Vitamin D in insulin sensitivity and obesity

VITAMIN D IN INSULIN SENSITIVITY AND OBESITY:
FACT OR FICTION?

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This thesis is dedicated to my beloved jewels Fadhlan & Fathima,
children of tomorrow. You both will shape the world someday.
So be brave, be kind, and be passionate with what you do.

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CHAPTER 1

General Introduction

Obesity pandemic

The World Health Organization (WHO) defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to human health (1). Currently, in population settings, obesity is defined by body mass index (BMI, calculated as weight in kilograms divided by the square of height in meters (kg/m^2)), a simple measure of body fatness based on height and weight that applies to adult men and women. A BMI ≥25 kg/m^2 is categorized as overweight and a BMI > 30 kg/m^2 is classified as obese (2). Recent findings indicate that BMI might not be considered as an accurate marker to define body fat distribution and cardiometabolic risk (3, 4).

Over the last decades, the prevalence of obesity worldwide has risen enormously and reached pandemic proportions. According to World Health Organization report in 2018, about 39% of adults aged 18 years and over (39% of men and 40% of women) were overweight. Overall, about 13% of the world's adult population (11% of men and 15% of women) were obese (1). According to a recent report based on data from the Organization for Economic Cooperation and Development (OECD) (5), obesity has reached epidemic proportions in both developed (USA (38.2%), UK (26.9%), The Netherlands (12.8%), and Japan (3.7%)) and developing (Brazil (20.8%), China (7.0%), India (5.0%) and Indonesia (5.7%)) countries (6). Taken together, it is now clear that prevalence of obesity expands in all age groups and in both sexes, irrespective of ethnicity, geographical locations and socioeconomic status (7).

Obesity is strongly associated with an increased risk of Type 2 Diabetes Mellitus (T2D), cardiovascular disease (e.g. dyslipidemia, stroke and hypertension), non-alcoholic fatty liver disease (NAFLD), musculoskeletal disorders, certain type of cancers and mental illness (8). Nowadays, not only the Western society is affected by obesity, also many low-middle-income countries have increased morbidity and mortality rates because of the increasing prevalence of obesity (9). Furthermore, obesity and its co-morbidities are associated with increased healthcare costs (particularly secondary health care) and a reduced ability for employment, indicating high impact on social-economic burden (10). Thus obesity and its related co-morbidities have now been well-recognized as a major health problem, and the prevalence of obesity is predicted to still increase in upcoming years thereby having a large impact on future public health (6).

Metabolic interorgan crosstalk in obesity and insulin resistance

The underlying cause of overweight and obesity is an imbalance between energy intake and energy expenditure, which is influenced by genetic, microbial, neuro-biological, behavioral, and environmental factors (11). Obesity is strongly associated with the development of insulin resistance which is defined as an impaired action of insulin on its metabolic target organs such as adipose tissue (AT), liver, skeletal muscle and pancreas (12, 13). **Figure 1** illustrates the inter-organ crosstalk between these tissues under obesity-induced insulin resistant conditions.

The adipose tissue is not only a passive fat storage organ, but is an active metabolic and endocrine organ (14, 15). The adipose tissue has two major functions in lipid metabolism, (1) to store lipid as triacylglycerol (TAG) from the uptake of meal-derived chylomicrons and

(2) the release of non-esterified fatty acids (NEFA) to supply energy into non-adipose tissue organs during conditions of increased energy demand (16). In the presence of a chronic positive energy balance, the fatty acid supply may exceed the storage capacity. This results in dysfunctional adipose tissue characterized by enlarged adipocytes (17, 18), an impaired differentiation of preadipocytes (i.e. adipogenesis) (19, 20), decreased adipose tissue blood flow (16, 21, 22), chronic low-grade local inflammation (23, 24) and an impaired lipid buffering capacity (25, 26). This impaired lipid buffering capacity is characterized by an reduced insulin-mediated TAG extraction and a reduced insulin-mediated inhibition of endogenous lipolysis. The impaired inhibition of endogenous lipolysis may be (partly) compensated by a relative increase in insulin secretion and thus increased circulating insulin concentrations (25). If the AT buffering capacity is exceeded, fatty acids can spillover in the circulation and will be accumulated in other insulin sensitive tissues such as liver, skeletal muscle, and pancreas, which may relate to impairment in insulin sensitivity and insulin secretion (12, 13). Additionally, adipose tissue inflammation may result in systemic low grade inflammation, which has also been linked to the development of insulin resistance (27).

In the liver, lipid accumulation may be explained by (i) an increased spillover of NEFA from subcutaneous adipose tissue (SAT) into circulation (28), (ii) an increased flux of FFA from visceral adipose tissue (VAT) through portal vein directing to the liver ("portal vein hypothesis") (29), and (iii) an increased uptake from lipoprotein remnants (28). An increased lipogenesis due to an increased hepatic lipid supply leads to an increase of very-low-density-lipoprotein (VLDL)-TAG secretion. Hepatic lipid accumulation interferes with insulin signaling in the liver, resulting in a diminished insulin-mediated suppression of VLDL production and secretion (26). Furthermore, hepatic insulin resistance is associated with an increased of endogenous glucose production and a reduced glycogen storage in the liver (30, 31).

The skeletal muscle is an important organ in peripheral insulin sensitivity since it contributes to 70-90% of the total glucose disposal under postprandial conditions (26). An elevated lipid supply from adipose tissue and liver to circulation may result in an increased muscle lipid uptake. The obese, insulin resistant state is characterized by a reduced capacity to adapt fat oxidation to fatty acid supply (32). This leads to an increased intramuscular lipid accumulation, in particular the accumulation of bioactive lipid metabolites, which contributes to impairments in the insulin signalling (33, 34), a subsequent reduced glucose transporter-4 (GLUT-4) translocation to the skeletal muscle membrane (35), and a reduced insulin-mediated glucose uptake (36).

In pancreas, chronic exposure to elevated levels of NEFAs results in a decreased insulin gene expression and blunted glucose-stimulated insulin secretion (GSIS) *in vitro* (37). Using proton-magnetic resonance spectroscopy (^{1}H-MRS), it has been demonstrated that pancreatic lipid accumulation was increased in individuals with an impaired glucose metabolism (38). This pancreatic fat accumulation may be associated with a decreased glucose-stimulated insulin secretion by the beta-cells (39).

As a consequence, obesity-related insulin resistance accompanied by impairments in insulin secretion can be progressing towards a prediabetic state and subsequently toward T2DM (40). Prediabetes can be classified as a state of impaired fasting glucose (IFG, fasting glucose > 5.6 mmol/L) and/or impaired glucose tolerance (IGT, 2h oral glucose tolerance test OGTT)-derived glucose 7.8 – 11.0 mmol/L) (41, 42). These two prediabetic states, may be representing different etiologies towards T2D (43).

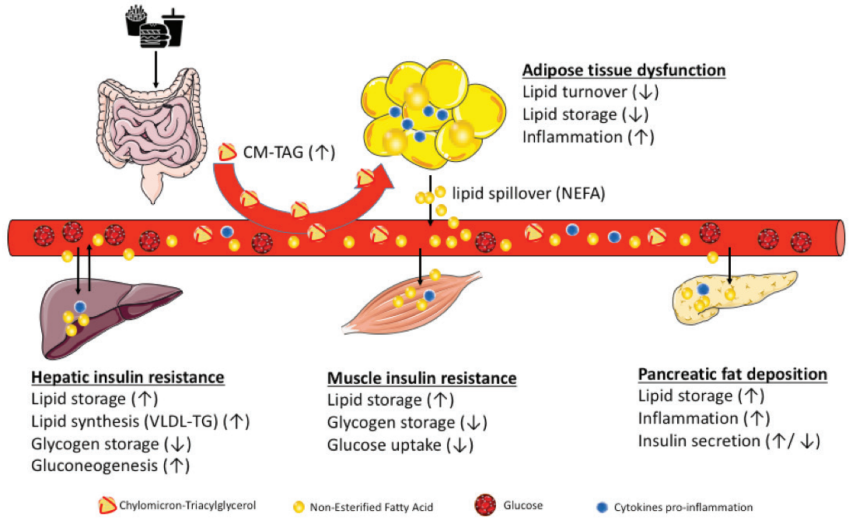

Figure 1. Inter-organ crosstalk in human obesity-related insulin resistance. Long-term positive energy balance results in systemic lipid overload and an increased lipid storage in the adipose tissue. When the buffering capacity in the AT is diminished, fatty acids (NEFA) spillover in non-adipose tissues such as liver, skeletal muscle, and pancreas. An impaired oxidation of lipids in these tissues, directly to increase lipid accumulation within these tissues and induce tissue-specific insulin resistance. Furthermore, cytokines pro-inflammation may also spillover from the AT towards other tissues and potentially decrease whole-body insulin sensitivity. Of note, an increased of insulin secretion has been suggested as an adaptive response to whole-body insulin resistance at the early stage, and it decreases along with chronic insulin resistance and beta-cell dysfunction. (↑) increase; (↓) decrease.

Measurement of insulin resistance

There are several methodologies for estimating insulin resistance. Homeostasis model assessment of insulin resistance (HOMA-IR) is a common-used surrogate marker describing glucose-insulin homeostasis by mathematically-derived nonlinear equations. It is derived from the use of the fasting insulin (FPI) and glucose (FPG) product, divided by a constant (22.5) (44). The HOMA-IR is a surrogate marker, which may reflect merely hepatic insulin resistance (45). In addition, Matsuda and DeFronzo have developed a model to estimate whole-body insulin sensitivity from the OGTT. Here the OGTT index of insulin sensitivity [ISI (composite)] was calculated using both the data (insulin and glucose) during 2-hours of the test (46).

Furthermore, Abdul-Ghani et al, has developed a model to estimate hepatic insulin resistance and muscle insulin sensitivity based on a multiple sampling points OGTT. The hepatic insulin resistance index (HIRI) is estimated using the square root of the product of the area under curves (AUCs) for glucose and insulin during the first 30 min of the OGTT, where as muscle insulin sensitivity index (MISI) can be estimated using the rate of decay of plasma glucose concentration during the OGTT (47). Among the tools to determine IR and measure whole-body insulin sensitivity, the hyperinsulinemic euglycemic clamp technique is recognized as the gold-standard method to estimate insulin sensitivity (44). In a two step of hyperinsulinemic euglycemic clamp with a [6,6-$^{2}H_{2}$]-glucose tracer infusion both hepatic and peripheral insulin sensitivity as well as adipose tissue insulin sensitivity can be assessed (48, 49).

Obesity, insulin resistance and vitamin D status

Skin synthesis of vitamin D_3 stimulated by sun exposure is the major source of vitamin D in humans. Vitamin D can also be ingested from the diet as vitamin D_2 and D_3 (50). In form vitamin D_2 is commonly found in mushroom (51), whereas vitamin D_3 can be found in oil-rich fish and eggs, meat, fortified foods (most often milk and dairy products, margarine and spreads and some breakfast cereals), as well as supplementation (50, 52). The circulating vitamin D 25-hydroxyvitamin D_3 [25(OH)D_3/calcifediol) is still inactive until this has been hydroxylated (1-α-hydroxylase enzyme/CYP27B1) into 1,25-dihydroxyvitamin D/1,25OH_2D_3 (calcitriol) in the kidney, which is the active vitamin D metabolite (53). Both of the active and inactive form of vitamin D have been shown to be lipophilic as indicated by recent studies which were able to detect both vitamin D metabolites in adipose tissue (54, 55). Vitamin D deficiency (defined as having [25(OH)D_3] levels <50 nmol/L or <20 ng/mL) (56) often seen in human obesity across age, ethnicity and geographical locations (57).

Vitamin D: skeletal function vs. extra-skeletal role in glucose and lipid metabolism

The causal role of vitamin D deficiency in bone health is well established. Vitamin D sufficiency is pivotal for normal skeletal development from utero until childhood, and also for maintaining bone health in adults (58). This is due to the fact that sufficient of vitamin D levels lead to adequate calcium-phosphorus concentrations resulting in an effective bone mineralization to prevent vitamin D deficiency and related diseases like rickets (59). In adults, low 25(OH)D_3 and high Parathyroid hormone (PTH) also lead to low serum calcium and phosphorus concentrations, resulting in osteomalacia, i.e., a defective mineralization of the collagen matrix causing a reduction of structural support and being associated with an increased risk of fracture (58).

During the last couple decades, studies suggest the link between vitamin D and chronic metabolic diseases or conditions like obesity, insulin resistance and T2DM. Observational human studies have extensively documented an inverse relationship between vitamin D status and obesity-related insulin resistance (60). Vitamin D has been shown to stimulate the expression of insulin receptor substrate in muscle tissue of HFD mice, (61). Vitamin D directly activates peroxisome proliferator activator receptor-ð (PPAR-ð) (62), a transcription factor involved in the regulation of fatty acid metabolism in skeletal muscle and adipose tissue. Vitamin D may have an effect on insulin secretion by maintaining extracellular calcium, ensuring normal calcium influx through cell membranes and adequate calcium pool in the pancreatic beta cells (63). The role of of vitamin D in human lipid and glucose metabolism and its deficiency in obesity-associated insulin resistance has been reviewed more extensively in **chapter 2** of this thesis.

Vitamin D and adipose tissue metabolism

As indicated above, the obese insulin resistant state is also often associated with low levels of serum vitamin D (*Hypovitaminosis* D) (64) both in children (65-67) and adult men and women (60, 68). Adipose tissue represents a major storage reservoir for

vitamin D, which is not surprising giving its characteristics as a fat-soluble vitamin, although the underlying mechanisms for the circulating deficiency in human obesity is not clearly understood. The enlarged pool of visceral and subcutaneous AT in human obesity probably sequestrates vitamin D and its metabolites, reducing their bioavailability (69). Beside vitamin D uptake by adipose tissue, there also evidence of release both in mice as well as in human adipocytes (70). A recent *ex vivo* data by Di Nisio et al demonstrated that a blunted adrenaline-mediated lipolytic response, a characteristic of the obese insulin-resistant state, was accompanied by an increased accumulation of $25(OH)D_3$ in human SAT from obese donors, possibly indicating a blunted vitamin D release (71). However, *in vivo* data from human is rather limited, as more extensively discussed in **chapter 2**.

Vitamin D-related genes, obesity, and insulin resistance

Next to environmental (sun exposure, season) and behavioral (food intake) factors, genetic factors may play an important role in determining serum $25(OH)D_3$ concentrations (72). A study in twin male showed that genetic variation in vitamin D related metabolism genes may explain more than 50% of the variation in vitamin D metabolite concentrations particularly during winter (73). However, genome-wide association studies have shown that genetic variants in vitamin D-related genes explain a modest (~10%) of variations in circulating vitamin D concentrations (74-77). Genetic variations of vitamin D receptor (VDR) is most often associated with vitamin D concentration and metabolic health outcomes (78).

Vitamin D receptor genetic variants

Human and animal data indicate that VDR is present at the RNA and protein level in adipose tissue (AT) (79) and other insulin sensitive tissues such as liver (80) and skeletal muscle (81), thereby contributing to vitamin D-mediated effects on glucose and lipid metabolism in these tissues. VDR is known as a member of the superfamily of the steroid hormone receptors and is also recognized as a nuclear transcription factor (82). The genomic responses to vitamin D result from the interactions between its active metabolite [$1,25(OH)_2D_3$] and its intracellular VDR. Then, after the binding of vitamin D, VDR forms a heterodimer with the retinoid-X receptor (RXR) that binds to specific vitamin D response elements (VDREs) on DNA sequences leading to expression or trans-repression of several VDR targets genes of which some are involved regulating lipid and glucose metabolism (e.g. PPAR-ð, insulin receptor substrate-1) (82, 83).

The human VDR gene is located on chromosome 12q, which encodes the full-length VDR protein. FokI, BsmI, ApaI and TaqI (SNPs) have been identified in VDR. Ye et al. (84) found that the VDR TaqI T allele was associated with obesity in French Caucasian individuals with early onset of type 2 diabetes. A study conducted in a Saudi Arabian cohort identified that the VDR TaqI G allele polymorphisms were significantly more frequent in obese individuals (84). In a Central-European population, it has been suggested that genetic variability in the VDR region (including FokI and ApaI polymorphisms) may be an important factor influencing anthropometric characteristics associated with obesity including BMI, Fat Mass and body fat distribution (85). Furthermore, a study in Poland showed that the BsmI variant may be associated with BMI in men (86), but not in women (87). However, larger

genome wide studies in UK (men and women) (88) and Chinese women (89) indicate that VDR genetic variants are unlikely a major determinant of obesity related phenotype such as BMI, waist circumference (WC), and waist-to-hip ratio (WHR). In addition, it has been suggested that FokI variant is related with insulin sensitivity (86). However, is it unknown whether these VDR SNPs are associated with insulin sensitivity and to what extent these SNPs may affect intervention outcome (vitamin D supplementation), as was explored in **chapter 2** of this thesis.

Vitamin D supplementation and insulin sensitivity

The link between obesity, insulin resistance and vitamin D deficiency may give a rational for vitamin D supplementation (90). Therefore, increasing circulating vitamin D levels through either vitamin D fortified foods or drinks (91-93) as well as oral supplementation (94) has been hypothesized to improve glucose metabolism and insulin sensitivity. Numerous randomized controlled trials (RCTs) have been conducted to investigate whether vitamin D supplementation has a causal effect on glucose homeostasis and insulin resistance. However, the results from these RCTs are still inconsistent and next to only fasting glucose measures, as well as indirect markers of insulin sensitivity are used. Some studies suggested a beneficial effect to improve insulin sensitivity (94-96), on the other hand, studies did not find any beneficial effects on glucose homeostasis and/or insulin sensitivity (97-99). Evidence of human interventional studies regarding the effect of vitamin D supplementation on glucose homeostasis and insulin sensitivity is described more detail in **chapter 2** and **chapter 6** of this thesis.

