary intervention alone despite the significant rise in the 25(OH)VitD levels in the first. In both groups (VitD Suppl vs Non-Suppl) triglycerides, HDL-C, LDL-C, fasting glucose, HbA_1c, HOMA index and DBP did not significantly change and even SBP that decreased by 3.7% in the VitD Suppl group versus 1.5% in the Non-Suppl group did not differ significantly between groups. But in the VitD Suppl group the serum 25(OH)VitD increase was inversely correlated with SBP decrease ($r = -0.398$, $p = 0.049$) which is of clinical importance.

However, treatment with VitD was not associated with any changes in emerging CVD risk factors. Patients in both groups had no significant changes in sdLDL-C levels, mean LDL size or $LpPLA_2$ activity, serum leptin and adiponectin levels and leptin to adiponectin ratio after VitD supplementation. No differences in the changes of the same parameters were noticed between groups.

Moreover, VitD administration plus dietary intervention in the same patients was not associated with meaningful reductions in oxidative stress markers compared with dietary intervention alone. Ox-LDL, PON-1 and ARYL did not change significantly at follow-up in both groups, except for urine 8-iso-PGF_{2a} levels that decreased by 22.7% in the VitD Suppl group ($p = 0.015$) versus 14.4% in Non-Suppl group (p=NS). But again no difference was noted in the reduction of the 8-iso-PGF_{2a} levels between the 2 groups.

4) VitD supplementation (2000 IU/day p.os for 3 months) in **adolescents** with obesity and VitD insufficiency/deficiency effectively increased their 25(OH)VitD levels and was associated with marginal decreases in HbA_1c and leptin as well as an increase in LDL-C levels. Other clinical and laboratory metabolic parameters (BMI, waist circumference, BP, TCHOL, HDL-C, triglycerides, glucose, insulin, HOMA index, PTH)

along with oxidative stress markers (ox-LDL, PON-1, ARYL and urine 8-iso-PGF_{2a}) remained unchanged.

5) High-dose rosuvastatin monotherapy as well as usual-dose rosuvastatin plus micronized fenofibrate and usual-dose rosuvastatin plus omega-3 fatty acids resulted to significant and similar increases in serum 25(OH)VitD levels in patients with mixed dislipidemia.

Also patients with primary hypercholesterolemia while achieving similar lower levels of LDL-C with 40 mg simvastatin alone or with 10/10 mg simvastatin/ezetimibe, they had more than double increase in their 25(OH)VitD levels with simvastatin monotherapy.

On the contrary neither the switch to high-dose rosuvastatin, nor add-on-statin ER-NA/LRPT or fenofibrate was found to cause any significant changes in 25(OH)VitD levels in patients with mixed dyslipidemia and not at goal while on treatment with a conventional statin dose.

Overall according to our results unlike rosuvastatin and simvastatin other lipid-lowering drugs like ezetimibe, fenofibrate, omega-3 fatty acids and nicotinic acid seem to have minimal if any effect on 25(OH)VitD serum concentrations in patients with dyslipidemia.

ΠΕΡΙΛΗΨΗ

ΕΙΣΑΓΩΓΗ

Την τελευταία 20ετία η έλλειψη VitD (25(OH)VitD <20 ng/mL) επανέρχεται ως νέο πρόβλημα δημόσιας υγείας Τα χαμηλά επίπεδα VitD έχουν συσχετιστεί με πολλά χρόνια σκελετικά αλλά και μη-σκελετικά νοσήματα που ξεκινούν από την παιδική/εφηβική ηλικία και εκδηλώνονται στην ενήλικο ζωή. Μεταξύ αυτών περιλαμβάνονται τα καρδιαγγειακά νοσήματα (ΚΑΝ), το μεταβολικό σύνδρομο (ΜΣ), η παχυσαρκία, η υπέρταση, η δυσλιπιδαιμία, ο σακχαρώδης διαβήτης αλλά και άλλα όπως κακοήθειες, λοιμώξεις, νευροψυχιατρικά και αυτοάνοσα νοσήματα. Ωστόσο η επίδραση της έλλειψης VitD στους αντίστοιχους καρδιαγγειακούς παράγοντες κινδύνου σε παιδιά και εφήβους έχει μελετηθεί πολύ λιγότερο σε σχέση με τους ενήλικες και με αντικρουόμενα αποτελέσματα.

Επιπρόσθετα βασικό στοιχείο της πρόληψης αλλά και θεραπείας των ΚΑΝ στους ενήλικες είναι η φαρμακευτική αντιμετώπιση της δυσλιπιδαιμίας. Βάσει κάποιων μελετών πιθανά οι στατίνες να επηρεάζουν τα επίπεδα της VitD στον ορό, ως μια νεα πλειοτροπική τους δράση, ενώ δεν υπάρχουν επαρκή δεδομένα για το ρόλο των άλλων υπολιπιδαιμικών φαρμάκων στο μεταβολισμό της VitD.

Η ανεύρεση τροποποιήσιμων καρδιαγγειακών παραγόντων κινδύνου (όπως πιθανά η έλλειψη/ανεπάρκεια VitD) το συντομότερο δυνατόν αποτελεί σημαντικό βήμα για την πρόληψη των ΚΑΝ. Ωστόσο μέχρι στιγμής οι μελέτες υποκατάστασης VitD σε παιδιά/εφήβους αλλά και οι μεγάλες τυχαιοποιημένες κλινικές δοκιμές σε ενήλικες με πρωτογενές καταληκτικό σημείο τα ΚΑΝ έχουν καταλήξει σε αντικρουόμενα αποτελέσματα ως προς το κλινικό όφελος της χορήγησής της.

ΣΚΟΠΟΣ – ΜΕΘΟΔΟΙ

Η παρούσα μελέτη αποτελείται από 2 μέρη: ένα συγχρονικό και ένα προοπτικό.

Σκοπός - Μέθοδοι της σύγχρονης μελέτης:

Ο προσδιορισμός των επιπέδων 25(OH)VitD ορού σε Έλληνες ασθενείς, α) ενήλικες με μεταβολικό σύνδρομο (ΜΣ) (N=52) και β) εφήβους με παχυσαρκία (N=69) καθώς και

σε αντίστοιχους ως προς την ηλικία και το φύλο μάρτυρες (58 ενήλικες, 34 έφηβοι). Επιπλέον η διερεύνηση της πιθανής συσχέτισης των επιπέδων της VitD με τα κριτήρια διάγνωσης του ΜΣ και άλλων βιοχημικών παραμέτρων που παρατηρούνται στα πλαίσια του.

Σκοπός - Μέθοδοι της προοπτικής μελέτης:

Α) Η μελέτη της επίδρασης υπολιπιδαιμικών φαρμάκων στα επίπεδα της 25(OH)VitD ορού ενήλικων ασθενών με δυσλιπιδαιμία, 3 μήνες μετά την έναρξη θεραπείας, βάσει των παρακάτω 3 θεραπευτικών πρωτοκόλλων:

i) Η επίδραση της ροσουβαστατίνης 40 mg (N=22) έναντι του συνδυασμού ροσουβαστατίνης 10 mg με φαινοφιμπράτη 200 mg (N=21) ή του συνδυασμού ροσουβαστατίνης 10 mg με ωμέγα-3 λιπαρά οξέα 2 g (N=17) στα επίπεδα 25(OH)VitD ορού ασθενών με μεικτή δυσλιπιδαιμία.

ii) Η επίδραση της σιμβαστατίνης 40 mg (N=25) έναντι του συνδυασμού σιμβαστατίνης 10 mg με εζετιμίμπη 10 mg (N=25) στα επίπεδα 25(OH)VitD ορού ασθενών με υπερχοληστερολαιμία.

iii) Η επίδραση της ροσουβαστατίνης 40 mg (N=17) έναντι του συνδυασμού ροσουβαστατίνης 10 mg με φαινοφιμπράτη 200 mg (N=14) ή του συνδυασμού ροσουβαστατίνης 10 mg με νικοτινικό οξύ/λαροπιπράντη 2 g (N=13) στα επίπεδα 25(OH)VitD ορού ασθενών με μεικτή δυσλιπιδαιμία, οι οποίοι δεν είχαν επιτύχει τους θεραπευτικούς στόχους ενώ ήδη έπαιρναν μια συμβατική δόση στατίνης (σιμβαστατίνη 10-40 mg ή ατορβαστατίνη 10-20 mg ή ροσουβαστατίνη 5-10 mg).

Β) Η επίδραση της χορήγησης χοληκαλσιφερόλης (VitD3) (2000 IU/ημέρα) στις μεταβολικές παραμέτρους ενηλίκων με ΜΣ και εφήβων με παχυσαρκία:

i) Ενήλικες με ΜΣ τυχαιοποιήθηκαν βάσει φύλου και ηλικίας να εφαρμόσουν είτε μόνο υγιεινο-διαιτητικές οδηγίες (N=25), είτε να λαμβάνουν 2000 IU VitD/ημέρα per os μαζί με υγιεινο-διαιτητικές οδηγίες (N=25) και εκτιμήθηκαν κλινικο-εργαστηριακά στην έναρξη της μελέτης και 3 μήνες μετά την παρέμβαση.

ii) Έφηβοι με παχυσαρκία (BMI= 35.0±7.9) και ανεπάρκεια VitD (25(OH)VitD <20 ng/mL) (N=15) έλαβαν 2000 IU VitD/ημέρα per os μαζί με υγιεινο-διαιτητικές οδηγίες και 3 μήνες μετά επανεκτιμήθηκαν.

Το πρωτογενές καταληκτικό σημείο ήταν οι μεταβολές στις παραμέτρους του ΜΣ 3 μήνες μετά την έναρξη της θεραπείας, και συγκεκριμένα:

- Στην περίμετρο μέσης
- Στην επίπτωση της αρτηριακής πίεσης (συστολικής και διαστολικής)
- Στα τριγλυκερίδια νηστείας
- Στα επίπεδα της HDL-C
- Στα επίπεδα της γλυκόζης νηστείας

Τα δευτερογενή καταληκτικά σημεία περιλαμβάνουν μεταβολές στα:

- Στα επίπεδα της 25(OH)VitD και της PTH ορού
- Στην ομοιοστασία του μεταβολισμού της γλυκόζης (HOMA index: fasting insulin x fasting glucose/405)
- Στα επίπεδα της γλυκοζηλιωμένης αιμοσφαιρίνης (HbA1c)
- Στα επίπεδα LDL-C ορού
- Στα υποκλάσματα της LDL-C (mean LDL-C particle size, small dence-LDL-C levels)
- Στα επίπεδα της high sensitivity C reacting protein (hsCRP)
- Στην ενεργότητα της Lp-PLA2 (lipoprotein-associated phopspholipase A2)
- Στην ενεργότητα της Paroxonase-1 (PON1) και της arylesterase (ARYL)
- Στην αξιολόγηση του οξειδωτικού στρες με μέτρηση των επιπέδων 8-isoprostane στα ούρα και των επιπέδων της oxidized LDL (oxLDL) στον ορό
- Στα επίπεδα αδιποκινών στον ορό (λεπτίνη, αδιπονεκτίνη, βισφατίνη)

ΑΠΟΤΕΛΕΣΜΑΤΑ

Αποτελέσματα της σύγχρονης μελέτης:

Α) Οι **ενήλικες με ΜΣ** είχαν σημαντικά χαμηλότερα επίπεδα 25(OH)VitD ορού σε σχέση με τους μάρτυρες (11.8 (0.6-48.3) ng/mL vs 17.2 (4.8-62.4) ng/mL, p=0.027). Συνολικά το 91.3% των συμμετεχόντων στη μελέτη είχε ανεπάρκεια VitD (25(OH)VitD <30 ng/mL), εκ των οποίων το 65% είχε σημαντική έλλειψη VitD (25(OH)VitD) <20 ng/mL). Επιπλέον η επίπτωση της έλλειψης VitD ήταν σχετικά μεγαλύτερη στους ασθενείς με ΜΣ (65.3%) σε σχέση με τους μάρτυρες (59.2%). [p=NS]

Στους ενήλικες με ΜΣ η μονοπαραγοντική ανάλυση (univariate analysis) έδειξε ότι τα επίπεδα της 25(OH)VitD ορού είχαν αρνητική συσχέτιση με τα τριγλυκερίδια (r=-0.416, p=0.003), αλλά όχι με τα άλλα διαγνωστικά κριτήρια του ΜΣ. Επιπλέον τα επίπεδα της 25(OH)VitD είχαν αρνητική συσχέτιση με τα επίπεδα των small dense LDL-C (sdLDL-C) (p=0.03) και της PTH (r=-0.376, p=0.04) αλλά όχι με τις άλλες παραμέτρους (LDL size, Lp-PLA_2 και hsCRP). Ωστόσο η πολυπαραγοντική ανάλυση (stepwise multivariate linear regression analysis) έδειξε ότι τα επίπεδα των sdLDL-C επηρεάζονταν σημαντικά μόνο από τα επίπεδα των τριγλυκεριδίων και όχι από τα επίπεδα της 25(OH)VitD.