Outline of thesis

This thesis describes the role of vitamin D in human overweight/obesity and whole body and tissue-specific insulin sensitivity. As described earlier in this thesis, the link between vitamin D deficiency, obesity-related insulin resistance is reviewed in more detail in **chapter 2**. In addition, this chapter provides and an extensive overview on the etiology of vitamin D deficiency in obesity-related insulin resistance, which covers vitamin D metabolism, the role of vitamin D in (tissue-specific) insulin resistance and in increased adiposity (both human observational and intervention studies). Vitamin D is known as a fat-soluble vitamin that may accumulate in adipose tissue.

In human with overweight/obesity, the expanded adipose tissue mass may take up and store vitamin D thereby representing as a reservoir (metabolic sink) for vitamin D, reducing its bioavailability. To investigate this, in **chapter 3** we analyzed the uptake and release of vitamin D metabolites [25(OH)D_3 and 1,25$(OH)_2D_3$] across abdominal subcutaneous adipose tissue in lean and obese men following an overnight fast and beta-adrenergic stimulation (to stimulate lipid hydrolysis) using state-of-the-art arterio-venous difference methodology. In **Chapter 4** investigates whether the circulating vitamin D metabolites [25(OH)D_3 and 1,25$(OH)_2D_3$] are associated with adipose tissue, muscle and liver insulin sensitivity using the gold standard hyperinsulinemic euglycemic clamp in individuals with overweight/obesity. Furthermore, vitamin D receptor (VDR) and vitamin D-metabolizing enzymes [cytochrome 450 (CYP)] expression in adipose tissue (AT) was studied in relation to AT insulin sensitivity.

Genetic variants in VDR have been suggested to link with obesity related insulin resistance. In **Chapter 5** extends the outcome from previous **chapter 4** and describes the association between genetic variations of VDR, adiposity and (tissue-specific) insulin resistance as well as weight loss and weight regain in human with overweight/obesity from DIOGENES cohort, a Pan-European, randomized, controlled dietary intervention study investigating the effects of dietary protein and glycemic index on weight loss and weight maintenance. Several meta-analyses on effect of vitamin D supplementation only focus on markers related fasting insulin and glucose concentrations.

In **chapter 2**, we briefly resumed recent meta-analysis study the effect of vitamin D supplementation on glucose metabolism in humans. However, most meta-analyses have been executed with fasting measures of glucose and insulin metabolism. In **chapter 6** we conducted a meta-analysis on the effect of vitamin D supplementation on insulin sensitivity derived from Matsuda index, insulin sensitivity index, as well as hyperinsulinemic euglycemic clamp methodologies. Finally, in **chapter 7** the results of the present thesis are discussed in broader perspective.

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CHAPTER 2

Vitamin D deficiency in the aetiology of obesity-related insulin resistance

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Summary

The obese insulin resistant state is often associated with low circulating concentration of vitamin D 25-hydroxyvitamin D_3 [25(OH)D_3]. Fat sequestration of vitamin D in the expanded obese adipose tissue mass has been pointed out as a plausible explanation for this circulating vitamin D deficiency. However, the putative mechanisms behind this hypovitaminosis D remain to be elucidated.

The presence of vitamin D receptor and vitamin D metabolizing enzymes in insulin-sensitive organs suggests that vitamin D may be involved in glucose and lipid metabolism and may be related to insulin sensitivity. Indeed, mainly *in vitro* studies support a role of vitamin D in regulating glucose and lipid metabolism in several insulin sensitive tissues including adipose tissue, skeletal muscle, liver, as well as pancreatic insulin secretion. A potential role of vitamin D in gut barrier function and metabolism has also been suggested. This review summarizes recent knowledge on vitamin D deficiency in the etiology of obesity related insulin resistance, and discusses potential underlying mechanisms. Finally, the role of vitamin D supplementation on insulin sensitivity and glycemic control will be discussed

Introduction

Obesity is a main contributor to the development of type 2 diabetes mellitus (T2D) and cardiovascular diseases (CVD) and has become a major public health problem worldwide (1, 2). The World Health Organization (WHO) has reported that more than 422 million people suffer from T2D worldwide.(3) In addition, the prevalence of prediabetes, an intermediate condition of fasting and/or postprandial hyperglycemia, has also been indicated to sharply increase in several countries (4-6). Much attention has been given to the prevention of T2D, with emphasis on the prediabetic state. Compared to normal glucose tolerant (NGT) people, 37% of the pre-diabetic individuals develop T2D within 4 years (7), so maintaining their blood glucose levels in the normal range may reduce the incidence of T2D (8, 9).

Obesity is generally described as a condition of excessive fat accumulation, with abdominal obesity being the leading risk factor for insulin resistance and T2D (10). Obesity–associated health complications are often accompanied by dysfunctional adipose tissue (AT) rather than an increased fat mass per se, which is characterized by impairments in lipid storage capacity, particularly in the postprandial state. Hence the surplus energy as triglyceride will flow over into the circulation and result in an increased lipid supply towards non-adipose tissue organs like the liver and the skeletal muscle (11, 12). Fat deposition in these non-adipose tissues may result in functional impairments that contribute to the development of whole-body insulin resistance leading to T2D (13).

Originally, the fat-soluble vitamin D has been well known as major regulator of calcium and phosphate homeostasis related to bone metabolism. There is considerable evidence that vitamin D 25-hydroxyvitamin D_3 (25OH)D_3) deficiency (concentration <50 nmol/L) is associated with osteoporosis and reduced bone health (14, 15) On the other hand, evidence is accumulating that vitamin D has much broader non-skeletal roles in the human body than previously thought. In line, vitamin D metabolizing enzymes and vitamin D receptors (VDR) are expressed in several insulin-sensitive tissues, suggesting extrarenal metabolism and activity of vitamin D (16, 17). Obesity, insulin resistance and T2D are often characterized by a relative circulating vitamin D deficiency (hypovitaminosis D), suggesting a role for vitamin D in the pathophysiology of obesity-related metabolic disorders (18-20). There is accumulating mechanistic evidence for a role of vitamin D in glucose and lipid metabolism in insulin sensitive tissues like the adipose tissue, skeletal muscle and liver (21-23). In this review, we discuss the latest knowledge on vitamin D metabolism as well as its role in glycemic control and lipid metabolism. Firstly, vitamin D metabolism, receptor expression and intracellular signaling will be discussed. Additionally, the role of the active vitamin D metabolite 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and VDR in controlling tissue substrate metabolism and insulin sensitivity will be addressed. Subsequently, data on vitamin D status in the obesity-related insulin resistant will be reviewed. Finally, an overview will be given from recently published meta-analyses on the effect of vitamin D supplementation to improve insulin sensitivity and blood glucose control.

Vitamin D metabolism

In human, vitamin D_3 can be synthesized from the skin following ultraviolet B (UVB)

light exposure (15). Vitamin D_2 and D_3 from diet also contribute to circulating levels of vitamin D (24), although their relative contribution to circulating vitamin D levels is still unknown. Vitamin D_3 has a higher affinity to bind to vitamin D binding protein (VDBP) and is therefore more likely to be hydroxylated in the liver (24). In the liver, the hydroxylase enzyme converts vitamin D_3 into 25-hydroxyvitamin D_3 ($25(OH)D_3$) at the endoplasmic reticulum (25). The mechanism by which $25(OH)D_3$ is released from the liver and subsequently bound to VDBP to be transported to the kidneys or extrarenal tissues is not well understood (25).

25-hydroxyvitamin D_3 in complex with its plasma carrier, VDBP, is filtered through the glomerulus and reabsorbed in the proximal tubules by the endocytic receptor megalin (26). Subsequently, $25(OH)D_3$ is converted by the 1-α-hydroxylase enzyme to biologically active $1,25(OH)_2D_3$ intracellularly.(26, 27) Dysfunction of these receptors, which is commonly found in patients with diabetic nephropathy, even at early stages, may explain why vitamin D deficiency is often observed in these patients (27).

Interestingly, recent *in vitro* studies have shown that megalin is involved in the uptake and release of vitamin D $25(OH)D_3$ in murine muscle cells (28), whereas cubilin is involved in uptake and release of vitamin D $25(OH)D_3$ in mice white adipocytes (29). The presence of functional VDR and vitamin D metabolizing enzymes was identified at the transcriptional and protein level in murine muscle (30) and adipose tissue (31) indicating extrarenal vitamin D metabolism. Uptake and release of $25(OH)D_3$, in mice adipose and muscle cells has been investigated (29, 32), which was regulated by VDR and its ligand ($1,25(OH)_2D_3$) in a time dependent-manner (33). Thus, vitamin D uptake and release in tissues like muscle and adipose tissue might be tightly regulated (29, 34).

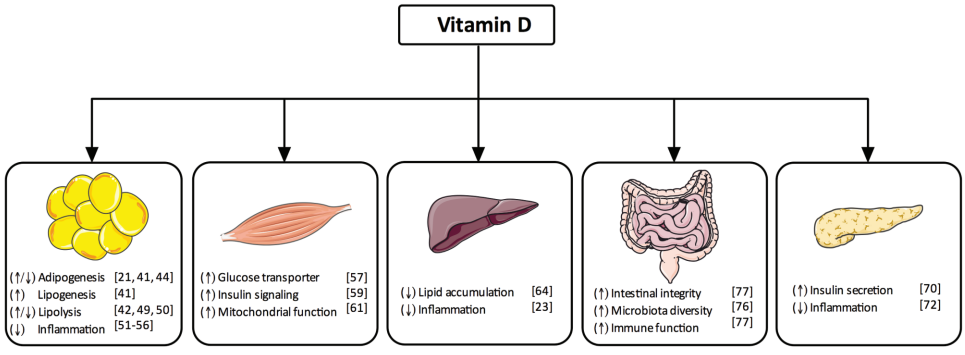

Figure 1. Metabolic effects of Vitamin D on insulin sensitive tissues including adipose tissue metabolism, muscle glucose/lipid metabolism, liver metabolism, pancreatic insulin secretion, and gut-intestinal function. Of note, majority of evidence for these functional effects are derived from in vitro and animal models which warrant further investigations in human. (↑) increase; (↓) decrease; references between brackets [].

The vitamin D receptor belongs to the nuclear receptor family, thereby controlling DNA transcription.(35) Briefly, the cytosolic VDR-$1,25(OH)_2D_3$ complex translocates to the nucleus where it subsequently interacts with retinoic acid X receptor (RXR) to form RXR-VDR complexes that bind to specific DNA regions called vitamin D response element (VDRE) regions, hence regulating VDR-targeted gene expression (36). Although the VDR primarily acts as nuclear transcription factor, it is postulated that non-genomic actions of vitamin D involve rapid binding of $1,25(OH)_2D_3$ to both cytosolic and membrane VDR which activates

several second messenger systems (37, 38). Interestingly, a VDRE was identified in the promoter of the human insulin receptor gene, indicating vitamin D might be involved in the transcriptional control of insulin signaling (39).

Role of vitamin D in adipose tissue lipid metabolism

VDR mRNA expression was identified in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) of lean and obese. In VAT, expression of VDR was higher in obese than in lean, where as no difference of VDR expression in SAT was observed (40). In addition, VDR protein expression has recently been demonstrated in primary adipocytes derived from obese donors (21), indicating the possible involvement of vitamin D in AT development and metabolism. Indeed, recent studies suggest a role of vitamin D in AT adipogenesis, lipogenesis, lipolysis, and inflammation (21, 41-43).

Adipogenesis

An *in vitro* study using 3T3-L1 preadipocytes has shown that treatment with $1,25(OH)_2D_3$ (10nM) suppresses the major adipogenic transcription CCAAT enhancer binding protein alpha/beta (CEBPα/β) and peroxisome proliferator activated receptor-γ (PPAR-γ) in a VDR dependent manner, resulting in inhibition of adipogenic differentiation (44). Of interest $1,25(OH)_2D_3$ was added to the medium in the early phase of differentiation. In contrast when 10 nM $1,25(OH)_2D_3$ was added in the late phase of differentiation, there was an increased mRNA expression of adipogenic markers like PPAR-γ, CEBPα/β, Fatty Acid Binding Protein 4 (FABP4), and Fatty Acid Synthase (FASN) in AT from human multipotent adipose-derived stem cells (hMADS) and subcutaneous pre-adipocytes (21, 41). A recent study has suggested $1,25(OH)_2D_3$ (10 nM) upregulated CEBP expression after 24h of induction of differentiation in human SGBS cells (45). It is likely that treating cell with vitamin D at different stages of differentiation could partly explain these opposite findings in human primary adipocytes (21, 41) as compared to murine cell model (44). Nevertheless, taken together, these data suggested that vitamin D might affect adipogenesis (**Figure 1**) at the transcriptional level, at least in a species specific manner.

Lipogenesis

Incubation of differentiated subcutaneous human adipocytes derived from male and female donors with a wide range of BMI (25.6–50.9 kg/m^2) with 10 nM $1,25(OH)_2D_3$ (for 24 hours) increased the protein expression of the lipogenic enzyme fatty acid binding protein 4 (FABP4), the mRNA expression of lipoprotein lipase (LPL), and increased triacylglycerol (TAG) accumulation as compared to control adipocytes (41). Furthermore, incubating human adipocytes with 5 nM $1,25(OH)_2D_3$ increased FAS protein level (46). Upregulation of FAS by $1,25(OH)_2D_3$ is likely mediated by the VDR(21) and VDR activity may be necessary for lipid accumulation in the late phase of adipogenesis (47). However, *in vivo* injection of $1,25(OH)_2D_3$ (1g/kg/day) in Sprague-Dawley rats (16 days) did not increase mRNA expression

of FAS in AT (48). These findings indicate that vitamin D may modulate lipogenic enzymes expression at least *in vitro* **(Figure 1)**, therefore human *in vivo* studies are required to clarify these findings *in vitro*.

Lipolysis

A study by Xue et al. showed that $1,25(OH)_2D_3$ (5 nM) inhibits basal and beta-adrenergically mediated lipolysis in human adipocytes, possibly via increased intracellular calcium levels resulting in decreased cAMP levels and a reduced hormone-sensitive lipase (HSL) phosphorylation (46, 49). In contrast, recent data by Chang et al. (42) and Larrick et al(50) showed that $1,25(OH)_2D_3$ increased glycerol release both under basal and beta-adrenergically stimulated conditions in murine 3T3-L1 adipocytes (42). This increased lipolysis was accompanied by increased mRNA expression of the lipase HSL, and markers of fat oxidation, and mitochondrial biogenesis/function like carnitine palmitoyltransferase 1 (CPT1α), PPARα, co-activator proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and sirtuin 1 (SIRT1) (42). Thus, data on the effect of vitamin D and lipolysis in human primary and murine adipocytes are still inconsistent **(Figure 1)**.

Inflammation: cytokine-adipokine production

Low-grade circulating inflammation and increased AT infiltration of immune cells are strongly associated with local adipose tissue and whole-body insulin resistance. *In vitro* studies using murine 3T3-L1 adipocytes and human pre-adipocytes have shown that pro-inflammatory markers (e.g. IL-6, MCP-1, IL-1β) and TNF-α-derived macrophage activity is reduced following treatment with 10 nM of $1,25(OH)_2D_3$, and this effect is partly mediated by VDR (51, 52) In line, recent *in vivo* findings in Sprague-Dawley male mice following 12-weeks combined High Fat and Low Vitamin D Diet (HFD+LVD) have demonstrated an increase in AT macrophage infiltration, IL-6 and TNF-α mRNA and protein expression compared to the HFD+1000 IU Vitamin D/Kg group (53). NFκB/MAPK mediated mechanisms have been described to explain the possible anti-inflammatory action of the VDR-ligands in murine adipocytes,(54, 55) as well as human preadipocytes (56).

In summary, the current available *in vitro* and animal data indicate that vitamin D may affect AT lipid buffering capacity by affecting adipogenic differentiation, lipogenesis, intracellular lipolysis, and oxidative capacity (the latter via effects on mitochondrial biogenesis/function). Furthermore, Vitamin D may have beneficial effects on local AT and systemic low-grade inflammation via activation of NFκB/MAPK mediated pathways **(Figure 1)**. Currently, there is a gap between data available from *in vitro* studies using animal-human derived cell models and well-controlled human intervention studies.

Role of Vitamin D on muscle glucose and lipid metabolism

As indicated above, vitamin D deficiency may affect AT lipid buffering capacity, thereby altering fatty acid supply to skeletal muscle and indirectly affecting muscle lipid content (preventing lipotoxicity). Additionally, direct effects of vitamin D on skeletal muscle

metabolism have also been described. For instance, L6 and C2C12 myotubes treated with 100 nM (24h) and 10nM of 1,25$(OH)_2D_3$ (72h) showed an elevated glucose transporter 4 (GLUT4), VDR and Insulin Receptor (IR) mRNA expression, but functional effects on glucose uptake were not determined (57, 58). In line, C2C12 mouse myotubes treated with 10nM 1,25$(OH)_2D_3$ for 48h ameliorated lipid-induced insulin resistance, through improved insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation and increased serine phosphorylation of protein kinase B (*PKB*), also known as *Akt* (59, 60). Additionally, 1,25$(OH)_2D_3$ increased mRNA content of Perilipin 2 (PLIN 2,) as well as adipose triglyceride lipase (ATGL) in C2C12 myotubes (22), indicating an involvement of vitamin D in muscle lipolysis and intramuscular lipid catabolism.

Furthermore, treatment of human primary muscle cells from healthy lean donors with 10 nM 1,25$(OH)_2D_3$ resulted in an improved mitochondrial morphology (volume and structure) and altered mRNA expression of genes involved in muscle glucose and lipid metabolism like pyruvate dehydrogenase kinase 4 (PDK4) and CPT1 (61), These in vitro animal and human data indicate that vitamin D may affect muscle insulin sensitivity via improvement of mitochondrial function, substrate oxidation, and lipid turnover **(Figure 1)**. However, future investigations are needed to elucidate the physiological importance of vitamin D in muscle substrate handling and insulin sensitivity in humans.

Role of Vitamin D in liver lipid and glucose metabolism

Obesity is often associated with excessive fat deposition in the liver causing inflammation and hepatic insulin resistance (62). An imbalance between lipid availability (from adipose tissue lipolysis or *de novo* lipogenesis) and lipid removal (via fatty acid oxidation or triglyceride-rich lipoprotein secretion) in the liver can result in hepatic lipid accumulation, leading to non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) (63).