Β) Οι **έφηβοι με παχυσαρκία** είχαν χαμηλότερα επίπεδα 25(OH)VitD ορού σε σχέση με τους φυσιολογικού βάρους μάρτυρες [12.0 (3.0-36.0) versus 34.0 (10.0-69.0) ng/mL αντίστοιχα, p=0.000]. Συνολικά το 74.7% των συμμετεχόντων είχαν ανεπάρκεια VitD [25(OH)VitD <30 ng/mL] εκ των οποίων το 52.4% είχε σημαντική έλλειψη VitD [25(OH)VitD) <20 ng/mL]. Επίσης η επίπτωση της έλλειψης VitD ήταν μεγαλύτερη μεταξύ των παχύσαρκων εφήβων (73.9%) σε σχέση με τους φυσιολογικού βάρους εφήβους (17.6%) (p=0.001).

Στους εφήβους με παχυσαρκία τα επίπεδα της 25(OH)VitD ορού βρέθηκε να έχει αρνητική συσχέτιση με τα επίπεδα της λεπτίνης ορού (r = -0.280, p=0.037) και αυτή η σχέση δεν έδειξε να επηρεάζεται από το BMI (r=-0.340, p=0.009).

Αποτελέσματα της προοπτικής μελέτης:

A.i) Τρεις μήνες μετά τη **φαρμακευτική αγωγή ενηλίκων** με μεικτή δυσλιπιδαιμία, τα επίπεδα της 25(OH)VitD ορού αυξήθηκαν σημαντικά και στις 3 ομάδες: στην ομάδα που έλαβε ροσουβαστατίνη 40 mg αυξήθηκαν κατά 53% (p=0.000), στην ομάδα που έλαβε ροσουβαστατίνη 10 mg μαζί με φαινοφιμπράτη κατά 64% (p=0.001), και στην ομάδα που έλαβε ροσουβαστατίνη 10 mg μαζί με ω3 λιπαρά οξέα κατά 61% (p=0.04). Οι αυξήσεις των επιπέδων 25(OH)VitD ορού ήταν συγκρίσιμες και στις 3 ομάδες φαρμακευτικής αγωγής.

A.ii) Τρεις μήνες μετά τη φαρμακευτική αγωγή ενηλίκων με υπερχοληστερολαιμία, τα επίπεδα της 25(OH)VitD ορού αυξήθηκαν σημαντικά και στις 2 ομάδες: σε αυτή που έλαβε σιμβαστατίνη/εζετιμίμπη 10/10 mg αυξήθηκαν κατά 36.7%, ενώ στην ομάδα που έλαβε σιμβαστατίνη 40 mg κατά 79.1%. Η αύξηση των επιπέδων της 25(OH)VitD

ήταν σημαντικά μεγαλύτερη στην ομάδα σε σιμβαστατίνη 40 mg σε σύγκριση με αυτή που παρατηρήθηκε σε ασθενείς που πήραν σιμβαστατίνη/εζετιμίμπη 10/10 mg (p=0.04).

A.iii) Τρεις μήνες μετά την τροποποίηση της φαρμακευτικής αγωγής ενηλίκων με μεικτή δυσλιπιδαιμία οι οποίοι δεν είχαν επιτύχει τους θεραπευτικούς στόχους με τη συμβατική δόση στατίνης, τα επίπεδα της 25(OH)VitD ορού δεν διαφοροποιήθηκαν σημαντικά και στις 3 ομάδες: στις δύο ομάδες που έλαβαν αντίστοιχα ροσουβαστατίνη 40 mg και ροσουβαστατίνη 10 mg με νικοτινικό οξύ/λαροπιπράντη παρατηρήθηκε μία τάση μείωσης των επιπέδων της 25(OH)VitD (-4.7% and -14.8%, αντίστοιχα) που δεν ήταν στατιστικά σημαντική, ενώ στην ομάδα που έλαβε ροσουβαστατίνη 10 mg με φαινοφιμπράτη διαπιστώθηκε μια αύξηση (+13%) η οποία επίσης δεν ήταν στατιστικά σημαντική. Οι ανωτέρω αλλαγές στα επίπεδα της 25(OH)VitD ορού δεν διέφεραν στατιστικά σημαντικά μεταξύ των 3 ομάδων.

B.i) Στους **ενήλικες με ΜΣ** που έλαβαν per os VitD, τα επίπεδα της 25(OH)VitD αυξήθηκαν κατά 91% (απο 16.0 (3.0-35.0) σε 30.6 (8.4-67.0) ng/mL, p<0.001), ενώ όσοι δεν έλαβαν VitD παρουσίασαν μια μη στατιστικά σημαντική αύξηση κατά 30% (από 10.0 (4.0-39.6) σε 13.0 (3.5-37.0) ng/mL, p=NS). Τα επίπεδα των TCHOL, TGs, HDL-C, LDL-C, ApoA1, ApoB, γλυκόζης και ινσουλίνης νήστεως, HbA1c, HOMA index and διαστολικής ΑΠ δεν μεταβλήθηκαν σημαντικά και στις 2 ομάδες. Στην ομάδα που έλαβε per os VitD η συστολική ΑΠ μειώθηκε κατά 3.7% (από 134±14 σε 129±13 mmHg, p=0.05), ενώ στην ομάδα που δεν έλαβε VitD μειώθηκε μόνο κατά 1.5% (από 132±13 σε 130±16 mmHg, p=NS). Στην ομάδα που έλαβε per os VitD η αύξηση της 25(OH)VitD συσχετίστηκε με τη μείωση της συστολικής ΑΠ (r = -0.398, p = 0.049).

Ωστόσο, δεν παρατηρήθηκαν σημαντικές μεταβολές στα επίπεδα των sdLDL-C, στο ποσοστό των sdLDL, στο μέσο μέγεθος LDL και στη δραστηριότητα $LpPLA_2$, όπως και στα επίπεδα λεπτίνης και αδιπονεκτίνης ορού καθώς και στο λόγο λεπτίνη/αδιπονεκτίνη και στις 2 ομάδες. Επίσης ούτε τα επίπεδα του ορού της ox-LDL-C και των δραστηριοτήτων paraoxonase και arylesterase της PON-1 μεταβλήθηκαν σημαντικά και στις 2 ομάδες. Αντίθετα τα επίπεδα των ισοπροστανίων των ούρων (urine 8-iso-PGF_{2a}) μειώθηκαν σημαντικά κατά 22.7% στην ομάδα που έλαβε per os VitD [από 48.8 (26.8 to 137.1) σε 37.7 (12.3-99.0) ng/mmol creatinine, p

= 0.015], ενώ μια τάση μείωσης κατά 14.4% διαπιστώθηκε στην ομάδα που δεν έλαβε VitD η οποία δεν ήταν στατιστικά σημαντική [από 45.8 (16.6 to 99.3) σε 39.2 (13.3-120.1) ng/mmol creatinine, p=NS]. Ωστόσο η μείωση των επιπέδων των 8-iso-PGF_{2a} ούρων δεν διέφερε στατιστικά σημαντικά μεταξύ των 2 ομάδων.

B.ii) Στους **εφήβους με παχυσαρκία και ανεπάρκεια VitD** πού έλαβαν per os VitD τα επίπεδα της 25(OH)VitD μετά 3 μήνες αυξήθηκαν σημαντικά κατά 88.4% [από 17.3 (12.5-27.8) σε 32.6 (14.3-68.0) ng/mL, p=0.005]. Παράλληλα διαπιστώθηκαν σημαντικές μειώσεις στα επίπεδα της HbA_1c (p=0.03) και της λεπτίνης (p=0.03), ενώ αντίθετα τα επίπεδα της LDL-C αυξήθηκαν (p=0.022). Οι άλλες κλινικές και εργαστηριακές παράμετροι που εξετάσθηκαν (BMI, περίμετρος μέσης, ΑΠ, TCHOL, HDL-C, τριγλυκερίδια, γλυκόζη, ινσουλίνη, HOMA index, PTH) και οι δείκτες οξειδωτικού στρες (oxidized-LDL, paraoxonase, arylesterase and isoprostanes ούρων) δεν έδειξαν σημαντικές διακυμάνσεις.

ΣΥΜΠΕΡΑΣΜΑΤΑ

1) Οι ενήλικες με ΜΣ είχαν σημαντικά χαμηλότερα επίπεδα 25(OH)VitD ορού σε σύγκριση με μάρτυρες της ίδιας ηλικίας και φύλου.

Οι έφηβοι με παχυσαρκία εμφάνισαν σημαντικά μεγαλύτερο ποσοστό ανεπάρκειας/έλλειψης VitD σε σχέση με φυσιολογικού βάρους μάρτυρες (73.9% vs 17.6% αντίστοιχα).

2) Στους ενήλικες με ΜΣ τα επίπεδα της 25(OH)VitD ορού βρέθηκαν να έχουν αρνητική συσχέτιση με τα επίπεδα των τριγλυκεριδίων και των sdLDL-C. Ωστόσο στην πολυπαραγοντική ανάλυση τα επίπεδα των sdLDL-C έδειξαν να επηρεάζονται μόνο από τα επίπεδα των τριγλυκεριδίων και όχι από τα επίπεδα της 25(OH)VitD. Επίσης δεν βρέθηκε συσχέτιση της 25(OH)VitD με τα άλλα διαγνωστικά κριτήρια του ΜΣ (περίμετρος μέσης, ΑΠ, HDL-C και γλυκόζη νηστείας), αλλά ούτε και με τα επίπεδα της $LpPLA_2$ και της hsCRP ορού.

Στους εφήβους με παχυσαρκία η 25(OH)VitD βρέθηκε να έχει αρνητική συσχέτιση με τα επίπεδα της λεπτίνης ορού (ακόμη και μετά τη διόρθωση για τον BMI). Αντίθετα δεν έδειξε να συχετίζεται με άλλες παραμέτρους, όπως το BMI, η ΑΠ, τα λιπίδια, η γλυκόζη, η ινσουλίνη, ο δείκτης HOMA index, η αδιπονεκτίνη, ο λόγος λεπτίνη/αδιπονεκτίνη και η βισφατίνη.

3) Στους ενήλικες με ΜΣ η χορήγηση pos 2000 IU VitD/ημέρα για 3 μήνες σε συνδυασμό με υγιεινο-διαιτητικές οδηγίες δεν οδήγησε σε βελτίωση των κλασσικών καρδιαγγειακών παραγόντων σε σύγκριση με αυτούς που δεν έλαβαν VitD, παρά τη σημαντική αύξηση των επιπέδων της 25(OH)VitD στους πρώτους. Και στις 2 ομάδες τα επίπεδα των τριγλυκεριδίων, της HDL-C, της LDL-C, της γλυκόζης νηστείας, της HbA_1c, του δείκτη HOMA και της διαστολικής ΑΠ δεν μεταβλήθηκαν σημαντικά μετά τους 3 μήνες. Ωστόσο στην ομάδα που έλαβε per os VitD η αύξηση των επιπέδων της 25(OH)VitD ορού βρέθηκε να συσχετίζεται με τη μείωση της συστολικής ΑΠ, ένα εύρημα που θεωρείται ότι έχει κάποια κλινική σημασία. Όμως η χορήγηση VitD δεν βρέθηκε να επηρεάζει τους «αναδυόμενους» καρδιαγγειακούς παράγοντες κινδύνου (επίπεδα sdLDL-C, mean LDL size ή $LpPLA_2$ activity, όπως και τα επίπεδα λεπτίνης και αδιπονεκτίνης και το λόγο λεπτίνη/αδιπονεκτίνη). Επίσης στις 2 ομάδες δεν παρατηρήθηκαν σημαντικές μεταβολές στα επίπεδα των δεικτών **του** οξειδωτικού στρες που μελετήθηκαν (ox-LDL, PON-1 και ARYL), με εξαίρεση τη μείωση των 8-iso-PGF_{2a} ούρων κατά 22.7% στην ομάδα που έλαβε VitD (p=0.015), έναντι της μείωσης κατά 14.4% στην ομάδα ελέγχου (p=NS), μια διαφορά που δεν ήταν στατιστικά σημαντική.