In vivo and *in vitro* studies in rodents have provided insight into the putative mechanisms through which vitamin D may lower liver lipid accumulation possibly via effects on the enterohepatic circulation and autophagy. Intrahepatic injection of 1,25$(OH)_2D_3$ (5 ng/g body weight, twice per week) for 4 weeks in HFD+VDD treated mice resulted in an upregulation of bile acids transport, suppressed gene expression related to hepatic lipogenesis and proinflammatory markers, thereby improving the NASH phenotype.(23) In line, intraperitoneal injection of 1,25$(OH)_2D_3$ (2.5 ng/g body weight 3 times per week) for 4 weeks significantly reduced liver lipid accumulation in mice (64). As shown in HepG2 cells, 1,25$(OH)_2D_3$ may ameliorate hepatic steatosis by upregulating autophagy-related (i.e. ATG16L1) mRNA expression, indicating that vitamin D might induce lipophagy via the autophagy-lysosomal pathway (64).

Nelson and colleagues showed that serum 25$(OH)D_3$ levels are inversely associated with hepatocyte damage, as reflected by hepatocellular ballooning degeneration, in obese men and women with NASH (65). However, another study has reported no correlation between serum vitamin D and liver fat accumulation nor liver insulin sensitivity and inflammation in obese men and women with NASH (66). These data indicate that studies on the association between vitamin D, liver fat and liver insulin sensitivity and inflammation in humans are inconsistent, despite accumulating mechanistic evidence from *in vitro* and *in*

vivo rodent models **(Figure 1)**.

Role of Vitamin D in insulin secretion

Obesity-associated insulin resistance may chronically elevate glucose and fatty acids in the circulation accompanied by a decline in beta cell function, resulting eventually in a reduced insulin secretion. Therefore, improving beta cell insulin secretion or responsiveness has a major effect on glycemic control (67, 68).

Incubation of beta cells derived from VDD mice with 1 nM $1,25(OH)_2D_3$, pretreated with high-glucose, resulted in a reduced mRNA and protein expression of Renin Angiotensin System (RAS) components (69), of which involvement in insulin secretion has been demonstrated in a T2D mice model (70). In addition, incubation of pancreatic cell islets from type 1 diabetic donors with $1,25(OH)_2D_3$ increased VDR expression and prevented cytokine-induced FAS expression (mRNA and protein), which is a mediator of beta-cells apoptosis (71). A recent *in vitro* study has suggested that a VDR-ligand (Calcipotriol/derivative of $1,25(OH)_2D_3$) may protect human ß-like cells (derived from human induced pluripotent stem cells/iPS) against interleukin 1ß-mediated inflammation (72). Together, these data suggest that vitamin D may affect pancreatic insulin secretion in a RAS dependent manner, via induction of beta-cell apoptosis, and may protect beta-cell from local inflammation **(Figure 1)**. Furthermore, the putative underlying mechanisms for the effect of vitamin D on insulin secretion and glycemic control in human pancreatic islets remain to be determined.

Role of Vitamin D in gut

It is well-known that intestinal calcium balance can be modulated by vitamin D through stimulating intestinal calcium absorption as shown in human and animal studies (73, 74). The presence of the VDR and the expression of CYP27B1 in small and large intestinal epithelial and immune cells indicate the biological and metabolic significance of vitamin D in the human gut (75). Interestingly, it has been shown that vitamin D_3 supplementation for 4 weeks results in an increased microbial diversity and a decrease in relative abundance of Gammaproteobacteria in the upper gastro-intestinal tract in healthy men and women (76). Furthermore, intraperitoneal injection of $1,25(OH)_2D_3$ (5 ng/g body weight) for one month attenuated metabolic endotoxemia (as evidenced by lower plasma lipopolysaccharides, LPS), and improved intestinal tight junction integrity, as well as Paneth cell defense in HFD+VDD fed mice (77). An elegant review by Zheng and colleagues showed that evidence is accumulating for a role of Vitamin D in improved beta cell function in Type 1 Diabetes (T1D), possibly via effects on the microbiome (78). Nevertheless, the underlying mechanisms still need further investigations. Overall, these findings indicate that vitamin D may have important roles in intestinal barrier function, local inflammation and modulation of microbial composition **(Figure 1)**.

Vitamin D status in the obese insulin resistant state

Inverse correlations between serum $25(OH)D_3$ and weight, BMI, and waist circumference have been shown in overweight and obese adults with different ethnic

backgrounds (79). Low circulating 25(OH)D_3 concentrations in human obesity might be due to increased uptake and sequestration of fat-soluble vitamin D in the increased fat mass, though the underlying mechanism is unclear (80). In line, dilution of vitamin D due to a higher volume distribution in obese has been proposed as underlying mechanism for the inverse correlation between body size (body weight) and serum 25(OH)D_3 concentrations (81). In addition, obese individuals may spend less time outdoors, which may result in reduced endogenous vitamin D production contributing to the lower circulating vitamin D levels observed in obesity (82). Interestingly, Wamberg and colleagues showed that VDR expression was comparable between VAT and SAT from obese individuals, but higher in VAT from obese individuals compared with VAT from lean individuals (40). The expression of 25-hydroxylase enzymes (CYP2J2, CYP27A1 and CYP27B1) was decreased in SAT from obese individuals (40). These data indicate an impaired adipose tissue vitamin D related metabolism of obese individuals **(Figure 2)**. However, it is still unclear whether this impaired adipose tissue vitamin D related metabolism is affected by circulating vitamin D concentrations, insulin resistance or adiposity per se in human obesity.

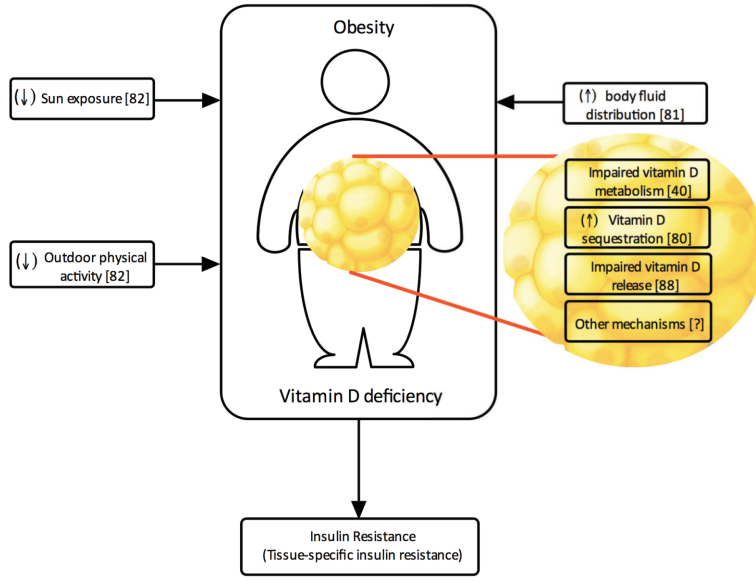

Figure 2. Obesity is often accompanied with low circulating vitamin D levels (vitamin D deficiency). External factors such as less sun exposure and less outdoor physical activity may be associated with low circulating vitamin D levels in human obesity. Furthermore, endogenous mechanisms, including increased body fluid distribution and adipose tissue dysfunction, might also contribute to increased vitamin D sequestration and an impaired vitamin D release in human obesity, which may ultimately lead to the development of insulin resistance. (↑) increase; (↓) decrease; references between brackets [].

Fat mass reduction has been considered as a strategy to increase circulating vitamin D in obesity. For instance, energy restriction using a very low-calorie diet (VLCD, 500 kcal per day) combined with physical activity increased plasma 25(OH)D_3 levels after 1 year intervention in obese individuals, which was inversely related with the reduction of VAT volume (83). In contrast, the intensive lifestyle Diabetes Prevention Program (DPP)

showed that a decrease total body fat was not associated with increased plasma 25(OH)D_3 concentration in prediabetic individuals (84). In addition to lifestyle intervention, weight-reducing surgery using gastric bypass only resulted in a temporal elevation of serum 25(OH)D_3 levels, which decreased again one month after surgery (85). Furthermore, two systematic reviews and one meta-analysis concluded that weight loss interventions showed only marginal changes in serum 25(OH)D_3 following significant weight and body fat loss (86, 87). Thus, the above studies provide inconsistent evidence on the relation between AT mass and the circulating levels of serum 25(OH)D_3. Therefore, it may be that the often observed circulating vitamin D deficiency in obesity is more complex than previously thought and cannot be solely explained by increased sequestration/dilution in the expanded AT mass.

Of interest, recent *ex vivo* data by Di Nisio et al (88) demonstrated that a blunted adrenaline mediated lipolytic response, a characteristic of the obese insulin resistant state was accompanied by increased accumulation of 25(OH)D_3 in human subcutaneous adipocytes from obese donors, possibly indicating a blunted vitamin D release. Thus, there may be an important link between an impaired lipid mobilization/turnover and an attenuated vitamin D release from the AT in obese-insulin resistant individuals which remains to be investigated in more detail.

Vitamin D, blood glucose control and insulin sensitivity

The potential effects of vitamin D on glucose homeostasis and insulin sensitivity have been studied in numerous human cross-sectional and intervention studies. The majority of these cross-sectional studies showed a positive association of serum 25(OH)D_3 levels with insulin sensitivity measures derived from a hyperinsulinemic euglycemic clamp test(89, 90) or indirect markers such as Matsuda index (91), or Quantitative Insulin Sensitivity Index (QUICKI)(92, 93). A negative association was observed with Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)(93) or 2-hour Oral Glucose Tolerance Test (2h OGTT) (94), However, in most studies the relationship between serum 25(OH)D_3 and markers of insulin sensitivity/resistance attenuated (95, 96) or even disappeared (94, 97) after adjustment for markers of adiposity (including BMI, and/or body fat percentage). This indicates that the association between vitamin D deficiency and degree of insulin resistance in obese individuals, may be mediated mainly by the increased body weight and changes in body composition.

Evidence from human intervention studies

Obesity-associated with circulating vitamin D deficiency may cause a decreased vitamin D bioavailability. Therefore, increasing circulating vitamin D levels by means of supplementation, is expected to affect tissue function and insulin sensitivity in the obese resistant state. As shown in **table 1**, eight meta-analyses published between 2012 and 2018 (98-105) of human intervention carried out between 1980 and 2017 showed an increased plasma vitamin D 25OHD_3 concentration following supplementation, but the majority of these meta-analyses suggested insufficient evidence to no effects on glycemic control and insulin sensitivity/resistance. The included interventions were performed across different ethnicities, in both sexes, and with a broad spectrum of vitamin D doses, modes of administration, treatment duration and most often used surrogate markers of insulin resistance.

Table 1. Meta-analyses of RCTs regarding the effect of vitamin D supplementation on insulin sensitivity and glucose metabolism

Investigators (Year)	N trials included	Study Populations	Markers of glucose & insulin (effect size)	Outcome
George et al. (2012)	15 RCTs from 1984 to 2010	Subjects with NGT and IFG	- FPG (SMD –0.02; 95% CI: –0.10 to 0.06; P=0.59) - HOMA-IR (SMD –0.07; 95% CI: –0.20 to 0.06; P = 0.30) - HbA1c (SMD 0.03; 95% CI: –0.18 to 0.23; P=0.81)	No effect
Jamka et al. (2015)	12 RCTs between 2007 and 2014	Subjects with Obesity	- FPG (SMD -0.10; 95% CI: –0.26 to 0.07; P=0.25) - HOMA-IR (SMD 0.04; 95% CI: –0.44 to 0.52; P=0.86)	No effect
Li et al. (2018)	20 RCTs between 2008 and 2017	Subjects with T2D	- FPG (WMD -3.59; 95%CI: -7.94 to 0.76; P=0.11) - HOMA-IR (SMD –0.57; 95%CI: −1.09 to –0.04; P=0.03) - HbA1c (WMD -0.11; 95%CI: -0.35 to 0.13; P=0.38)	Insufficient evidence
Mirhosseini et al.(2017)	24 RCTs between 2009 and 2016	Subjects with T2D	- FPG (SMD -0.27; 95%CI: -8.1 to -1.6; P=0.003) - HOMA-IR (SMD: -0.66; 95%CI: -1.06 to -0.26; P = 0.001) - HbA1c (SMD: -0.30; 95%CI: -0.45 to -0.15; P<0.001)	Sufficient evidence
Mirhosseini et al.(2018)	28 RCTs between 2009 and 2017	Prediabetes	- FPG (WMD –0.46; 95% CI: –0.74 to –0.19; P=0.001) - HOMA-IR (WMD –0.39; 95% CI: –0.68 to –0.11; P=0.007) - HbA1c (WMD –0.48; 95% CI: –0.79 to –0.18; P=0.002)	Sufficient evidence
Poolsup et al. (2016)	10 RCTs between 2007 and 2014	Subjects with Prediabetes (based on IFG or IGT)	- FPG (MD –0.10; 95% CI: –0.18 to –0.03; P = 0.006) - HbA1C (MD –0.89; 95% CI: –1.54 to –0.23; P = 0.008) - HOMA-IR (MD –0.06; 95% CI: –0.36 to 0.24; P = 0.69) - 2hOGTT (MD –0.23; 95% CI: –0.65 to 0.19; P=0.28)	Insufficient evidence
Seida et al. (2014)	36 RCTs between 1984 and 2013	Subjects with NGT, Prediabetes, T2D	- HOMA-IR (MD –0.04; 95% CI: –0.03 to 0.22; P = 0.77) - HbA1c (MD –0.05; 95% CI: –0.12 to 0.03; P=0.20)	No effect
Wu et al. (2017)	24 RCTs between 2007 and 2016	Subjects with T2D	- FPG (SMD −0.14; 95%CI: −0.31 to 0.03; P=0.10) - HbA1c (SMD −0.25; 95%CI: −0.45 to −0.05; P=0.001)	Insufficient evidence

Abbreviations: 2OGTT: 2-hour Oral Glucose Tolerance Test; FPG: Fasting Plasma Glucose; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; HbA1C: Glycated Haemoglobin; IGT: Impaired Glucose Tolerance; MD: Mean Difference; NGT: Normal Glucose Tolerance; SMD: standardized mean difference; WMD: weighted mean difference; T2D: Type 2 Diabetes.

Focusing on humans with obesity, a meta-analysis in 1181 subjects with overweight/obesity (100), including sub-analysis based on dosage (high vs. low), duration (long term vs. short term), and group (vitamin D deficient vs. non-deficient) showed no effect of vitamin D supplementation on glucose metabolism and insulin resistance. Furthermore, subgroup analysis suggested that insulin resistance is improved in non-obese as compared to obese subjects (102-104). Together, these data may point towards no direct causality between circulating vitamin D concentration and development of insulin resistance in humans with obesity.

Conclusion and future recommendations

This review reports that obesity and insulin resistance are often associated with circulating vitamin D deficiency, which may be partly explained by an increased sequestration/dilution in the expanded AT mass. Additionally, vitamin D metabolism within the AT may also be altered as reflected by a down-regulation of vitamin D metabolizing enzymes and the finding of an impaired (lipolysis-mediated) release of vitamin D 25(OH)D_3. However, further studies are needed to determine the underlying mechanism in humans with obesity.

The role of vitamin D in tissue specific glucose and lipid metabolism is still under debate in humans. Data from mostly murine and *in vitro* studies indicate that vitamin D possibly contributes to the improvement of AT lipid buffering or fat storage capacity and may reduce the pro-inflammatory state, but data are not entirely consistent and human evidence is lacking. Vitamin D has also been implicated in muscle insulin signaling, intramuscular lipid partitioning as well as liver inflammation and steatosis. However, these (murine) *in vitro* findings have not been followed by consistent human *in vivo* evidence on whole-body

and peripheral (muscle, liver and adipose) insulin sensitivity. Overall, the current published meta-analyses do not support the use of vitamin D supplementation for improving glycemic control and insulin sensitivity in overweight/obese humans. Nevertheless, it should also be noted that a universal approach (one size fits all) for vitamin D deficiency related-insulin resistance may not be appropriate. Perhaps ethnic/geographical location and genetic variations of vitamin D-related metabolism (106, 107) may influence responses to, and benefits of, vitamin D supplementation (104, 108). Therefore, further evaluation of genetic determinants of vitamin D status among different ethnic/geographical groups and metabolic phenotype may enable the identification of population subgroups, which may benefit from vitamin D supplementation. Finally, combining vitamin D supplementation with other modes of intervention (e.g. diet, exercise, polyphenols, or other micronutrients) might provide new strategies for the treatment and prevention of insulin resistance in humans but this needs to be investigated under well-controlled human settings.

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Author contributions

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Declaration of interest

The authors declare that they have no conflict of interest.

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CHAPTER 3

Vitamin D release across abdominal adipose tissue in lean and obese men: the effect of ß-adrenergic stimulation

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Abstract

Obesity is characterized by a blunted lipolytic response in abdominal subcutaneous adipose tissue (SAT) and low circulating vitamin D levels. Here, we investigated whether an impaired SAT lipolytic response coincides with an impaired SAT vitamin D release in eight lean and six obese men. 25-hydroxyvitamin D_3 [25(OH)D3] and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] fluxes across SAT were measured using arterio-venous blood sampling in combination with AT blood flow measurements after an overnight fast and during 1-hour intravenous infusion of the non-selective ß-adrenergic agonist isoprenaline (20 ng.kg FFM^{-1}.min^{-1}). $1,25(OH)_2D_3$ was released across abdominal SAT during isoprenaline infusion in lean [-0.01 (-0.04 to 0.00) pmol*100g $tissue^{-1}$*min^{-1}, P=0.017 vs. zero flux], but not in obese men [0.01 (0.00 to 0.02) pmol*100g $tissue^{-1}$*min^{-1}, P=0.116 vs. zero flux], and accompanied by an impaired isoprenaline-induced lipolytic response in abdominal SAT of obese versus lean men. Isoprenaline had no significant effects on net $25(OH)D_3$ release across abdominal SAT and plasma vitamin D metabolites in lean and obese men. To conclude, a blunted isoprenaline-mediated lipolysis is accompanied by reduced release of $1,25(OH)_2D_3$ vitamin D across abdominal SAT in obesity.