4) Στους εφήβους με παχυσαρκία και ανεπάρκεια VitD η χορήγηση pos 2000 IU VitD/ημέρα για 3 μήνες σε συνδυασμό με υγιεινό-διαιτητικές οδηγίες αύξησε σημαντικά τα επίπεδα της 25(OH)VitD στον ορό και η οποία συσχετίστηκε με οριακή μείωση των επιπέδων της HbA_1c και της λεπτίνης αλλά και με αύξηση των επιπέδων της LDL-C. Οι άλλες κλινικές και εργαστηριακές παράμετροι (BMI, περίμετρος μέσης, ΑΠ, TCHOL, HDL-C, τριγλυκερίδια, γλυκόζη, ινσουλίνη, HOMA index, PTH), όπως και οι δείκτες του οξειδωτικού στρες (ox-LDL, PON-1, ARYL και 8-iso-PGF_{2a} ούρων) δεν μεταβλήθηκαν σημαντικά.

5) Η μονοθεραπεία με υψηλή δόση ροσουβαστατίνης όπως και η συνδυασμένη θεραπεία με συμβατική δόση ροσουβαστατίνης και φαινοφιμπράτης ή με ω3 λιπαρά οξέα συσχετίστηκαν με σημαντική και παρόμοιου μεγέθους αύξηση των επιπέδων της 25(OH)VitD ορού ασθενών με μεικτή δυσλιπιδαιμία.

Ασθενείς με πρωτοπαθή υπερχοληστερολαιμία οι οποίοι πέτυχαν παρόμοια μείωση των επιπέδων της LDL-C με λήψη σιμβαστατίνης 40 mg ή με συνδυασμό σιμβαστατίνης/εζετιμίμπης 10/10 mg παρουσίασαν πάνω από διπλάσια αύξηση των επιπέδων της 25(OH)VitD με τη μονοθεραπεία με σιμβαστατίνη.

Αντίθετα σε ασθενείς με μεικτή δυσλιπιδαιμία οι οποίοι δεν είχαν καταφέρει να επιτύχουν τους θεραπευτικούς στόχους με λήψη συμβατικής δόσης στατίνης, η αλλαγή σε χορήγηση υψηλής δόσης ροσουβαστατίνης ή η προσθήκη φαινοφιμπράτης ή νικοτινικού οξέως/λαροπιπράντης μαζί με τη συμβατική δόση ροσουβαστατίνης δεν οδήγησαν σε σημαντικές αλλαγές στα επίπεδα της 25(OH)VitD.

Συνολικά βάσει των ευρημάτων μας στους ασθενείς με δυσλιπιδαιμία, αντίθετα με τη ροσουβαστατίνη και τη σιμβαστατίνη που φαίνεται να οδηγούν σε αύξηση των επιπέδων της 25(OH)VitD στην κυκλοφορία, τα άλλα υπολιπιδαιμικά φάρμακα (εζετιμίμπη, φαινοφιμπράτη, ω3 λιπαρά οξέα και νικοτινικό οξύ) φαίνεται ότι δεν επηρεάζουν σημαντικά τα επίπεδα.

ABSTRACT

Background: Hypovitaminosis D has been associated with various cardiovascular disease CVD risk factors beginning in childhood/adolescence which manifest later in adulthood. Also statins may affect serum VitD levels, but there are insufficient data on the effect of other hypolipidemic drugs.

Aims & Methods: The present study consists of 2 parts:

Aims & Methods of cross-sectional study:

Determination of serum 25(OH)VitD levels in Greek a) adults with Metabolic Syndrome (MetS) (N=52) and b) adolescents with obesity (N=69) and corresponding controls (58 adults, 34 adolescents). Investigation of the possible correlation between VitD levels and MetS diagnostic criteria and other biochemical parameters.

Aims & Methods of prospective study:

A) Assessment of the effect of hypolipidemic drugs on 25(OH)VitD serum levels in adult patients with dyslipidemia, at the beginning and 3 months after the start of treatment, based on 3 treatment protocols.

B) Assessment of the effect of cholecalciferol (VitD3) administration on metabolic parameters in adults with MetS, randomized to apply either only healthy-dietary guidelines (N=25) or to receive 2000 IU VitD/day pos along with healthy-dietary guidelines (N=25), and in adolescents with obesity (BMI=35.0±7.9) and VitD deficiency (25(OH)VitD <20 ng/mL) (N=15) who received 2000 IU VitD/day pos along with healthy-dietary guidelines and were re-assessed 3 months later.

Results:

Results of the cross-sectional study:

a) **Adults with MetS** had significantly lower serum 25(OH)VitD levels than controls [11.8 (0.6-48.3) ng/mL vs 17.2 (4.8-62.4) ng/mL, p=0.027]. In adults with MetS, univariate regression analysis showed that serum 25(OH)VitD levels correlated negatively with triglycerides, but not with other diagnostic criteria of MetS. In addition, 25(OH)VitD had a negative correlation with sdLDL-C and PTH levels, but not with LDL size, Lp-PLA_2 activity and hsCRP. However, stepwise multivariate linear regression analysis

showed that sdLDL-C levels were significantly affected only by triglyceride levels and not by 25(OH)VitD levels.

b) **Adolescents with obesity** had lower serum 25(OH)VitD levels than normal-weight controls [12.0 (3.0-36.0) versus 34.0 (10.0-69.0) ng/mL respectively, p = 0.000]. In obese adolescents, serum 25(OH)VitD levels were found to be negatively correlated with serum leptin levels, independently of BMI.

Results of the prospective study:

A.i) Three months after **drug treatment** of adults with mixed dyslipidemia, serum 25(OH)VitD levels increased significantly in all 3 treatment groups: in the group receiving rosuvastatin 40 mg they increased by 53%, in group receiving rosuvastatin 10 mg plus phenofibrate by 64%, and in the group receiving rosuvastatin 10 mg plus omega-3 fatty acids by 61%. Increases in serum 25(OH)VitD levels were comparable in all 3 treatment groups.

A.ii) Three months after drug treatment of adults with primary hypercholesterolemia, serum 25(OH)VitD levels increased significantly in both groups: in the recipients of simvastatin/ezetimibe 10/10 mg they increased by 36.7%, while in the group receiving simvastatin 40 mg by 79.1%. The increase in 25(OH)VitD levels was significantly higher in the simvastatin 40 mg group compared to that in simvastatin/ezetimibe 10/10 mg group.

A.iii) Three months after the modification of medication in adults with mixed dyslipidemia who had not achieved the therapeutic goals with a conventional statin dose, serum 25(OH)VitD levels did not change significantly in all 3 treatment groups: in both groups receiving rosuvastatin 40 mg and rosuvastatin 10 mg plus nicotinic acid/laropiprant, a tendency to decrease was observed in 25(OH)VitD serum levels (-4.7% and -14.8%, respectively), which was not statistically significant. In the group that received rosuvastatin 10 mg plus phenofibrate an increase in 25(OH)VitD serum levels (+13%) was found, which was also statistically non-significant. The above changes in serum 25(OH)VitD levels did not differ statistically significantly between the 3 groups.

B.i) In **adults with MetS** who received pos VitD supplementation, 25(OH)VitD levels increased by 91%, while those who did not receive VitD showed a statistically non-significant increase of 30%. In the group that received VitD, systolic BP decreased by

3.7%, while in the group that did not receive VitD it decreased by only 1.5% (p=NS in both). In the group that received VitD, the increase in 25(OH)VitD was significantly associated with a decrease in systolic BP Also, the urine isoprostane levels decreased significantly by 22.7% in the group receiving pos VitD, while a downward trend of 14.4% was found in the group that did not receive VitD, but it was not statistically significant. However, the reduction in urine isoprostane levels did not differ significantly between the 2 groups.

B.ii) In **adolescents with obesity and VitD insufficiency** who received pos VitD, 25(OH)VitD levels increased significantly by 88.4%. At the same time, significant reductions were found in HbA1c and leptin levels, while LDL-C levels increased.

Conclusions: Adults with MetS had significantly lower 25(OH)VitD levels compared with those without MetS. VitD deficiency was also more prevalent among Greek adolescents with obesity compared with controls. In adults with MetS 25(OH)VitD levels were inversely associated with triglycerides. In adolescents with obesity 25(OH)VitD was inversely associated with leptin. VitD supplementation plus dietary intervention in adults with MetS was not associated with any significant change in classic and emerging CVD risk factors, compared with dietary intervention alone. VitD supplementation in adolescents with obesity and hypovitaminosis D effectively increased their 25(OH)VitD levels and was associated with marginal decreases in HbA_1c and leptin as well as an increase in LDL-C levels. Also, unlike rosuvastatin and simvastatin other lipid-lowering drugs like ezetimibe, fenofibrate, omega-3 fatty acids and nicotinic acid seem to have minimal if any effect on 25(OH)VitD serum concentrations in patients with dyslipidemia.

ΣΥΝΤΟΜΗ ΠΕΡΙΛΗΨΗ

Εισαγωγή: Η υποβιταμίνωση D έχει συσχετιστεί με διάφορους παράγοντες κινδύνου καρδιαγγειακής νόσου. Επίσης, οι στατίνες μπορεί να επηρεάσουν τα επίπεδα VitD στον ορό, αλλά δεν υπάρχουν επαρκή στοιχεία για την επίδραση άλλων υπολιπιδαιμικών φαρμάκων.

Σκοπός και μέθοδοι: Η παρούσα μελέτη αποτελείται από 2 μέρη:

Σκοπός και μέθοδοι σύγχρονης μελέτης: Προσδιορισμός επιπέδων 25(OH)VitD στον ορό Ελλήνων α) ενηλίκων με μεταβολικό σύνδρομο (MetS) (N = 52) και β) εφήβων με παχυσαρκία (N = 69) και σε αντίστοιχους μάρτυρες (58 ενήλικες, 34 έφηβοι). Διερεύνηση της πιθανής συσχέτισης των επιπέδων VitD με τα διαγνωστικά κριτήρια MetS και άλλες βιοχημικές παραμέτρους.

Σκοπός και μέθοδοι προοπτικής μελέτης:

Α) Αξιολόγηση της επίδρασης των υπολιπιδαιμικών φαρμάκων στα επίπεδα 25(OH)VitD ορού σε ενήλικες ασθενείς με δυσλιπιδαιμία, στην αρχή και 3 μήνες μετά την έναρξη της θεραπείας, με βάση 3 θεραπευτικά πρωτόκολλα.

Β) Αξιολόγηση της επίδρασης της χοληκαλσιφερόλης (VitD3) στις μεταβολικές παραμέτρους ενηλίκων με MetS, που τυχαιοποιήθηκαν να εφαρμόσουν είτε μόνο οδηγίες υγιεινής διατροφής (N = 25), είτε να λαμβάνουν 2000 IU VitD/ημέρα pos μαζί με οδηγίες για υγιεινή διατροφή (N = 25). Επίσης μελετήθηκαν έφηβοι με παχυσαρκία (BMI = 35,0 ± 7,9) και ανεπάρκεια VitD [25(OH)VitD <20 ng / mL] (N = 15) που έλαβαν 2000 IU VitD/ημέρα pos μαζί με οδηγίες υγιεινής διατροφής και επανεξετάστηκαν 3 μήνες αργότερα.

Αποτελέσματα:

Αποτελέσματα της σύγχρονης μελέτης:

Α) Οι ενήλικες με ΜΣ είχαν σημαντικά χαμηλότερα επίπεδα 25(OH)VitD ορού σε σχέση με τους μάρτυρες [11.8 (0.6-48.3) ng/mL vs 17.2 (4.8-62.4) ng/mL, p=0.027]. Στους ενήλικες με ΜΣ η μονοπαραγοντική ανάλυση έδειξε ότι τα επίπεδα της 25(OH)VitD ορού είχαν αρνητική συσχέτιση με τα τριγλυκερίδια, αλλά όχι με τα άλλα διαγνωστικά κριτήρια του ΜΣ. Επιπλέον τα επίπεδα της 25(OH)VitD είχαν αρνητική συσχέτιση με τα επίπεδα

των sdLDL-C και της PTH. Ωστόσο η πολυπαραγοντική ανάλυση έδειξε ότι τα επίπεδα των sdLDL-C επηρεάζονταν σημαντικά μόνο από τα επίπεδα των τριγλυκεριδίων και όχι από τα επίπεδα της 25(OH)VitD.

Β) Οι έφηβοι με παχυσαρκία είχαν χαμηλότερα επίπεδα 25(OH)VitD ορού σε σχέση με τους φυσιολογικού βάρους μάρτυρες [12.0 (3.0-36.0) versus 34.0 (10.0-69.0) ng/mL αντίστοιχα, p=0.000]. Στους εφήβους με παχυσαρκία τα επίπεδα της 25(OH)VitD ορού βρέθηκε να έχει αρνητική συσχέτιση με τα επίπεδα της λεπτίνης ορού, ανεξαρτήτως του BMI.