Introduction

Obesity is associated with adipose tissue (AT) dysfunction, which is characterized by adipocyte hypertrophy, AT inflammation and impaired lipid metabolism, thereby contributing to insulin resistance (1). Noteworthy, obesity is often characterized by low circulating vitamin D levels (2). In line, studies have described an inverse association between body mass index (BMI) and circulating concentration of the inactive vitamin D metabolite 25-hydroxyvitamin D_3 ($25(OH)D_3$) (3). Obesity-associated insulin resistance is often accompanied by dysfunctional adipose tissue (AT), which might also contribute to increased vitamin D sequestration and an impaired vitamin D release in human obesity (4).

Uptake and sequestration of vitamin D in the expanded obese AT mass may partly contribute to the relatively low vitamin D concentrations in the circulation in obesity (5), although the underlying mechanism is not yet clearly understood. Furthermore, evidence in rodents suggests that vitamin D may also be released from adipose tissue into the circulation (6). Obesity is characterized by increased lipid storage in the form of triacylglycerol (TAG), mainly in adipose tissue. Catecholamine stimulation leads to an increase in adipose tissue lipolysis, thereby resulting in the hydrolysis of TAG stored in lipid droplets (1). Since vitamin D is a lipophilic vitamin that has been postulated to accumulate in adipose tissue, deliberation of TAG may coincide with release/mobilization of vitamin D metabolites from adipose tissue (7). In line with our hypothesis, Di Nisio et al have shown that adipose tissue derived from obese individuals releases less vitamin D ex vivo when stimulated with adrenaline compared to lean subjects. In the latter study, impaired mobilization of vitamin D coincided with blunted catecholamine-induced lipolytic response, determined by glycerol release into the medium. Nevertheless, other mechanisms like competition between some free fatty acids and vitamin D for binding sites to vitamin D binding protein (DBP) (8) or a role of adipose tissue blood flow (ATBF) in relation to both lipolysis and vitamin D release may possibly be involved. Therefore, it is tempting to speculate that low circulating vitamin D levels in human obesity might be due to an increased uptake and/or a blunted release of vitamin D across abdominal subcutaneous AT (SAT), which may occur concurrently with the often observed blunted catecholamine-mediated lipolysis (9). However, it remains to be determined whether ß-adrenergic stimulation induces vitamin D $25(OH)D_3$ [inactive metabolite] as well as $1,25(OH)_2D_3$ [active metabolite] release across human SAT *in vivo*.

In the present study, using arterio-venous methodology, we investigated (1) the effect of ß-adrenergic stimulation on net release of vitamin D $25(OH)D_3$ [inactive] and $1.25(OH)_2D_3$ [active metabolite] across abdominal SAT in lean and obese men, and (2) whether an impaired release of vitamin D across obese abdominal SAT is accompanied by a blunted lipolytic response in obese men.

Methods

Study participants

The participants of this study were a subset of previous study (Jocken et al, 2008). Eight lean (BMI <25 kg/m^2) and six obese (BMI >30 kg kg/m^2) men were included in this analysis. Inclusion criteria for both groups were that participants had to be weight-stable (weight change <3.0 kg) for at least 3 months prior to the study, were in good health as

assessed by medical history, were free of any medication and spent not more than 3h of organized sports activities a week. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, use of medication known to affect body weight and glucose metabolism, or untreated hypertension. The Medical Ethical Committee of Maastricht University (MEC-03-179) approved the study which was performed according to the procedures set by the latest version of the Declaration of Helsinki, and written informed consent was obtained from all participants.

Study Design

In this study, participants were allowed to perform only light-intensity physical activity (for examples: walking slowly (in the office), sitting in front of computer/TV, and no sports activities) 2-3 prior to the test day. All participants were asked to refrain from drinking alcohol and to perform no strenuous exercise for 24 h before the study. Participants came to the university and underwent arterio-venous (A-V) blood sampling across abdominal SAT after an overnight fast and after 1 hour intravenous infusion of the nonselective ß-adrenergic agonist isoprenaline (20 ng (kg FFM)$^{-1}$ min^{-1}), as previously described (9). Circulating concentrations and fluxes across abdominal SAT of glycerol and vitamin D were measured following a 3 hour primed (3µmol.kg-1) constant infusion of [$^{2}H_{5}$]glycerol (0.2 umol.kg-1.min-1). Blood samples were taken simultaneously from the arterialised venous blood was sampled from a superficial dorsal hand vein and adipose vein at three baseline time points (*t*90, *t*105 and *t*120 min) and at three time points during the last 30 min of isoprenaline infusion (*t*150, *t*165 and *t*180 min). Adipose tissue blood flow (ATBF) was monitored continuously using the ^{133}Xe wash-out technique. Lean and obese subjects were studied throughout the year in random order, thus the comparison between groups was not confounded by seasonal variation.

Laboratory analysis

Blood samples were transferred into ice-chilled polypropylene tubes and were centrifuged (1000 g, 4 °C, 10 min). Plasma was immediately frozen in liquid nitrogen and safely stored at -80 °C until analyses. It has been previously demonstrated that when immediately frozen (at -80°C), vitamin D metabolites are stable for many years (10-14). Vitamin D 25(OH)D_3 and 1,25$(OH)_2D_3$ levels were measured in arterialized and venous plasma samples. Plasma samples at time-points t90, t105 and t120 min (steady state for lipolysis at baseline) and t150, t165 and t180 min (steady state for lipolysis during ISO) were pooled because of a lack of sample material at the same time point for all subjects. There was a steady state at baseline as well as during ISO so that pooling was justified. Vitamin D metabolites were analyzed using liquid-chromatography tandem-mass spectrophotometry (LC-MS/MS) (15). Stable isotope enrichment of glycerol was measured using GC-MS as described previously (9).

Calculations

Net vitamin D 25(OH)D_3 and 1,25$(OH)_2D_3$ fluxes across SAT were calculated by multiplying arterial-venous (A-V) concentration differences by adipose tissue plasma flow,

as described in other contexts (9, 16). Tissue blood flow during baseline is an average of time-points t90, t105, t120 min and during ISO is an averaged of t150, t165 and t180 min. Plasma flow was calculated as tissue blood flow multiplied by (1 – haematocrit/100), with haematocrit expressed as a fraction. Positive fluxes indicate net uptake from the circulation, whereas negative fluxes indicate net tissue release into the circulation. The SAT total glycerol uptake was calculated as described previously according to the steady state Steele's equation. Abdominal SAT total glycerol release was calculated by subtracting abdominal SAT net glycerol flux to abdominal SAT total glycerol uptake (9).

Statistical Analysis

Subjects characteristics were normally distributed, data are presented as mean ± standard deviation (SD), and differences between lean and obese were tested using Student's unpaired t test. Since vitamin D metabolites, net fluxes, and adipose tissue blood flow were not normally distributed, a non-parametric Mann-Whitney test was used for group comparisons. The effects of ß-adrenergic stimulation within groups were tested using the Wilcoxon signed-rank test, and data presented as median (range). Kruskal Wallis test was performed in order to analyze the difference between groups. A Spearman correlation was performed to analyse the relationships between vitamin D fluxes, glycerol, NEFA and circulating vitamin D levels. Statistical calculations were performed with SPSS for Macintosh (version 21.0; SPSS, Chicago, IL, USA).

RESULTS

Subject characteristics

Table 1 shows that lean and obese subjects had a comparable age. By definition, BMI, body fat percentage, body fat mass as well as homeostasis model assessment for insulin resistance (HOMA-IR) were significantly higher in obese compared with lean participants (all $P<0.01$).

Adipose tissue blood flow (ATBF)

Table 1. Characteristics of participants

Characteristics	Lean (N=8)	Obese (N=6)
Age (years)	50 ± 9	53 ± 9
BMI(kg/m)	23.7 ± 1.3	32.3 ± 2.2**
Waist (cm)	88.9 ± 3.1	110.2 ± 7.3**
WHR	0.9 ± 0.03	1.0 ± 0.03*
BF(%)	21.5 ± 3.0	31.8 ± 1.6**
FM (kg)	16.2 ± 2.0	31.4 ± 4.5**
HOMA-IR	1.8 ± 0.7	3.6 ± 1.0*

**P<0.01; **P<0.001, values are mean ± SD*
BF, body fat; BMI, body mass index; FM, fat mass; HOMA-IR, homeostatic model assessment for insulin resistance; WHR*, Waist to Hip Ratio..*

Adipose tissue blood flow (ATBF) was comparable between lean and obese individuals at baseline ($P=0.108$). As expected, Isoprenaline significantly increased ATBF both

in lean [1.8(1.3–2.9) vs. 4.8(2.6–11.1) ml (100 g tissue)$^{-1}$min^{-1}; P=0.01) and obese [1.3(1.2–2.4) vs. 3.2(2.1–6.2) ml (100 g tissue)$^{-1}$min^{-1}; P=0.03] men. Importantly, this increase was not different between groups (P=0.245).

Systemic (arterialized) concentrations and net vitamin D release across abdominal SAT

Vitamin D 25(OH)D_3

Plasma arterialized 25(OH)D_3 did not differ between lean and obese individuals at baseline (56.1(16.0–84.5) vs 49.6(33.5–63.4) nmol/L, respectively, P=0.852). Additionally, no net 25(OH)D_3 release across abdominal SAT was observed at baseline in both lean [0.02(-5.2–5.0) pmol*100g tissue^{-1}*min^{-1}, P=1.000 vs. zero flux] and obese [0.70(-4.9–1.7) pmol*100g tissue^{-1}*min^{-1}, P=0.917 vs. zero flux].

Following ß-adrenergic stimulation plasma (arterialized) 25(OH)D_3 level was not significantly increased in lean [baseline: 56.1(16.0–84.5) vs. isoprenaline 55.0(17.4–80.0), P=0.499] (**Fig.1A**) or obese men [baseline: 49.6(33.5–63.4) vs. 49.3(34.6–64.6), P=0.917] (**Fig.1B**). In line, ß-adrenergic stimulation did not significantly induce net 25(OH)D_3 release across abdominal SAT in lean [-1.9(-10.8–3.2) pmol*100g tissue^{-1}*min^{-1}, P=0.208 vs. zero flux] **(Fig.1C)** and obese [-3.4(-12.1–2.4) pmol*100g tissue^{-1}*min^{-1}, P=0.249 vs. zero flux] **(Fig.1D)**.

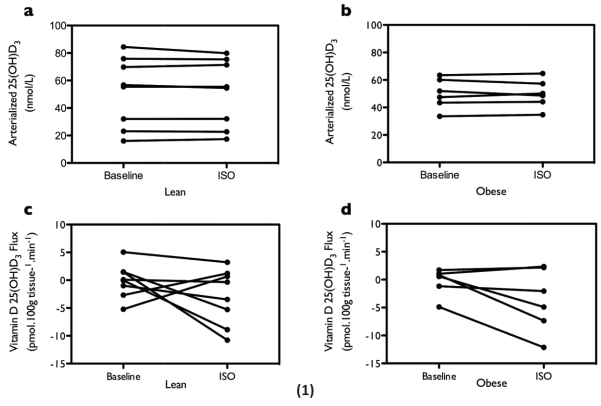

Figure 1. Panel a and b depict plasma (arterialized) Vitamin D 25(OH) D3 concentration at baseline and following ISO in lean (n = 8) and obese (n = 6). Net vitamin D 25(OH)D3 release (flux) across abdominal SAT in lean (Panel c) and obese (Panel d)

Vitamin D 1,25(OH)$_2$$D_3$

Plasma arterialized 1,25(OH)$_2$$D_3$ did not differ between lean and obese individuals at baseline (96.6(60.7–185.7) vs 108.2(80.5–147.7) pmol/L, respectively, P=0.755). Furthermore, no net 1,25(OH)$_2$$D_3$ release across SAT was observed under baseline conditions in lean [0.00(-0.01–0.03) pmol*100g tissue^{-1}*min^{-1}, P=0.889 vs. zero flux] and obese men [0.00(-0.09–0.00) pmol*100g tissue^{-1}*min^{-1}, P=0.345 vs. zero flux]. Following ß-adrenergic stimulation plasma (arterialized) 1,25(OH)$_2$$D_3$ was not significantly increased in lean [baseline: 96.6(60.7–185.7) vs. isoprenaline 97.0(51.9–165.2), P=0.889] (**Fig.2A**) or obese men [baseline: 108.2(80.5–147.7) vs. 111.1(90.1–139.3), P=0.528] (**Fig.2B**). However,

the ISO induced change in 1,25$(OH)_2D_3$ fluxes was significantly different between groups (P=0.007). An increased net 1,25$(OH)_2D_3$ release across abdominal SAT was observed in lean [ISO: -0.01(-0.04–0.00), P=0.017 vs. zero flux] (**Fig.2C**), but not in obese men [ISO: 0.01(0.00–0.02), P=0.116 vs. zero flux] (**Fig.2D**).

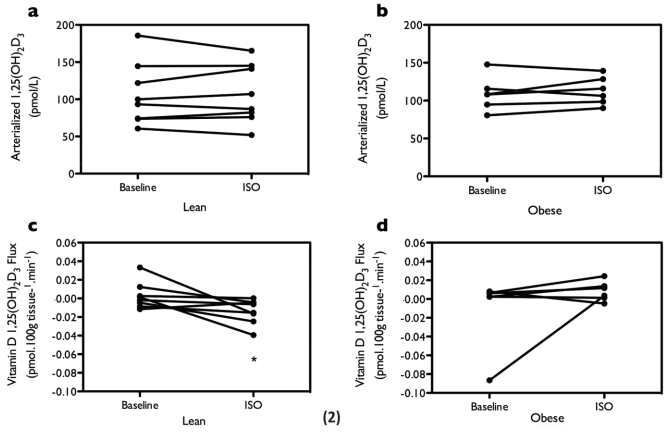

Figure 2. Plasma (arterialized) vitamin D 1,25$(OH)_2D_3$ concentration at baseline and following ISO in lean (Panel a) versus obese (Panel b). Net vitamin D 1,25$(OH)_2D_3$ release (flux) across abdominal SAT in lean (Panel c) and obese (Panel d).

(*) p < .05 versus zero flux.

Relationship between AT lipolysis and vitamin D (arterialized) concentrations

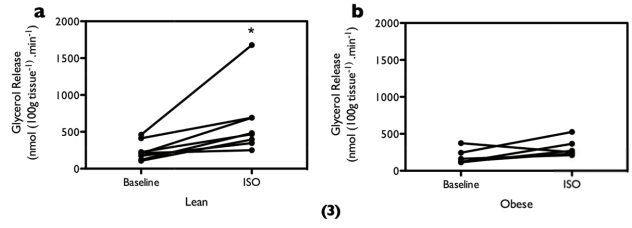

Figure 3. Total glycerol release across abdominal SAT at baseline and during ß-adrenergic stimulation in lean (n = 8) versus obese (n = 6). Total glycerol release following ß-adrenergic stimulation was significantly higher in lean (Panel a) versus obese (Panel b). (*) p < .05

Next, we investigated whether the observed vitamin D release across SAT was associated with changes in local lipolytic responses and systemic vitamin D concentration. As reported previously, a blunted ß-adrenergic mediated increase in total glycerol release across abdominal SAT was observed in obese [glycerol baseline vs. ISO obese: 143.9(114.4–373.5) vs. 260.5(213.2–526.1) nmol.100g $tissue^{-1}.min^{-1}$; (P=0.11, **Fig.3B**)] compared to lean men [glycerol baseline vs. ISO lean: 209.6(170.8–460.2) vs. 474.8(250.8–1678.2) nmol.100g $tissue^{-1}.min^{-1}$); (P=0.01, **Fig.3A**)]. However, in present study, net vitamin D 1,25$(OH)_2D_3$ release (flux) was not correlated with plasma (arterialized) glycerol levels during ß-adrenergic stimulation nor with circulating (arterialized) non-esterified fatty acid (NEFA) and 1,25$(OH)_2D_3$ levels. (P>0.05 for all, Supplemental Table S1).

Discussion

Obesity is often associated with vitamin D deficiency, which has been suggested to relate to insulin resistance and an impaired metabolic health (4). Recent studies in humans have demonstrated that vitamin D (including its metabolites) accumulates and might be metabolised in adipose tissue (17-19). It has been shown that at least 35% of circulating vitamin D is likely sequestered in human subcutaneous adipose tissue (20). It was recently demonstrated that beside vitamin D uptake by adipose tissue there also can be significant release both in mice as well as human adipocytes (21). The latter findings suggest that beside an increased sequestration of vitamin D in AT, also an impaired release (22), may contribute to the accumulation of vitamin D in adipose tissue and to the often observed reduced circulating vitamin D concentrations in obesity.

In the present study, we investigated *in vivo* fluxes of $25(OH)D_3$ and $1,25(OH)_2D_3$ across abdominal SAT for the first time after an overnight fast and during short-term ß-adrenergic stimulation in lean and obese men using the arterio-venous balance methodology. We observed that net vitamin D $1,25(OH)_2D_3$ release across abdominal SAT during ß-adrenergic stimulation was significantly higher in lean as compared to obese men, suggesting a blunted vitamin D $1,25(OH)_2D_3$ release across abdominal SAT in obese men *in vivo*. In contrast, no significant $25(OH)D_3$ release across SAT was observed in lean or obese men following an overnight fast or during ß-adrenergic stimulation. The latter findings may be in contrast with recent *ex vivo* data, which showed a more pronounced reduction in $25(OH)D_3$ content in SAT derived adipocytes in lean compared to obese donors following adrenaline stimulation, possibly pointing towards a blunted $25(OH)D_3$ release in obese SAT (22). Unfortunately, in that study only measured vitamin D $25(OH)D_3$ tissue content and not $25(OH)D_3$ or release in the medium. By using *ex vivo* cultures, the adipocytes are withdrawn from their natural local hormonal microenvironment, which may also partly explain the differences with the present *in vivo* findings.