Αποτελέσματα της προοπτικής μελέτης:

Α.i) Τρεις μήνες μετά τη φαρμακευτική αγωγή ενηλίκων με μεικτή δυσλιπιδαιμία, τα επίπεδα της 25(OH)VitD ορού αυξήθηκαν σημαντικά και στις 3 ομάδες: στην ομάδα που έλαβε ροσουβαστατίνη 40 mg αυξήθηκαν κατά 53%, στην ομάδα που έλαβε ροσουβαστατίνη 10 mg μαζί με φαινοφιμπράτη κατά 64%, και στην ομάδα που έλαβε ροσουβαστατίνη 10 mg μαζί με ω3 λιπαρά οξέα κατά 61%. Οι αυξήσεις των επιπέδων 25(OH)VitD ορού ήταν συγκρίσιμες και στις 3 ομάδες φαρμακευτικής αγωγής.

Α.ii) Τρεις μήνες μετά τη φαρμακευτική αγωγή ενηλίκων με υπερχοληστερολαιμία, τα επίπεδα της 25(OH)VitD ορού αυξήθηκαν σημαντικά και στις 2 ομάδες: σε αυτή που έλαβε σιμβαστατίνη/εζετιμίμπη 10/10 mg αυξήθηκαν κατά 36.7%, ενώ στην ομάδα που έλαβε σιμβαστατίνη 40 mg κατά 79.1%. Η αύξηση των επιπέδων της 25(OH)VitD ήταν σημαντικά μεγαλύτερη στην ομάδα σε σιμβαστατίνη 40 mg σε σύγκριση με αυτή που παρατηρήθηκε σε ασθενείς που πήραν σιμβαστατίνη/εζετιμίμπη 10/10 mg.

Α.iii) Τρεις μήνες μετά την τροποποίηση της φαρμακευτικής αγωγής ενηλίκων με μεικτή δυσλιπιδαιμία οι οποίοι δεν είχαν επιτύχει τους θεραπευτικούς στόχους με τη συμβατική δόση στατίνης, τα επίπεδα της 25(OH)VitD ορού δεν διαφοροποιήθηκαν σημαντικά και στις 3 ομάδες: στις δύο ομάδες που έλαβαν αντίστοιχα ροσουβαστατίνη 40 mg και ροσουβαστατίνη 10 mg με νικοτινικό οξύ/λαροπιπράντη παρατηρήθηκε μία τάση μείωσης των επιπέδων της 25(OH)VitD (-4.7% and -14.8%, αντίστοιχα) που δεν ήταν στατιστικά σημαντική, ενώ στην ομάδα που έλαβε ροσουβαστατίνη 10 mg με φαινοφιμπράτη διαπιστώθηκε μια αύξηση (+13%) η οποία επίσης δεν ήταν στατιστικά σημαντική. Οι ανωτέρω αλλαγές στα επίπεδα της 25(OH)VitD ορού δεν διέφεραν στατιστικά σημαντικά μεταξύ των 3 ομάδων.

B.i) Στους **ενήλικες με ΜΣ** που έλαβαν per os VitD, τα επίπεδα της 25(OH)VitD αυξήθηκαν κατά 91%, ενώ όσοι δεν έλαβαν VitD παρουσίασαν μια μη στατιστικά σημαντική αύξηση κατά 30%. Στην ομάδα που έλαβε per os VitD η συστολική ΑΠ μειώθηκε κατά 3.7%, ενώ στην ομάδα που δεν έλαβε VitD μειώθηκε μόνο κατά 1.5% (p=NS και στις 2). Στην ομάδα που έλαβε per os VitD η αύξηση της 25(OH)VitD συσχετίστηκε με τη μείωση της συστολικής ΑΠ. Επίσης, τα επίπεδα των ισοπροστανίων ούρων μειώθηκαν σημαντικά κατά 22.7% στην ομάδα που έλαβε per os VitD, ενώ μια τάση μείωσης κατά 14.4% διαπιστώθηκε στην ομάδα που δεν έλαβε VitD, η οποία δεν ήταν στατιστικά σημαντική. Ωστόσο η μείωση των επιπέδων των ισοπροστανίων ούρων δεν διέφερε στατιστικά σημαντικά μεταξύ των 2 ομάδων.

B.ii) Στους **εφήβους με παχυσαρκία και ανεπάρκεια VitD** πού έλαβαν per os VitD τα επίπεδα της 25(OH)VitD αυξήθηκαν σημαντικά κατά 88.4% 3 μήνες μετά. Παράλληλα διαπιστώθηκαν σημαντικές μειώσεις στα επίπεδα της HbA_1c και της λεπτίνης, ενώ αντίθετα τα επίπεδα της LDL-C αυξήθηκαν.

Συμπεράσματα: Οι ενήλικες με MetS είχαν σημαντικά χαμηλότερα επίπεδα 25(OH)VitD σε σύγκριση με εκείνους χωρίς MetS. Η ανεπάρκεια VitD ήταν επίσης πιο διαδεδομένη στους Έλληνες εφήβους με παχυσαρκία σε σύγκριση με τους μάρτυρες. Σε ενήλικες με MetS τα επίπεδα 25(OH)VitD συσχετίστηκαν αντίστροφα με τα τριγλυκερίδια. Σε εφήβους με παχυσαρκία, τα επίπεδα 25(OH)VitD συσχετίστηκαν αντίστροφα με τη λεπτίνη. Η υποκατάσταση VitD και η διατροφική παρέμβαση σε ενήλικες με MetS δεν συσχετίστηκαν με καμία σημαντική αλλαγή στους κλασικούς και αναδυόμενους παράγοντες καρδιαγγειακού κινδύνου, σε σύγκριση με τη διατροφική παρέμβαση μόνο, παρά τη σημαντική αύξηση στα επίπεδα 25(OH)VitD στην πρώτη ομάδα. Η υποκατάσταση VitD σε εφήβους με παχυσαρκία και έλλειψη VitD αύξησε αποτελεσματικά τα επίπεδα 25(OH)VitD ορού και συσχετίστηκε με οριακές μειώσεις της HbA_1c και της λεπτίνης καθώς και με αύξηση των επιπέδων LDL-C. Επίσης, σε αντίθεση με τη ροσουβαστατίνη και τη σιμβαστατίνη, τα άλλα υπολιπιδαιμικά φάρμακα όπως η εζετιμίμπη, φαινοφιμπράτη, ωμέγα-3 λιπαρά οξέα και νικοτινικό οξύ φαίνεται να έχουν ελάχιστη ή καμία επίδραση στα επίπεδα 25(OH)VitD ορού σε ασθενών με δυσλιπιδαιμία.