The reason for the blunted SAT release of $1,25(OH)_2D_3$ and not $25(OH)D_3$ in obese individuals remains to be determined. Factors like variation in ATBF theoretically resulting in a differential supply of vitamin D carriers to SAT or differences in circulating NEFA, interfering with the binding of vitamin D to its carrier could possibly explain the difference in vitamin D release. However, the ISO-induced increase in blood flow as well as circulating NEFA were not different between groups. Moreover, the above mechanisms would not explain why the differential release is only observed for $1,25(OH)_2D_3$ and not for $25(OH)D_3$. Vitamin D $1,25(OH)_2D_3$ is a ligand for vitamin D receptor (VDR), and it has been shown that vitamin D receptor (VDR) expression is increased in SAT of individuals with obesity (23). From the latter, it could be speculated that vitamin D $1,25(OH)_2D_3$ binds to a higher extent to VDR within SAT in individuals with obesity, resulting in less spillover of vitamin D $1,25(OH)_2D_3$ in the circulation in obese individuals but not in lean, which still needs further investigation.

Of interest, the observed blunted release of $1,25(OH)_2D_3$ across abdominal SAT that we found in the present study was accompanied by (but not correlated with) a blunted glycerol release in SAT of obese men. The latter finding as well as the fact that the blunted lipolysis only coincides with a blunted release of $1,25(OH)_2D_3$ and not $25(OH)D_3$ does not support the idea that it is in the obese individuals observed impaired TAG hydrolysis that drives a blunted release of vitamin D metabolites. The exact relationship between the impaired ISO-induced lipolysis and $1,25(OH)_2D_3$ release and to what extent they are co-

regulated remains to be determined.

Furthermore, although net $1,25(OH)_2D_3$ release was observed during acute ß-adrenergic stimulation, no changes in plasma vitamin D $1,25(OH)_2D_3$ (arterialized) concentration were observed. It is likely that following this short-term (1 hour) ISO infusion, the contribution of $1,25(OH)_2D_3$ release per unit adipose tissue may be relatively too small to induce significant changes in circulating vitamin D concentrations. We have estimated this based on several assumptions for the amount of total body water (72% of fat free mass and 10% of fat mass is water) and extra cellular water (38% of total body water) (24) and we took into account the half-life of $1,25(OH)_2D_3$ (25). Based on these assumptions, the estimated percentage contribution of total vitamin D $1,25(OH)_2D_3$ release across adipose tissue to plasma concentrations during isoprenaline stimulation ranged between 0 and 4%. This relatively small contribution might partly explain why no significant increase in plasma vitamin D $1,25(OH)D_3$ after ß-adrenergic stimulation was observed in the present study.

We have measured vitamin D $25(OH)D_3$ and $1,25(OH)_2D_3$ metabolites based on their proposed importance in human metabolism and health (26). However, there are several other vitamin D metabolites such as 24,25-dihydroxyvitamin D [$24,25(OH)_2D_3$] (27) and 3-epi-25(OH)D (28). The role of the latter vitamin D metabolite (29) in human is currently unknown and warrants investigation. A limitation of the study is that we were, unfortunately, unable to measure adipose tissue vitamin D content, which would be interesting to take into account in future studies. Whether long-term interventions that activate SAT lipolysis and vitamin D release (e.g. weight loss (30) and exercise (7)) might affect circulating vitamin D concentrations needs to be investigated in more detail. In conclusion, our unique *in vivo* data show that ß-adrenergic stimulation induces release of active vitamin D metabolite across abdominal SAT. In the present study, a blunted catecholamine-mediated lipolysis was accompanied by a decreased $1,25(OH)_2D_3$ (active metabolite) release across abdominal SAT in obese men. Future studies are warranted to elucidate to what extent this blunted vitamin D release may affect circulating vitamin D concentrations in obese humans.

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Disclosure statement

The authors declare that they have no conflict of interest.

Author Contributions

A.P. and J.W.E.J. designed the study and collected data. A.P. analyzed data. Data interpretation was performed by A.P., J.W.E.J., G.H.G., and E.E.B. The manuscript was written by A.P. and was revised by J.W.E.J., G.H.G., and E.E.B. All authors reviewed and approved the final manuscript.

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Supplemental Table S1. Spearman correlation coefficient between net vitamin D release and arterialized glycerol, arterialized NEFA levels, arterialized vitamin D 1,25$(OH)_2D_3$ during ISO concentrations.

	Arterialized Glycerol	**Arterialized NEFA**	**Arterialized 1,25$(OH)_2D_3$**
1.25$(OH)_2D_3$ flux during ISO-stimulation	0.402 (P=0.154)	0.341 (P=0.233)	0.240 (P=0.409)

Data are presented as correlation coefficient (P value).

CHAPTER 4

Vitamin D and tissue-specific insulin sensitivity in humans with overweight/ obesity

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Abstract

Context: Vitamin D deficiency in obesity has been linked to insulin resistance. However, studies that examined the association between plasma 25-hydroxyvitamin D_3 [25(OH)D_3] as well as plasma 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and tissue-specific insulin sensitivity are scarce. Furthermore, vitamin D receptor (VDR) and vitamin D-metabolizing enzymes (CYPs) expression in adipose tissue (AT) might affect AT insulin sensitivity.

Objective: To investigate the association between BMI and plasma 25(OH)D_3, plasma 1,25$(OH)_2D_3$, and AT VDR; between plasma 25(OH)D_3 ,1,25$(OH)_2D_3$, and AT VDR and tissue-specific insulin sensitivity in overweight/obese individuals.

Design and Patients: This analysis included 92 adult individuals (BMI: >25 kg/m^2). A two-step hyperinsulinemic-euglycemic clamp with a [6,6-$^2H_2$]-glucose tracer was performed to assess tissue-specific insulin sensitivity. Abdominal subcutaneous AT (SAT) mRNA expression of VDR and CYP was determined using qRT-PCR.

Setting: University Medical Centre.

Main Outcome Measures: Plasma 25(OH)D_3, 1,25$(OH)_2D_3$, 1,25$(OH)_2D_3$/25(OH)D_3 ratio, SAT AT VDR and CYPs mRNA, and tissue-specific insulin sensitivity.

Results: BMI was inversely associated with plasma 25(OH)D_3 (ß= -0.274, P=0.011) but not with plasma 1,25$(OH)_2D_3$. Plasma 25(OH)D_3 was not related to CYPs or VDR expression in SAT. Plasma 1,25$(OH)_2D_3$ and 25(OH)D_3 were not related with tissue-specific insulin sensitivity. Interestingly, SAT VDR mRNA was negatively associated with AT insulin sensitivity (ß= -0.207, P=0.025),

Conclusions: BMI was inversely associated with 25(OH)D_3 concentrations, which could not be explained by alterations in SAT VDR and CYP enzymes. Plasma vitamin D metabolites were not related to tissue-specific insulin sensitivity. However, VDR expression in SAT was negatively associated with AT insulin sensitivity.

Introduction

Obesity has reached epidemic proportions in both developed and developing countries (1). Body fat accumulation and in particular an impaired adipose tissue (AT) function, which is commonly observed in obesity, has been linked to the development of metabolic syndrome, insulin resistance, type 2 diabetes and cardiovascular diseases (2, 3). Of interest, obesity is often characterized by low plasma 25-hydroxyvitamin D_3 [25(OH)D_3] levels (<50 nmol/L). This plasma vitamin D deficiency in obese individuals has been suggested to contribute to obesity-related metabolic disorders such as insulin resistance and type 2 diabetes (4, 5). However, evidence from human observational data is not consistent. Recent studies have shown a negative association between plasma or serum vitamin D 25(OH)D_3 levels and whole-body insulin resistance, as estimated from the Homeostatic Model for Insulin Resistance (HOMA-IR), which was independent of body mass index (BMI) in both men and women (6, 7). In contrast, other studies have shown no association between serum 25(OH)D_3 and insulin resistance (HOMA-IR) in obese humans (8, 9). It has been hypothesized that mainly adiposity itself may determine the observed relationship between plasma vitamin D deficiency and insulin resistance, due to sequestration of vitamin D in the enlarged AT mass (10). Furthermore, it has been proposed that the difference in vitamin D concentrations between lean and obese individuals may be explained by volumetric dilution in obesity, with body weight explaining 13% of the variance in vitamin D concentrations (11). Finally, it has been postulated that an altered vitamin D metabolism in AT may contribute to vitamin D deficiency and insulin resistance (12).

Indeed, AT is actively involved in vitamin D metabolism, which is underlined by the expression of vitamin D-metabolizing enzymes (CYP) and the vitamin D receptor (VDR) in AT (13). The vitamin D receptor (VDR) belongs to the nuclear receptor family and mediates effects of vitamin D at the gene transcription level (14). In murine adipocytes, the active vitamin D metabolite ($1,25(OH)_2D_3$) may increase sirtuin 1 (SIRT1) and the NAD^+/NADH, thereby affecting cellular energy metabolism (15). Furthermore, it has previously been reported that VDR expression is comparable in visceral AT (VAT) and subcutaneous AT (SAT) of obese individuals (16), although the expression of vitamin D-metabolizing enzymes (CYP) was decreased in SAT of obese individuals (16). Currently, it is not clear whether VDR expression is altered in the obese insulin resistant state and whether alterations in AT expression of VDR or vitamin D metabolizing enzymes relate to vitamin D deficiency and/or AT insulin resistance. Recent meta-analyses have shown that vitamin D supplementation had no significant effects on whole-body insulin resistance and insulin secretion in overweight/obese individuals (17, 18). Taken together, the contribution of vitamin D deficiency to whole-body and tissue-specific insulin resistance remains to be established, and it is unclear whether a possible association is independent of body mass index (BMI) and/or body fat percentage (19, 20). Moreover, most studies that examined the association between vitamin D and insulin sensitivity did not use the gold-standard hyperinsulinemic euglycemic clamp to determine insulin sensitivity (21). Additionally, most of the studies on the relationship between vitamin D and insulin sensitivity have measured the inactive metabolite of vitamin D, 25(OH)D_3 (7, 9, 22), instead of its active metabolite, $1,25(OH)_2D_3$ or the active/inactive metabolite ratio. Finally, data on AT vitamin D metabolism in the obese insulin resistant state is scarce.

Therefore, in the present study, we investigated vitamin D metabolites (both inactive

and active forms) as well as AT expression of genes involved in vitamin D metabolism in overweight/obese men and women who underwent a 2–step hyperinsulinemic euglycemic clamp with a [6,6-$^2H_2$]-glucose tracer to investigate adipose tissue, liver and skeletal muscle insulin sensitivity. Firstly, we investigated whether obesity (BMI) is related to plasma active, inactive vitamin D concentrations or the ratio between both. Subsequently, we investigated whether putative alterations in plasma vitamin D metabolites in obesity may be mediated by an altered expression of AT VDR or vitamin D-metabolizing enzymes. Secondly, we investigated whether plasma vitamin D and AT expression of VDR relates to tissue-specific insulin sensitivity and, if so, whether these relationships are independent of BMI, age and sex. Finally, we investigated whether an altered AT vitamin metabolism may relate to AT insulin sensitivity.

Subjects and Methods

Subjects

The study participants were men and women with Caucasian ethnicity, recruited at the Maastricht University Medical Center+, Maastricht, The Netherlands. Inclusion and exclusion criteria of these participants are described elsewhere in more detail (23, 24). The subjects gave written informed consent for their participation, and the study was reviewed and approved by the local Medical Ethical Committee of Maastricht University Medical Center+.

Two-step hyperinsulinemic-euglycemic Clamp

A two-step hyperinsulinemic–euglycemic clamp combined with a [6,6-$^2H_2$]-glucose tracer (Cambridge Isotope Laboratories) was performed to analyze the insulin-mediated suppression of Free Fatty Acids (FFAs; representing AT insulin sensitivity) (25), insulin-stimulated rate of disappearance (Rd) of glucose (peripheral/muscle insulin sensitivity), and insulin-mediated suppression of Endogenous Glucose Production (EGP) (hepatic insulin sensitivity) (26). A bolus injection of 2.4 mg [6,6-$^2H_2$]-glucose/kg was administered, continued with [6,6-$^2H_2$]-glucose infusion at 0.04 mg/ (kg x min). After 2 hr, low-dose insulin was infused at 10 mU/m^2/min for 2 hr (27), followed by high-dose insulin at 40 mU/m^2/min for 2 hr (28). By variable co-infusion of a 17.5% glucose solution, enriched by 1.1% tracer, plasma glucose concentrations were maintained at 5.0 mmol/l. For calculation of steady-state kinetics, additional blood samples were taken in the last 30 min of each step (0, 10, and 40 mU/m2/min insulin) (23). The Rd was calculated during the 0 and 40 mU/m^2/min insulin infusion, whereas calculations for insulin-mediated suppression of EGP and free fatty acids was performed during 0 and 10 mU/m^2/min insulin infusion, as relative percentage (%) of suppression during 10 compared with 0 mU/m^2/min (24). Blood samples were taken from a superficial dorsal hand vein, which was arterialized by using a hot-box (~50^{o}C).

Adipose Tissue Biopsy and Analysis

An abdominal subcutaneous AT biopsy was taken under local anesthesia following an overnight fast, as described before (23). The AT sample was washed in sterile saline,

immediately frozen in liquid nitrogen, and maintained at - 80°C until further analysis. For RNA isolation, the tissue was placed in Trizol (Qiagen) and then mixed by means of an ultra turrax homogenizer. Total RNA was extracted using phenol-chloroform extraction method and described in more detail elsewhere (16).

Adipose tissue gene expression

For real-time quantitative PCR, cDNA was generated using an iScript cDNA synthesis kit, according to the instructions of the manufacturer (Bio-Rad Laboratories). PCR-master mix, containing the specific primers, was added and real-time quantification of genes was performed by SYBR-green real-time reverse transcription PCR assay (Bio-Rad iQ SYBR green mix) using a MyIQ Real time PCR cycler (Bio-Rad). The primer pairs that were used are described in **Supplemental Table S1**. 18S was used as a housekeeping gene and data were calculated using delta CT method (16).

Biochemical analysis

Blood was collected into pre-chilled tubes and centrifuged at 1,000 g, and plasma was snap-frozen and stored at ~80°C until analyses. Tracer enrichment of rate of disappearance (Rd) and endogenous glucose production (EGP) were determined using gas chromatography-mass spectrometry (26). Plasma glucose and FFA were determined using commercial available colorimetric assays on a Cobas Fara auto-analyzer (Roche, Switzerland). Plasma insulin was measured with a double-antibody radioimmunoassay (Millipore). Plasma concentrations of vitamin D 25(OH)D_3 and 1,25$(OH)_2D_3$ were determined using liquid-chromatography tandem-mass spectrophotometry (LC-MS/MS), as described before (29).

Statistical Analysis

All continuous variables were checked for normal distribution. Variables with a skewed distribution were square root transformed (plasma vitamin D concentration and SAT VDR mRNA) or natural logarithmic (ln) transformed (SAT vitamin D enzymes, CYP2J2, CYP27A1, CYP27B1, and CYP24A1) to satisfy condition of normality.

First, simple regression analysis was performed with plasma vitamin D metabolites or VDR and vitamin D-metabolizing enzymes as dependent variables, and BMI as independent variable. Subsequently, multiple regression analysis was performed with age and sex added as independent variables.

Secondly, simple regression analysis was performed with hepatic, peripheral, or AT insulin sensitivity as dependent variables, and plasma 25(OH)D_3,1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$/25(OH)D_3 ratio as independent variables (Model 1). Next, BMI, age, and sex were added to the model as independent variables (Model 2). Finally, simple regression analysis was performed to relate SAT VDR gene expression and AT insulin sensitivity. Subsequently, multiple regression analysis was conducted to test whether SAT VDR gene expression might relate to AT insulin sensitivity, independent of age, BMI and sex. Interaction between covariates in the multiple regression analyses was also tested, and no interaction was found. All data were analyzed using SPSS for Mac version 22.0 (SPSS, Chicago, IL, USA)

and statistical significance was set at $p < 0.05$.

Results

Characteristics of study participants

Anthropometric and metabolic characteristics for all participants are presented in **Table 1.** The majority of study participants were men (78.3%), with age ranged between 19 and 69 years, and BMI ranged between 25.4 and 38.6 kg/m^2. The mean plasma 25(OH)D_3 (inactive metabolite) concentration was 56.0 nmol/L (range: 12.6 - 122.6 nmol/L), with 45.7% of all participants (n=42) classified as vitamin D deficient, based on the Endocrine Society cut off (< 50 nmol/L) (30).

Table 1. Characteristics of the Study Participants (n = 92)

Variable	Mean ± SD	Range
Age (years)	50.3 ± 13.6	19 - 69
Sex (Males/Females)	72 M / 20 F	
BMI (kg/m^2)	30.6 ± 2.9	25.4 – 38.6
Waist circumference (cm)	103 ± 12	77 – 126
W/H ratio	0.9 ± 0.1	0.7 – 1.2
Fasting insulin (mU/l)	13.9 ± 6.8	2.8 – 30.3
Fasting glucose (mmol/l)	5.6 ± 0.6	4.4 – 7.5
2h-glucose (mmol/l)	6.5 ± 1.8	3.4 – 11.2
HOMA-IR	3.6 ± 1.9	0.7 – 8.8
Insulin-mediated suppression of EGP (%)	50.6 ± 18.9	5.2 – 97.9
Insulin-mediated suppression of FFA (%)	52.9 ± 20.3	6.1 – 90.6
Insulin-stimulated Rd (μmol kg^{-1} min^{-1})	27.0 ± 10.8	9.8 – 53.9
25(OH)D_3 (nmol/L, inactive metabolite)	56.0 ± 21.6	12.6 – 122.6
1,25(OH)$_2D_3$ (pmol/L, active metabolite)	106.3 ± 26.4	54.9 – 205.0
The active/inactive metabolite ratio	2.2 + 0.9	1.0 – 6.5

Unless otherwise noted, values are the mean 6 SD. All participants were Caucasian. Abbreviations: EGP, endogenous glucose production; FFA, free fatty acid; HOMA-IR, homeostasis model assessment insulin resistance; Rd, rate of glucose disappearance.

Association between BMI and plasma vitamin D concentration

Table 2. Determinants of plasma 25(OH)D_3, 1,25(OH)$_2D_3$ and the 1,25(OH)$_2D_3$/25(OH)D_3 ratio

Dependent Variables	Plasma 25(OH)D_3 (inactive metabolite)				Plasma 1,25(OH)$_2D_3$ (active metabolite)				1,25(OH)$_2D_3$/25(OH)D_3			
	ß	95% CI	P value	Adj.R^2	ß	95% CI	P value	Adj.R^2	ß	95% CI	P value	Adj.R^2
Model 1												
BMI	-0.234	-0.44 (-0.02)	0.031	0.043	-0.095	-0.31 (0.12)	0.386	-0.003	0.140	-0.08 (0.36)	0.201	0.008
Model 2												
BMI	-0.274	-0.48 (-0.06)	0.011	0.104	-0.088	-0.31 (0.13)	0.434	-0.023	0.056	-0.01 (0.12)	0.099	0.067
Age	0.321	0.07 (0.57)	0.012		0.046	-0.22 (0.31)	0.734		-0.101	-0.18 (-0.02)	0.014	
Sex :												
Men	0	0			0	0			0	0		
Women	0.172	-0.43 (0.77)	0.570		0.185	-0.45 (0.83)	0.576		-0.060	-0.25 (0.13)	0.536	

BMI was negatively associated with plasma 25(OH)D_3 (std ß = -0.234, 95%CI -0.44 (-0.02), P=0.031), but did not relate with plasma 1,25(OH)$_2D_3$ and the ratio. Multiple regression analysis showed that the relationship between BMI and plasma 25(OH)D_3

remained significant after adjustment for age and sex (std ß= -0.274, P=0.011) (**Table 2**).

Association between BMI and vitamin D-related gene expression in SAT

BMI was positively associated with SAT VDR gene expression (std ß =0.223, 95%CI 0.00 (0.44), P=0.047, **Table 3**). It is well established that VDR expression is regulated by $1,25(OH)_2D_3$ (31). However, the strength of the association between BMI and AT VDR gene expression did not change (std ß = 0.213, 95%CI -0.11 (0.44), P=0.062) after adjustment for plasma $1,25(OH)_2D_3$ (active metabolite), age and sex. No significant associations were found between BMI and SAT expression of vitamin D metabolizing enzymes (CYP2J2, CYP27A1, CYP27B1, CYP24A1) (**Supplemental Table S2**).

Table 3. Determinants of vitamin D receptor expression in abdominal SAT

	SAT VDR gene expression			
	ß	95% CI	*P* value	Adj. R^2
Model 1				
BMI	0.223	0.00 (0.44)	0.047	0.036
Model 2				
BMI	0.213	-0.11 (0.44)	0.062	0.138
Plasma $1,25(OH)_2D_3$	0.019	-0.19 (0.23)	0.855	
Age	0.251	-0.01 (0.51)	0.057	
Sex :				
Men	0	0		
Women	-0.327	-0.99 (0.34)	0.332	

Plasma vitamin D $25(OH)D_3$ and SAT expression of VDR and vitamin D enzymes

Table 4. Relationship between plasma vitamin D deficiency and SAT expression of VDR and Vitamin D-Metabolizing enzymes

	Plasma $25(OH)D_3$ (inactive metabolite)			
	ß	95% CI	P Value	Adj. R^2
Model 1				
BMI	-0.234	-0.44 (-0.02)	0.031	0.043
Model 2				
BMI	-0.234	-0.48 (0.09)	0.059	0.028
SAT VDR mRNA	0.142	-0.09 (0.38)	0.232	
Model 3				
BMI	-0.252	-0.54 (0.03)	0.083	0.018
CYP2J2 mRNA	0.022	-0.23 (0.28)	0.861	
Model 4				
BMI	-0.254	-0.52 (0.01)	0.061	0.029
CYP27A1 mRNA	0.105	-0.13 (0.34)	0.373	
Model 5				
BMI	-0.189	-0.48 (0.10)	0.203	0.009
CYP27B1 mRNA	0.139	-0.12 (0.39)	0.282	
Model 6				
BMI	-0.248	-0.54 (0.04)	0.092	0.021
CYP24A1 mRNA	0.079	-0.18 (0.33)	0.539	

Next, we investigated the relationship between plasma $25(OH)D_3$ levels and the expression of VDR and enzymes related to vitamin D metabolism in SAT. Abdominal SAT expression of VDR and vitamin D-metabolizing enzymes was not associated with plasma $25(OH)D_3$ level (**Supplemental Table S3**). Additionally, we investigated whether the negative relationship between BMI and plasma $25(OH)D_3$ may be mediated by alteration in AT expression of VDR or vitamin D-metabolizing enzymes. As indicated in **Table 4**,

further adjustment for SAT VDR expression or SAT vitamin D metabolizing enzymes did not significantly alter the relationship between BMI and plasma 25(OH)D_3.

Plasma vitamin D metabolite levels and tissue-specific insulin sensitivity

Neither plasma 25(OH)D_3 (inactive metabolite) nor plasma 1,25$(OH)_2D_3$ (active metabolite) or the active/inactive metabolite ratio were associated with hepatic, peripheral or AT insulin sensitivity, as shown in **Table 5**.

Table 5. Relationship between plasma vitamin D metabolites and hepatic, peripheral, adipose tissue insulin sensitivity

Dependent variables:	Hepatic IS				Peripheral IS				Adipose Tissue IS			
	ß	95% CI	P	Adj.R^2	ß	95% CI	P	Adj.R^2	ß	95% CI	P	Adj.R^2
Model 1												
Plasma 25(OH)D_3	0.073	-0.15 (0.29)	0.512	-0.007	0.138	-0.08 (0.36)	0.219	0.007	-0.027	-0.26 (0.20)	0.816	-0.012
Plasma 1,25$(OH)_2D_3$	0.059	-0.17 (0.29)	0.614	-0.009	0.041	-0.19 (0.27)	0.727	-0.011	0.135	-0.10 (0.37)	0.264	0.003
Ratio plasma Vitamin D	-0.214	-0.92 (0.50)	0.551	-0.008	-0.373	-1.09 (0.34)	0.304	0.001	0.393	-0.35 (1.13)	0.293	0.002
Model 2												
Plasma 25(OH)D_3	0.106	-0.11 (0.32)	0.322	0.195	0.091	-0.11 (0.29)	0.362	0.318	-0.015	-0.21 (0.18)	0.876	0.385
BMI	-0.126	-0.34 (0.08)	0.236		-0.429	-0.62 (-0.23)	<0.001		-0.224	-0.41 (-0.03)	0.022	
Age	-0.101	-0.36 (0.15)	0.433		-0.064	-0.30 (0.17)	0.596		-0.030	-0.26 (0.20)	0.799	
Sex												
Men	0	0			0	0			0	0		
Women	0.847	0.26 (1.43)	0.005		0.639	0.09 (1.18)	0.022		1.319	0.78 (1.85)	<0.001	
Model 3												
Plasma 1,25$(OH)_2D_3$	0.001	-0.21 (0.21)	0.992	0.184	-0.050	-0.25 (0.15)	0.613	0.312	0.051	-0.14 (0.24)	0.593	0.387
BMI	-0.152	-0.36 (0.05)	0.145		-0.457	-0.65 (0.27)	<0.001		-0.214	-0.40 (-0.03)	0.025	
Age	-0.065	-0.31 (0.18)	0.604		-0.029	-0.26 (0.20)	0.805		-0.039	-0.26 (0.18)	0.730	
Sex												
Men	0	0			0	0			0	0		
Women	0.866	0.28 (1.46)	0.005		0.669	0.12 (1.22)	0.018		1.302	0.77 (1.84)	<0.001	
Model 4												
Ratio plasma Vitamin D	-0.390	-1.06 (0.28)	0.249	0.198	-0.397	-1.02 (0.22)	0.207	0.325	0.263	-0.34 (0.87)	0.392	0.391
BMI	-0.136	-0.34 (0.07)	0.190		-0.435	-0.62 (-0.24)	<0.001		-0.231	-0.42 (-0.05)	0.015	
Age	-0.105	-0.36 (0.15)	0.411		-0.074	-0.31 (0.16)	0.535		-0.008	-0.24 (0.22)	0.949	
Sex												
Men	0	0			0	0			0	0		
Women	0.845	0.26 (1.43)	0.005		0.633	0.09 (1.18)	0.023		1.331	0.80 (1.86)	<0.001	

Models 2 to 4 include BMI, age, and sex; model 2 includes plasma 25(OH)D_3; model 3 includes plasma 1,25$(OH)_2D_3$; Model 4 includes ratio vitamin D instead of plasma 25(OH)D_3 and 1,25$(OH)_2D_3$ in addition to independent variables BMI, age, and sex. **Abbreviation:** IS, insulin sensitivity.

Subcutaneous AT VDR, vitamin D-metabolizing enzyme expression and adipose tissue insulin sensitivity

Table 6. Relationship between SAT VDR expression and adipose tissue insulin sensitivity

	Adipose Tissue Insulin Sensitivity			
	ß	95% CI	P Value	Adj. R^2
Model 1				
SAT VDR mRNA	-0.366	-0.56 (-0.17)	<0.001	0.140
Model 2				
SAT VDR mRNA	-0.207	-0.39 (-0.03)	0.025	0.369[b]
BMI	-0.169	-0.34 (0.05)	0.057	
Age	0.036	-0.17 (0.25)	0.729	
Sex:				
Men	0	0		
Women	1.176	0.64 (1.71)	<0.001	

Finally, the association between AT VDR gene expression and AT insulin sensitivity was examined. SAT VDR expression was negatively correlated with AT insulin sensitivity (std

ß = -0.207, 95% CI -0.39 (-0.03), P=0.025), which was independent of age, sex, and BMI (**Table 6**). No associations were found between SAT expression of vitamin D-metabolizing enzymes and AT insulin sensitivity (data not shown).

Discussion

In the present study, we demonstrated for the first time that (1) BMI was negatively associated with of plasma 25(OH)D_3 but not with plasma 1,25$(OH)_2D_3$, nor its ratio; (2) Plasma vitamin D 25(OH)D_3 deficiency was neither related to changes in SAT vitamin D-metabolizing enzymes nor SAT VDR gene expression; (3) plasma 25(OH)D_3, 1,25$(OH)_2D_3$, and the active/inactive metabolite ratio were not significantly associated with hepatic, peripheral or AT insulin sensitivity, (4) SAT VDR gene expression was negatively associated with AT insulin sensitivity.

We found that BMI was one of the major determinants of plasma 25(OH)D_3 concentration (inactive metabolite), whilst BMI was neither associated with plasma 1,25$(OH)_2D_3$ concentration (active metabolite) nor with the active/inactive metabolite ratio. This might be explained by the increased leptin concentrations in obese individuals, which may suppress renal conversion of vitamin D 25(OH)D_3 to 1,25$(OH)_2D_3$ indirectly by stimulation of osteoblast and/or osteocyte FGF23 production (32)**.** In line, a recent large meta-analysis, which included 21 adult cohorts, clearly demonstrated that a higher BMI is accompanied by lower 25(OH)D_3 status (33). In addition, previous data in morbidly obese women has also shown that BMI was inversely related with 25(OH)D_3 level but not with 1,25$(OH)_2D_3$ (29). It has been postulated that these low plasma 25(OH)D_3 levels in obese may be partly explained by increased blood volume due to the larger body size(i.e. volumetric dilution) or sequestration of vitamin D in the expanded body fat depot (10, 11). However, vitamin D may affect skeletal muscle mass (34) as well as bone mass (35). Therefore, it is important to distinguish between the relationship between vitamin D and BMI or adipose tissue mass. Unfortunately, in the present study we did not assess body composition. Therefore, it is unclear whether the observed association between Vitamin D and BMI reflects the relationship with adipose tissue mass per se. The latter needs to be investigated in more detail in future research.

Alterations in AT vitamin D metabolism might be implicated in plasma vitamin D deficiency (36). However, in the present study, we did not find a significant association between SAT VDR gene expression and plasma 25(OH)D_3 concentration. Furthermore, the expression of the vitamin D-metabolizing enzymes (CYP) in SAT was not significantly related to plasma 25(OH)D_3 levels. In contrast, previous finding have shown a positive correlation between CYP27A1 expression in VAT and plasma 25(OH)D_3 (16). In addition, Wamberg et al.(16) showed that obesity is characterized by a decreased expression of the 25-hydroxylase CYP2J2 and the 1a-hydroxylase CYP27B1 in SAT. Whereas the catabolic CYP24A1 does not differ between lean and obese women. However, the expression of catabolic CYP24A1 increased after weight loss (16), suggesting a higher vitamin D turnover in the SAT of obese subjects following weight loss. We cannot exclude that gene expression of vitamin D-metabolizing enzymes may be different between the SAT and VAT (16), which might partly explain the lack of association between plasma 25(OH)D_3 with SAT gene expression in the present study. In the present study, SAT VDR expression was positively associated with BMI which is in line with previous findings in VAT (7), indicating that VDR expression is increased

in both SAT and VAT in obesity. However, the physiological relevance of this upregulation needs to be elucidated in future research. The SAT is by far the body's largest fat depot, and an important mass effect on vitamin D metabolism, is therefore, more likely compared to the VAT.

Recent meta-analyses have shown no effect of oral vitamin D3 supplementation on glucose metabolism and insulin resistance/secretion (17, 37). Here, we found that plasma $25(OH)D_3$, $1,25(OH)_2D_3$ concentration, and the $1,25(OH)_2D_3/25(OH)D_3$ ratio were not significantly associated with hepatic, peripheral, or AT insulin sensitivity in overweight/obese men and women. Taken together, our data suggest that plasma vitamin D deficiency is not related to insulin resistance in overweight/obese men and women, assessed using the gold-standard hyperinsulinemic-euglycemic clamp. Finally, we observed that higher SAT VDR gene expression was associated with lower AT insulin sensitivity independent of BMI, age, and sex, and explained 14% of the variance in AT insulin sensitivity. Recent study has suggested that the association between insulin resistance related diseases and VDR polymorphisms was more pronounced in dark-pigmented Caucasians and Asians but not in white-pigmented Caucasian (38). However, it needs to be investigated whether these genetic and our observed transcriptional differences also translate into functional changes at the protein level. Furthermore, it has been shown that VDR mRNA expression is positively associated with local inflammation in human SAT and VAT (39). However, further studies are needed to investigate whether the observed association between SAT VDR expression and AT insulin sensitivity is at least partly explained by AT inflammation.

In conclusion, obesity is associated with lower plasma $25(OH)D_3$ (inactive metabolite), but not with plasma $1,25(OH)_2D_3$ (active metabolite) nor $1,25(OH)_2D_3/25(OH)D_3$ ratio. This plasma vitamin D deficiency was not related changes in AT expression of vitamin D metabolizing enzymes (CYP) nor VDR. Neither plasma $25(OH)D_3$, $1,25(OH)_2D_3$ concentration or $1,25(OH)_2D_3/25(OH)D_3$ ratio were associated with hepatic, adipose tissue, and peripheral insulin sensitivity in overweight/obese men and women. Interestingly, SAT VDR gene expression was negatively associated with AT insulin sensitivity, and future studies are needed to unravel the molecular mechanisms by which nuclear and membrane-bound VDR interact with insulin action in abdominal SAT.

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Disclosure Summary:

The authors have nothing to disclose.

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Supplemental Tables

Supplemental Table S1. Primer sets used for real time PCR

Genes	Primers (forward)	Primers (reverse)
VDR	5'- GGCCCAACTCCAGACACACT-3'	5'-GGGTCACAGAAGGGTCATCTGA-3'
CYP2J2	5'-TCCATCCTCGGACTCTCCTAC-3'	5'-GCGCCGTCTTTTGAGAAAGT-3'
CYP27A1	5'-CGGCAACGGAGCTTAGAGG-3'	5'-GGCATAGCCTTGAACGAACAG-3'
CYP27B1	5'-GAGACTGGGACCAGATGTTTG-3'	5'-CAGGCAACTCTTCCCGGAAC-3'
CYP24A1	5'- GGTGGCGAGACTCAGAACG-3'	5'- GTCGTGCTGTTTCTTGAGACC-3'
18S	5'-AGTTAGCATGCCAGAGTCTGG-3'	5'-TGCATGGCCGTTCTTAGTTG-3'

Supplemental Table S2. Determinants of subcutaneous AT vitamin D-metabolizing enzymes mRNA

	CYP2J2 expression				CYP27A1 expression				CYP27B1 expression				CYP24A1 expression			
	ß	95% CI	P Value	Adj. R^2	ß	95% CI	P Value	Adj. R^2	ß	95% CI	P Value	Adj. R^2	ß	95% CI	P Value	Adj. R^2
BMI	0.058	-0.22 (0.33)	0.674	0.002	0.01	-0.28 (0.29)	0.968	0.05	0.02	-0.25 (0.29)	0.885	0.002	0.01	0.27 (0.29	0.943	0.011
Age	0.152	-0.14 (0.44)	0.302		0.37	0.07 (0.68)	0.018		0.252	-0.04 (0.54)	0.086		0.28	0.15 (0.58	0.063	
Sex :																
Men	0	0			0	0			0	0			0	0		
Women	0.283	-0.63 (1.12)	0.538		0.51	-0.45 (1.48)	0.29		0.465	-0.44 (1.37)	0.308		0.4	0.53 (1.33	0.395	

Supplemental Table S3. Relationship between SAT vitamin D metabolizing enzymes and plasma inactive vitamin D concentration

	Plasma 25(OH)D_3 (inactive metabolite)		
	ß	95% CI	P
CYP2J2 mRNA	-0.011	-0.27 (0.24)	0.930
CYP27A1 mRNA	0.080	-0.16 (0.32)	0.506
CYP27B1 mRNA	0.122	-0.13 (0.38)	0.345
CYP24A1 mRNA	0.077	-0.18 (0.34)	0.555

CHAPTER 5

The association between vitamin D receptor polymorphisms and tissue-specific insulin resistance in human obesity

Adriyan Pramono, Johan W.E. Jocken, Michiel E. Adriaens, Mads F. Hjorth, A. Astrup, Wim H. Saris and Ellen E. Blaak

Submitted

Abstract

Aims/hypothesis: This study aimed to: (1) investigate the association of four single-nucleotide polymorphisms (SNPs) in the VDR gene (TaqI/rs731236, ApaI/rs7975232, FokI/rs10735810, and BsmI/rs1544410) with adiposity, insulin sensitivity indexes at baseline; (2) identify the effect of the VDR SNPs in the SAT transcriptome in overweight/obese Caucasians of the DiOGenes cohort; and (3) determine the association of the VDR SNPs with change of body weight and insulin sensitivity indexes after weight loss and weight maintenance (regain) intervention.