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PUBLISHED ABSTRACTS IN INTERNATIONAL CONGRESSES RELATED TO THIS DOCTORAL THESIS

1) «Vitamin D status and its relation with emerging risk factors in metabolic syndrome» στο 15th International meeting on fat soluble vitamins, Καλαμπάκα, 22-24 Μαρτίου 2012

2) «Association of vitamin D with emerging risk factors in subjects with metabolic syndrome», 80th European Atherosclerosis Society Congress, Milan, Italy, 25-28 May 2012

3) «Effect of rosuvastatin monotherapy and in combination with fenofibrate or omega-3 fatty acids on serum vitamin D levels»

- 80th European Atherosclerosis Society Congress, Milan, Italy, 25-28 May 2012 and

- European Society o f Cardiology Congress, Munich, Germany, 25-29 August 2012

4) «Effect of Simvastatin/Ezetimibe 10/10 mg versus simvastatin 40 mg on serum Vitamin D levels» 81st European Atherosclerosis Society Congress, June 2-5 2013, Lyon, France

5) «Effect of switch to the highest dose of rosuvastatin vs add-on - nicotinic acid vs add-on - fenofibrate on serum Vitamin D levels in patients with mixed» 82nd European Atherosclerosis Society Congress, May 31 – June 03, 2014; Madrid, Spain

PRESENTED ABSTRACTS IN HELLENIC CONGRESSES RELATED TO THIS DOCTORAL THESIS (in greek language)

1) «Συσχέτιση της βιταμίνης D με αναδυόμενους παράγοντες κινδύνου σε άτομα με μεταβολικό σύνδρομο»

- στο 4ο Συμπόσιο των Ομάδων Εργασίας της Ελληνικής Εταιρείας Αθηροσκλήρωσης, Αθήνα, 2-3 Δεκεμβρίου 2011
- στο 2ο Πανελλήνιο Συνέδριο του Ινστιτούτου μελέτης, έρευνας και εκπαίδευσης για το Σακχαρώδη διαβήτη και τα μεταβολικά νοσήματα, Αθήνα, 27-29/4/2012
- στο 38ο Ετήσιο Πανελλήνιο Ιατρικό Συνέδριο, Αθήνα, 16-19 Μαΐου 2012
- στο 8ο Πανελλήνιο Συμπόσιο των Ομάδων Εργασίας της Ελληνικής Εταιρείας Αθηροσκλήρωσης, Αθήνα, 20-30.11.2019

2) «Μελέτη της συνεργικής επίδρασης της παχυσαρκίας και των επιπέδων της βιταμίνης D στο μεταβολισμό των υδατανθράκων κατά την εφηβική ηλικία»

- στο 50ο Πανελλήνιο Παιδιατρικό Συνέδριο, Ιωάννινα, 1-3/6/2012
- στο 5ο πανελλήνιο συνέδριο Αθηροσκλήρωσης, Αθήνα, 28.11-1.12.2012
- στο 25ο συνέδριο κοινωνικής παιδιατρικής, Ιθάκη, 29.8-1.9.2013
- στο 1ο συνέδριο Ινστιτούτου Μελέτης Μεταβολικών Νοσημάτων, Ιωάννινα, 4-6.4.2014

3) «Επίδραση της ροσουβαστατίνης ως μονοθεραπεία και ως συνδυαστική θεραπεία με φενοφιβράτη ή Ω-3 λιπαρά οξέα στα επίπεδα βιταμίνης D ορού»

- στο 2ο Πανελλήνιο Συνέδριο του Ινστιτούτου μελέτης, έρευνας και εκπαίδευσης για το σακχαρώδη διαβήτη και τα μεταβολικά νοσήματα, Αθήνα, 27-29/4/2012
- στο 38ο Ετήσιο Πανελλήνιο Ιατρικό Συνέδριο, Αθήνα, 16-19 Μαΐου 2012

4) «Μελέτη της χορήγησης συνδυασμού σιμβαστατίνης/εζετιμίμπης 10/10 mg έναντι της μονοθεραπείας με σιμβαστατίνη 40 mg στα επίπεδα της βιταμίνης D του ορού.»

- στο 1ο συνέδριο Ινστιτούτου Μελέτης Μεταβολικών Νοσημάτων, Ιωάννινα, 4-6.4.2014
- στο 5ο πανελλήνιο συνέδριο Αθηροσκλήρωσης, Αθήνα, 28.11-1.12.2012

5) «Επίδραση της αλλαγής της υπολιπιδαιμικής αγωγής σε μέγιστη δόση ροσουβαστατίνης έναντι της προσθήκης νικοτινικού οξέως ή φαινοφιμπράτης στα επίπεδα 25(OH)VitD ορού ασθενών με μεικτή δυσλιπιδαιμία» στο 6ο Πανελλήνιο Συνέδριο Ελληνικής Εταιρείας Αθηροσκλήρωσης, Αθήνα, 4-6.12.2014

6) «Μελέτη της επίδρασης χορήγησης Βιταμίνης D σε διάφορες μεταβολικές παραμέτρους ατόμων με μεταβολικό σύνδρομο» στο 6ο Πανελλήνιο Συνέδριο Ελληνικής Εταιρείας Αθηροσκλήρωσης, Αθήνα, 4-6.12.2014

7) « Επίδραση της χορήγησης 25(OH)VitD στα επίπεδα των 8-ισοπροστανίων στα ούρα σε ασθενείς με μεταβολικό σύνδρομο»

- στο 8ο Πανελλήνιο Συνέδριο ελευθέρων ριζών και οξειδωτικού στρες, Θεσσαλονίκη, 12-14/10/2012 (1ο Βραβείο καλύτερης εργασίας εις μνήμην Νικολάου Δημητρίου)
- στο 5ο πανελλήνιο συνέδριο Αθηροσκλήρωσης, Αθήνα, 28.11-1.12.2012

AWARDS RELATED TO PRESENTATIONS IN GREEK CONGRESSES BASED ON THIS DOCTORAL THESIS

1) « Επίδραση της χορήγησης 25(OH)VitD στα επίπεδα των 8-ισοπροστανίων στα ούρα σε ασθενείς με μεταβολικό σύνδρομο»

- στο 8ο Πανελλήνιο Συνέδριο ελευθέρων ριζών και οξειδωτικού στρες, Θεσσαλονίκη, 12-14/10/2012 (1ο Βραβείο καλύτερης εργασίας εις μνήμην Νικολάου Δημητρίου)

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