Methods: We included 553 adult obese individuals (mean BMI 34.8 kg/m^2), men (n=197) and women (n=356), which were studied at baseline, following an 8-week weight loss intervention and 26-weeks weight maintenance (regain). Genotyping was performed using an Illumina 660W-Quad SNP chip on the Illumina iScan Genotyping System. Tissue-specific insulin sensitivity was determined using Hepatic Insulin Resistance Index (HIRI), Muscle Insulin Sensitivity Index (MISI) and Adipose Tissue Insulin Resistance Index (Adipo-IR). Expression quantitative trait loci (eQTL) analysis was performed using SAT gene sequencing data to determine the effect of SNPs on SAT gene expression.

Results: The genetic variants, AG and GG in the *VDR* TaqI or CA and AA in the VDR ApaI were associated with higher BMI, waist and fat mass (P<0.05 for all), even after adjustment for age, sex, and center. None of the VDR polymorphisms were associated with HIRI or MISI. Interestingly, carriers of the G-allele of VDR FokI showed higher Adipo-IR (GG+GA 7.8 ± 0.4 vs. AA 5.6 ± 0.5, P=0.010) and higher systemic Free Fatty Acids (FFA) (GG+GA: 637.8 ± 13.4 vs. AA: 547.9 ± 24.7 µmol/L, P=0.011) as compared to non-carriers, even after adjustment with age, sex, center and fat mass. However, eQTL analysis showed minor to no effect of these genotypes on the transcriptional level in SAT. Also, VDR polymorphisms were not related to changes in body weight and insulin resistance as result of dietary intervention (P> 0.05 for all parameters).

Conclusions/interpretation: the VDR TaqI and ApaI variants are associated with markers of adiposity at baseline. The VDR FokI variant is associated with elevated circulating FFA and Adipo-IR in obese Caucasians at baseline. Nevertheless, the VDR SNPs had minor to no effect on the transcriptional level, at least in SAT, indicating that the putative mechanisms of action remain to be determined. The VDR polymorphisms did not relate to body weight and insulin resistance as result of dietary intervention.
DiOGenes was registered at clinicaltrials.gov as NCT00390637.

Introduction

The obesity prevalence has increased dramatically reaching epidemic proportions worldwide (1). Overweight and obesity have been shown as major risk factors for the development of insulin resistance, type 2 diabetes mellitus (T2D), and cardiovascular diseases (CVDs) (2). Next to economic, social, and physical environment, genetic factors play an important role in obesity development and its comorbidities (3). Previous studies have shown that human obesity is often characterized by vitamin D deficiency (circulating vitamin 25-hydroxyvitamin D_3/25OHD < 50 nmol/L) (4) and increased vitamin D receptor (VDR) expression within subcutaneous adipose tissue (SAT) (5). Interestingly, it has been suggested that 1,25-dihydroxyvitamin D3 (hydroxylated form of 25OHD in the kidney), the active form of vitamin D, has independent effects on features of the metabolic syndrome (6).

The actions of 1,25-dihydroxyvitamin D3 are mediated through VDR, a ligand-activated nuclear receptor that drives gene transcription involved in bone metabolism and also extra-skeletal cell cycle regulation (7). Interestingly, VDR is expressed not only in the kidney, but also in major insulin-sensitive tissues such as adipose (8), muscle and liver (9). Additionally, adipose tissue overexpression of human VDR in mice leads to an increased fat mass, a decreased glucose tolerance and energy expenditure (10). Of interest, our recent data indicated that VDR mRNA expression in subcutaneous adipose tissue (SAT) was positively related with body mass index (BMI) (5) and adipose tissue insulin resistance (Adipo-IR) derived from a hyperinsulinemic euglycemic clamp (5), indicating a possible role for VDR in regulating adipose tissue function.

Genetic variations of VDR (ApaI, TaqI, BsmI, and FokI) have previously been related with measures of adiposity. Some studies have shown that the ApaI, TaqI, BsmI, and FokI VDR variants are associated with markers of adiposity (11-13). In contrast, others reported a lack of association between VDR variants and adiposity (14, 15). Thus, evidence for the relationship between VDR genetic variants and obesity remains inconclusive. Of note, the majority of these studies determined adiposity based only on body mass index (BMI) (11-14), and therefore did not take a more precise determination of body composition into account. Furthermore, it has been shown that VDR genetic variants may be associated with whole body insulin resistance (16-18) and the development of T2D(19). Interestingly, it has been shown that the effects of vitamin D supplementation on insulin sensitivity (i.e. HOMA-IR) are affected by VDR genetic variation (20, 21). The latter may suggest that metabolic effects of vitamin D and intervention outcome, particularly insulin sensitivity, may also be influenced by genetic variation in the VDR.

Studies investigate the relationship between VDR polymorphisms, adiposity and tissue-specific insulin sensitivity (including muscle, liver and adipose tissue) are currently lacking. Also, whether these VDR variants may also affect human SAT at the transcriptional level is unknown. Here, we investigated the possible association of the VDR TaqI, ApaI, BsmI and FokI polymorphisms with markers of adiposity including body composition and tissue-specific insulin resistance in the adult Caucasian obese/overweight population of the DiOGenes study. Additionally, we investigated in the DIOGenes study whether these polymorphisms affected body weight loss as well as change in (tissue-specific) insulin sensitivity after an 8-week weight loss intervention followed by 26-weeks weight maintenance. To gain mechanistic insight, we also determined whether these VDR variants

affect abdominal subcutaneous adipose tissue (SAT) at the transcriptional level.

Methods

Study Design

The DiOGenes study is a Pan-European multicenter, randomized, controlled dietary intervention study, designed to assess the efficacy of moderate fat diets that vary in protein content and glycemic index for preventing weight regain and obesity related risk factors after weight loss (for details see Larsen et al (22)). The study involved 8 European countries (8 centers: Denmark, Netherlands, United Kingdom, Germany, Spain, Bulgaria, Czech Republic, and Greece). In total, 938 overweight or obese, nondiabetic adults free of cardiovascular disease [age 18-65 years, body mass index (BMI) 27-45 kg/m^2] ~~w~~ere recruited. More details on recruitment, inclusion and exclusion criteria, and study design are described elsewhere (22).

The analyses described here mainly focusses baseline data of 553 participants (men=197 and women=356), prior to any intervention, for whom VDR polymorphisms (TaqI/rs731236, ApaI/rs7975232, FokI/rs10735810, and BsmI/rs1544410) and detailed information of body composition such as body mass index (BMI), waist circumference (WC), and fat mass (FM), as well as glucose and insulin concentrations during an oral glucose tolerance test (OGTT) were available. Subsequently, we analyzed how the indicated VDR polymorphisms related to changes in body weight and (tissue-specific) insulin sensitivity during a weight loss and subsequent weight maintenance period.

For this, after the first clinical investigation day (pre–low-calorie diet) with baseline measurements (CID1), eligible adults followed an 8-week low calorie diet (Modifast, Nutrition et Sante´, France) consisting of 800 kcal/d. Adults who achieved a weight loss of ≥8% after 8-weeks underwent the second clinical investigation day (post–low-calorie diet), was randomized to ad libitum diets for 26 weeks of weight maintenance (22). After weight loss and weight maintenance the clinical investigation day was repeated (CID2 and CID3, respectively) The Medical Ethical Committees of the respective countries approved the study protocol. All participants gave written informed consent and the study was conducted in accordance with the principle of the Declaration of the Helsinki II.

Body composition and blood sampling

Body composition was determined and blood samples were collected after an overnight fast. We included the following baseline anthropometric parameters: body weight, body mass index (BMI), waist circumference (WC), and fat mass (FM). BMI was calculated by dividing the mass in kg by squared height (in meter). Body composition was determined by Bioimpedance analysis. Additionally, glucose, free fatty acids (FFA) (automatic spectrophotometric enzymatic techniques) and insulin (radioimmunoassay) were measured from fasting blood samples (22).

Estimates of insulin resistance indexes

Participants underwent a standard 5-point oral glucose tolerance test (OGTT) at baseline. Briefly, after an overnight fast, venous blood was collected before (t0) and after a 75 g glucose load was ingested. Blood samples were taken at 0, 30, 60, 90 and 120 minutes to determine glucose and insulin concentrations (22). Muscle insulin sensitivity index (MISI) and hepatic insulin resistance index (HIRI) were estimated using the methods of Abdul-Ghani *et al* (23).

The muscle insulin sensitivity index (MISI) was calculated according to the following formula: MISI= (dG/dt) / mean plasma insulin concentrations during the OGTT. Here, dG/dt is the rate of decay of plasma glucose concentrations during the OGTT, calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir (23). The decline in plasma glucose concentration after 60 minute primarily reflects glucose uptake by peripheral tissue mainly skeletal muscle.

The hepatic insulin resistance index (HIRI) was calculated using the square root of the product of the area under curves (AUCs) for glucose and insulin during the first 30 minute of the OGTT. The formula of HIRI's calculation was SQRT ($glucose_{0\text{-}30}$ [AUC in mg/d x h] x $insulin_{0\text{-}30}$ [AUC in µU/mL x h]). This index has been developed and validated against the product of fasting plasma insulin and endogenous glucose production in hyperinsulinemic euglycemic clamp studies in a Mexican-American population (non-Caucasian) (23).

The Adipose tissue insulin resistance index (Adipo-IR) was calculated for 485 participants (FFA data available) using the method of Søndergaard *et al* (24). The Adipo-IR was calculated using fasting insulin and fasting free fatty acid (FFA) concentrations (fasting insulin [µIU/mL] x fasting FFA [µmol/L]/1000). This formula has been strongly associated with suppression of lipolysis derived from palmitate flux (IC_{50}) as measured by the multistep pancreatic clamp technique (24).

SNP selection and genotyping

Buffy coats were collected for DNA extraction and genetic single nucleotide polymorphisms (SNPs) analysis. The four VDR SNPs: TaqI(rs731236), ApaI(rs7975232), BsmI(rs1544410), and FokI(rs10735810) were evaluated by allelic discrimination real-time PCR using an Illumina 660W-Quad SNP chip on the Illumina iScan Genotyping System (Illumina, San Diego, CA, USA).

Abdominal SAT RNAseq analysis

Total RNA was extracted from abdominal SAT biopsies as described before (25). Gene expression was examined by using 100-nucelutide long paired-end RNA sequencing with an Illumina HiSeq 2000 of libraries prepared by using the Illumina TruSeq kit following the manufacturer's standard protocols. Sequencing was performed using baseline SAT prior to any weight-loss intervention. For each sample, the number of reads mapping onto genes was retrieved by using Genomic Alignments as previously described (26).

Statistical analysis

All continuous variables were checked for normal distribution. Variables with a skewed distribution were natural logarithmically transformed (BMI, WC, FM, MISI, HIRI, and Adipo-IR) to satisfy the condition of normality. The data were back-transformed and presented as mean ± SE.

Allele frequency and Hardy-Weinberg Equilibrium (HWE) were calculated for all VDR SNPs. The non-random association of alleles at different loci of VDR polymorphisms (pairwise linkage disequilibrium (LD)) analysis was analyzed using SNPStat (27) (available online at https://www.snpstats.net/start.htm). Coefficient D' was used to describe pairwise LD, where a D' value close to 1 indicated high LD and D' value close to 0 suggested weak LD.

Analysis of covariance (ANCOVA) was performed to examine the differences in body composition (BMI, waist, and fat mass) between the genotypes adjusted for age, sex, and center. The changes in body weight between genotypes following weight loss intervention were adjusted for initial body weight, while the differences in body weight between genotypes following weight maintenance were corrected for weight loss during LCD and mean body weight pre and post LCD. Furthermore, ANCOVA was also conducted to analyze the differences in tissue-specific insulin resistance index (MISI, HIRI, and Adipo-IR) between the genotypes adjusted for age, sex, center and FM at baseline. The changes in MISI, HIRI, and Adipo-IR between the genotypes following weight loss intervention were adjusted for MISI, HIRI, and Adipo-IR at baseline, respectively. The changes in MISI, HIRI, and Adipo-IR between the genotypes following weight maintenance were adjusted for the changes MISI, HIRI, and Adipo-IR during LCD and mean of MISI, HIRI, Adipo-IR pre and post LCD, respectively. In addition, dominant, recessive, over and co-dominant models were selected. Briefly, a dominant model compares homozygote dominant versus heterozygote–homozygote recessive, whereas the recessive model compares heterozygote–homozygote dominant versus homozygote recessive. An over-dominant model compares homozygote dominant–recessive versus heterozygote where this model assumes the heterozygote has the strongest impact on the outcome. On the other hand, co-dominant models hypothesize that each genotype may be associated with the outcomes (28).

Expression quantitative trait loci (eQTL) analysis (29) was conducted to analyze the association between the VDR gene variants and abdominal SAT tissue gene expression (RNA-seq). We considered three sets of genes for the eQTL analysis: (i) the VDR gene (*cis*), (ii) all VDR target genes (*trans*) and (iii) all genes in *cis*, defined as any gene within 1 Mb upstream or downstream of the genomic location of the respective SNP. To test for eQTL associations, we first recoded the genotype of each SNP to the alternative allele dosage (i.e. 0, 1 or 2 copies). To construct the set of VDR target genes, we manually performed a search using PubMed (search criteria combined with Boolean operators AND/OR: Vitamin D, Vitamin D receptor (VDR), target genes, obesity). From the list of articles retrieved (10, 30-44), abstracts were scanned, and only those that reported vitamin D, gene name and obesity and/or insulin resistance were further analyzed resulting in a list of VDR target genes. Next, for each gene in a set, a linear additive model was created with gene expression as the dependent variable and the alternative allele dosage as the independent variable, while correcting for age, sex and center. Correction for multiple testing was performed by means of a Bonferroni correction and for eQTL analysis we performed the false discovery rate (FDR) correction. All analyses were performed in the statistical programming language R (version

3.3.1) (45).

Results

The main demographic, anthropometric and clinical characteristics of participants are presented in **Table 1**. In our population genotypes of TaqI rs731236, ApaI rs7975232, BsmI rs1544410, and FokI rs10735810 were predominantly heterozygous and all SNPs were compatible with HWE as shown in **Supplemental table S1.**

Table 1. Participants' demographic, anthropometric and clinical characteristics.

	Baseline (CID1) (N=553)		**After weight loss (CID2) (N=491)**		**After weight maintenance (regain) (CID3) (N=356)**	
	Mean ± SE	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**
Age (years)	41.5 ± 0.3	24 – 63	41.7 ± 0.3	24 – 63	42.5 ± 0.3	24 – 63
Sex (M/F)	197/356		178/313		120/236	
Weight (kg)	100.6 ± 0.8	66.6 – 168.6	89.4 ± 0.9	59 – 150	89.7 ± 0.7	57 – 154
BMI (kg/m^2)	34.8 ± 0.2	26.7 – 52.0	30.9 ± 0.2	22.2 – 45.7	30.9 ± 0.2	21.8 – 43.9
WC(cm)	109 ± 0.6	64.0 – 155.0	98.8 ± 0.7	70.0 – 133.0	99.3 ± 0.6	71.0 – 138.0
FM (kg)	40.8 ± 0.5	15.6 – 82.6	32.2 ± 0.6	9.9 – 58.8	33.2 ± 0.5	11.9 – 68.7
Fasting glucose (mmol/L)	5.2 ± 0.1	3.4 – 8.7	4.9 ± 0.0	4.0 – 8.0	5.0 ± 0.0	4.0 – 7.0
Fasting insulin µIU/mL	11.9 ± 0.4	2.0 – 134.0	8.3 ± 0.3	2.0 – 87.0	9.3 ± 0.5	2.0 – 100.0
Fasting FFA (µmol/L)	627 ± 12†	162 – 2226	712 ± 11††	153 – 1727	555 ± 12†††	109 – 1419
HIRI	33.4 ± 0.5^{f}	11.9 – 94.3	26.4 ± 0.7^{ff}	7.4 – 73.3	32.0 ± 0.9^{fff}	6.5 – 89.6
MISI	0.06 ± 0.00¶	0.01 – 0.53	0.06 ± 0.00¶¶	0.01 – 0.30	0.07 ± 0.00¶¶¶	0.01 – 0.36
Adipo-IR	7.0 ± 0.2†	1.0 – 39.3	5.8 ± 0.3††	0.7 – 43.9	5.1 ± 0.3†††	0.4 – 58.5

Abbreviations: Adipo-IR, adipose tissue insulin resistance index; BMI, body mass index; F, female; FM, fat mass; FFA, free fatty acid; HIRI, hepatic insulin resistance index; HOMA-IR, homeostatic model assessment for insulin resistance; M, male; MISI, muscle insulin sensitivity index. †N=485; ††N=420; †††N=281; ^{f}N=539; ^{ff}N=383; ^{fff}N=303; ¶N=519; ¶¶N=383; ¶¶¶N=300

VDR variants and anthropometry measurements at baseline

Variants in VDR TaqI in the dominant model (AA *versus* AG+GG) were related to BMI, WC, and FM (P=0.007; P=0.08; P=0.006, respectively) (Table 2). Furthermore, variants in VDR TaqI were associated with BMI, WC, and FM (P=0.009, P=0.013, P=0.006, respectively) in the over-dominant model (AA+GG *versus* AG). Overall, G allele carriers (AG+GG) of the VDR TaqI had higher BMI, WC, and FM as compared to non-carriers (AA) (**Table 2**).

In the dominant model VDR ApaI (CC *versus* CA+AA) was associated with higher BMI and FM (P=0.023; P=0.028, respectively) (Table 2). Further analysis in the over-dominant model showed that CC+AA variants in VDR ApaI were related to BMI and FM (P=0.012; P=0.008, respectively) as compared to the CA variant. Overall, A allele carriers (CA+AA) of VDR ApaI had higher BMI and FM compared to non-carriers (CC). However, neither VDR

BsmI nor FokI were related to BMI, WC, and FM in this cohort (P>0.05 for all parameters).

Table 2. The relationship between VDR polymorphisms and body composition.

TaqI rs731236	Genotype	N	Mean	SE	*P*-value	Mean	SE	*P*-value	Mean	SE	*P*-value
	AA	209	34.1	0.3		107	0.9		39.1	0.7	
Co-dominant	AG	251	35.4	0.3	0.017	110	0.9	0.045	42.1	0.8	0.012
	GG	93	34.9	0.5		107	1.4		41.1	1.2	
Dominant	AA	209	34.1	0.3	0.007	107	0.9	0.08	39.1	0.7	0.006
	AG+GG	344	35.3	0.3		109	0.7		41.8	0.7	
Recessive	AA+AG	460	34.8	0.2	0.99	109	0.6	0.17	40.8	0.5	0.74
	GG	93	34.9	0.5		107	1.4		41.1	1.2	
Over-dominant	AA+GG	302	34.3	0.3	0.009	107	0.7	0.013	39.7	0.6	0.006
	AG	251	35.4	0.3		110	0.9		42.1	0.8	
ApaI rs7975232	**Genotype**	**N**	**Mean**	**SE**	***p*-value**	**Mean**	**SE**	***p*-value**	**Mean**	**SE**	***p*-value**
	CC	206	34.1	0.3		107	0.9		39.4	0.7	
Co-dominant	CA	250	35.4	0.3	0.033	110	0.9	0.11	42.1	0.8	0.026
	AA	97	34.8	0.5		106	1.3		40.5	1.2	
Dominant	CC	206	34.1	0.3	0.023	107	0.9	0.36	39.4	0.7	0.028
	CA+AA	347	35.3	0.3		109	0.8		41.7	0.6	
Recessive	CC+CA	456	34.9	0.2	0.56	109	0.6	0.15	40.9	0.5	0.38
	AA	97	34.8	0.5		106	1.3		40.5	1.2	
Over-dominant	CC+AA	303	34.3	0.3	0.012	107	0.7	0.056	39.8	0.6	0.008
	CA	250	35.4	0.3		110	0.9		42.1	0.8	
BsmI rs1544410	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**Mean**	**SE**	***P*-value**	**Mean**	**SE**	***P*-value**
	TT	166	34.9	0.4		108	1.0		41.1	0.9	
Co-dominant	TC	263	35.1	0.3	0.18	110	0.9	0.40	41.2	0.7	0.44
	CC	124	34.1	0.4		108	1.1		39.6	1.0	
Dominant	TT	166	34.9	0.4	0.78	108	1.0	0.43	41.1	0.9	0.87
	TC+CC	387	34.8	0.3		109	0.7		40.7	0.6	
Recessive	TT+TC	429	35.1	0.2	0.07	109	0.7	0.45	41.2	0.6	0.21
	CC	124	34.1	0.4		108	1.1		39.6	1.0	
Over-dominant	TT+CC	290	34.6	0.3	0.21	108	0.8	0.18	40.5	0.7	0.37
	TC	263	35.1	0.3		110	0.9		41.2	0.7	
FokI rs10735810	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**Mean**	**SE**	***P*-value**	**Mean**	**SE**	***P*-value**
	GG	227	34.9	0.3		109	0.9		40.5	0.8	
Co-dominant	GA	259	34.9	0.3	0.94	109	0.8	0.85	41.6	0.7	0.14
	AA	67	34.8	0.6		108	1.5		39.2	1.4	
Dominant	GG	227	34.9	0.3	0.96	109	0.9	0.76	40.5	0.8	0.57
	GA+AA	326	34.8	0.3		109	0.7		41.1	0.6	
Recessive	GG-GA	486	34.9	0.2	0.76	109	0.6	0.73	41.1	0.5	0.10
	AA	67	34.8	0.6		108	1.5		39.2	1.4	
Over-dominant	GG+AA	294	34.8	0.3	0.80	109	0.8	0.59	40.2	0.7	0.11
	GA	259	34.9	0.3		109	0.8		41.6	0.7	

Abbreviations: BMI, body mass index; FM, fat mass; WC, waist circumference. P-value was corrected for age, sex, and center.

Linkage disequilibrium of VDR variants and haplotype analysis

Pairwise Linkage Disequilibrium (LD) between SNPs was assessed, and LD analysis revealed strong LD between VDR TaqI, ApaI, and BsmI (D'=0.9754, D'=0.9944, D'=0.9943,

respectively see Figure 1). In contrast, weak LD (D' values close to 0, see **Figure 1**) was observed between these variants (TaqI, ApaI, BsmI) and FokI. However, subsequent haplotype analysis revealed no significant associations between different haplotypes and BMI, WC and FM, even after adjustment for age, sex, and center (except uncommon haplotypes (frequency <1%) (**Supplemental Table S2**). Further haplotype analysis showed no association with HIRI, MISI or Adipo-IR (data not shown).

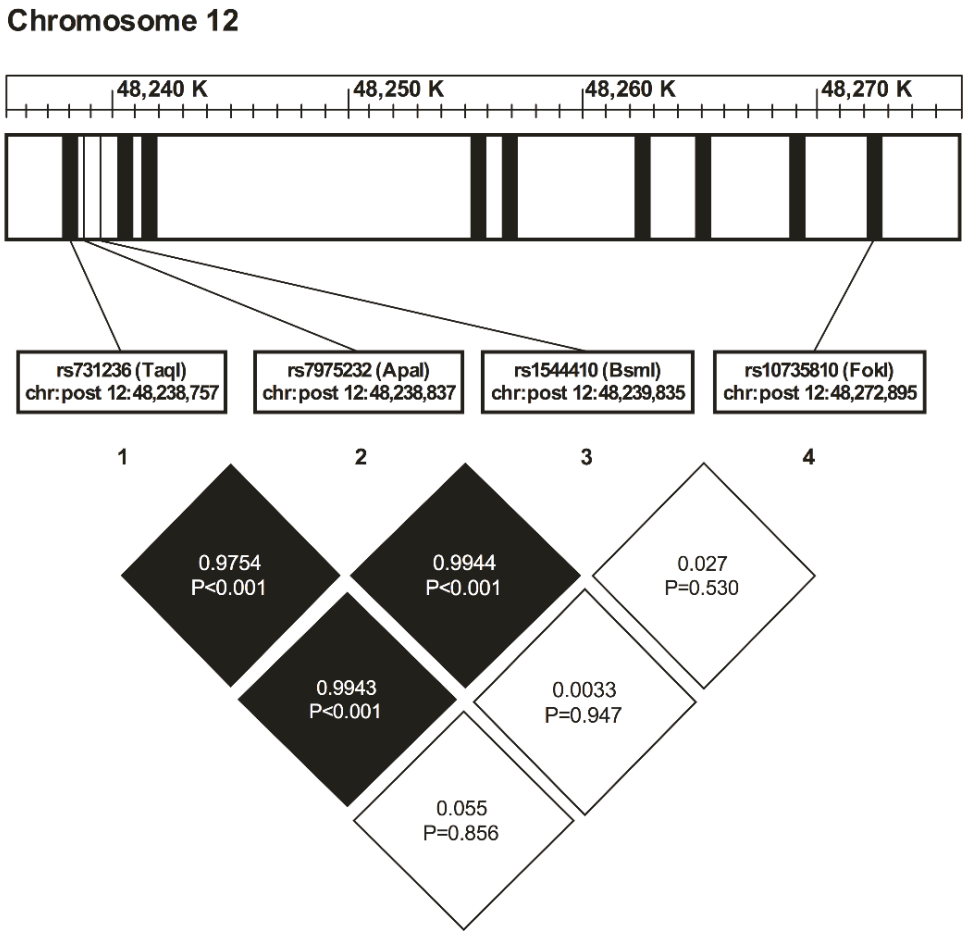

Figure 1. The VDR gene on chromosome 12. The approximate locations of the observed polymorphisms are indicated by arrows. The blocks represented pairwise linkage disequilibrium (LD) pattern. Each squares described the D' values and the p values between the pairs of VDR polymorphisms. The black blocks are proportional to D' values which indicates high LD between polymorphisms. The white blocks indicate low LD between polymorphisms.

VDR variants and tissue-specific insulin resistance index

The analysis of tissue-specific insulin resistance according to VDR genotypes as described in **Table 3**, shows no significant associations between VDR variants, hepatic insulin

resistance (HIRI) and muscle insulin sensitivity (MISI) (p>0.05 for all). Of interest, G allele carriers of VDR FokI showed higher Adipo-IR (GG+GA 7.8 ± 0.4 vs. AA 5.6 ± 0.5, P=0.010) even after adjustment for age, sex, center and FM.
Moreover, circulating FFA but not fasting insulin, were significantly higher in G allele carriers of VDR FokI as compared to non-carriers (GG+GA: 637.8 ± 13.4 (µmol/L) vs. AA: 547.9 ± 24.7, P=0.011) (**Supplemental Table S3**).

Table 3. The association between VDR polymorphisms and tissue-specific insulin resistance index.

		HIRI†				MISI‡				Adipo-IR#			
TaqI rs731236	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	AA	205	32.6	0.7		200	0.05	0.0		183	6.7	0.3	
Co-dominant	GA	243	33.8	0.7	0.89	233	0.06	0.0	0.54	225	8.1	0.5	0.81
	GG	91	34.1	1.5		86	0.05	0.0		77	7.7	1.2	
Dominant	AA	205	32.6	0.7	0.64	200	0.05	0.0	0.31	183	6.7	0.3	0.63
	GA+GG	334	33.9	0.7		319	0.06	0.0		302	8.0	0.5	
Recessive	AA+GA	448	33.3	0.5	0.90	433	0.06	0.0	0.44	408	7.5	0.3	0.80
	GG	91	34.1	1.5		86	0.05	0.0		77	7.7	1.2	
Over-dominant	AA+GG	296	33.1	0.7	0.72	286	0.05	0.0	0.68	260	6.9	0.4	0.52
	GA	243	33.8	0.7		233	0.06	0.0		225	8.1	0.5	
ApaI rs7975232	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	CC	201	32.6	0.7		200	0.05	0.0		181	6.7	0.4	
Co-dominant	CA	243	33.7	0.7	0.71	233	0.06	0.0	0.73	223	8.0	0.5	0.83
	AA	95	34.2	1.4		86	0.05	0.0		81	7.8	1.1	
Dominant	CC	201	32.6	0.7	0.41	200	0.05	0.0	0.42	181	6.7	0.4	0.56
	CA+AA	338	33.9	0.7		319	0.06	0.0		304	7.9	0.5	
Recessive	CC+CA	444	33.2	0.5	0.65	433	0.06	0.0	0.76	404	7.4	0.3	0.71
	AA	95	34.2	1.4		86	0.05	0.0		81	7.8	1.1	
Over-dominant	CC+AA	296	33.1	0.7	0.43	286	0.05	0.0	0.58	262	7.1	0.4	0.77
	CA	243	33.7	0.7		233	0.06	0.0		223	8.0	0.5	
BsmI rs1544410	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	TT	163	34.3	1.1		154	0.05	0.0		141	8.5	0.9	
Co-dominant	TC	255	33.2	0.7	0.59	246	0.06	0.0	0.52	236	7.2	0.3	0.59
	CC	121	32.6	0.9		119	0.06	0.0		108	6.8	0.5	
Dominant	TT	163	34.3	1.1	0.37	154	0.05	0.0	0.41	141	8.5	0.9	0.32
	TC+CC	376	33.0	0.6		365	0.06	0.0		344	7.1	0.3	
Recessive	TT+TC	418	33.6	0.6	0.43	400	0.06	0.0	0.30	377	7.7	0.4	0.56
	CC	121	32.6	0.9		119	0.06	0.0		108	6.9	0.5	
Over-dominant	TT+CC	284	33.6	0.7	0.87	273	0.06	0.0	0.90	249	7.8	0.6	0.67
	TC	255	33.2	0.7		246	0.06	0.0		236	7.2	0.3	
FokI rs10735810	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	GG	219	33.8	0.8		213	0.06	0.0		202	7.7	0.5	
Co-dominant	GA	254	33.5	0.7	0.54	244	0.06	0.0	0.60	225	7.8	0.5	**0.038**
	AA	66	31.8	1.4		62	0.07	0.0		58	5.6	0.5	
Dominant	GG	219	33.8	0.8	0.64	213	0.06	0.0	0.49	202	7.7	0.5	0.69
	GA+AA	320	33.2	0.7		306	0.06	0.0		283	7.4	0.4	
Recessive	GG+GA	473	33.6	0.5	0.27	457	0.06	0.0	0.36	427	7.8	0.4	**0.010**
	AA	66	31.8	1.4		62	0.07	0.0		58	5.6	0.5	
Over-dominant	GG+AA	285	33.3	0.7	0.80	275	0.06	0.0	0.92	260	7.2	0.4	0.29
	GA	254	33.5	0.7		244	0.06	0.0		225	7.8	0.5	

Abbreviations: Adipo-IR, adipose tissue insulin resistance index; HIRI, hepatic insulin resistance index; MISI, muscle insulin sensitivity index. P-value was corrected for age, sex, center, and fat mass. (†) N=539; (‡) N=519; (#) N=485.

Association between VDR polymorphisms and SAT gene transcription level

Expression quantitative trait loci (eQTL) analysis was conducted to analyze the association between the VDR gene variants and abdominal SAT tissue gene expression. Hoverer, *cis* and *trans* eQTL analysis showed that VDR gene expression did not significantly differ between genotypes of any of the VDR SNPs (TaqI, ApaI, BsmI, and FokI). We observed no significance difference of the VDR SNPs with gene expression of VDR target genes in *cis* and *trans* after FDR correction ($p > 0.05$) **(Supplemental Table S4 and S5)**.

VDR polymorphisms and change of body weight, change of tissue-specific IR following weight loss and maintenance

No effects of VDR genetic variants on the change of body weight following weight loss and weight maintenance were observed (**Supplemental Table S6**). Furthermore, there were no effects of VDR polymorphisms on the change of tissue-specific insulin resistance indexes (HIRI, MISI, and Adipo-IR) following weight loss intervention and weight maintenance (regain) (**Supplemental Table S7 and S8).**

Discussion

In this study, we showed that variants in VDR TaqI and ApaI are associated with elevated BMI (contributing 0.9 kg/m2 per risk allele), WC (3 cm per risk allele), or FM at baseline (2 kg per risk allele) at baseline. Secondly, variants in FokI VDR were associated with Adipo-IR as well as elevated circulating FFA at baseline (79 µmol/L per risk allele). Thirdly, *cis* and *trans* eQTL analysis demonstrated no major effects of these VDR polymorphisms on the SAT transcriptome at baseline. Finally, there was no relationship between VDR polymorphisms and changes in body weight and tissue-specific insulin resistance during weight loss and weight maintenance.

In the present study, we found that BMI, WC, and FM were significantly higher in individuals that carried the VDR TaqI G allele (AG and GG genotype) compared to non- carriers (AA genotype). In addition, BMI and FM of VDR ApaI A allele carriers were considerably higher than those of non-carriers (CC genotype). In line with this, Al Daghri et al. showed that TaqI (G allele) and ApaI (A allele) were associated with higher BMI in a dark-pigmented Caucasian population (11). Additionally, the TaqI polymorphism was also associated with higher BMI in a Greek population (12). In contrast, Vimaleswaran et al. and Walsh et al. showed no association between variants in VDR TaqI,, BMI (46) and FM (47) in male and female, lean/overweight and obese Caucasians. Regarding BsmI and FokI, we did not find any association with BMI, WC, or FM, which is in line with a previous finding by Dorjgochoo et al. and Walsh et al. showing no relationship between BsmI and FokI variants and markers of body composition (BMI (48) or FM (47)) in a Caucasian population.

Furthermore, pairwise LD analysis showed strong LD between TaqI, ApaI, and BsmI in our obese/overweight Caucasian population. However, haplotype analysis revealed no significantly associations with BMI, FM and tissue-specific insulin resistance, even after adjustment for age, sex, and center. These results, are in contrast with Al-Daghri et al. showing that in Dark-pigmented Caucasian individuals carriers of both G allele (TaqI) and

A allele (ApaI) had significantly higher BMI independent from age and sex (11). Differences in study populations (i.e. broader BMI vs. overweight/obese BMI) and ethnic-pigmentation (Dark-pigmented versus White-pigmented Caucasian) might partly explain this discrepancy (16), which still needs further investigation.

With respect to tissue specific insulin resistance, we did not find any associations between VDR polymorphisms (TaqI, ApaI, BsmI, and FokI), HIRI or MISI estimated from 5 time-points OGTT. Of interest, G-allele Carriers of VDR FokI showed a significant higher Adipo-IR and elevated fasting FFA concentrations, independent of age and sex. Moreover, recent studies in Asian populations with dyslipidemia suggested an association between FokI variants elevated triglyceride (TG) (49) and low-density lipoprotein (LDL) (50). These findings may indicate that VDR FokI variants are merely related to dyslipidemia and an impaired liver lipid metabolism (51), which needs to be investigated in more detail. Furthermore, the FokI polymorphism is located on the exon in the coding region of the VDR gene, resulting in different translation initiation sites (TIS) and giving rise to a full-length VDR protein or a three amino acid shorter VDR protein variant (52), having higher transcriptional activity (53, 54). Therefore, we studied whether variants in VDR FokI were associated with changes in the abdominal SAT transcriptome (targeted gene expression related to adipose tissue glucose, and lipid metabolism as well as inflammation). However, our *cis* and *trans* eQTL analysis suggested no effect of VDR FokI variants on SAT gene transcription, suggesting a minor contribution of VDR polymorphisms on adipose tissue function in overweight/obese men and women.

Further analysis, showed no effect of TaqI, ApaI and FokI VDR genetic variants on the change of body weight and tissue specific insulin resistance following weight loss and weight regain. Thus although these polymorphisms significantly contribute to adiposity and Adipo-IR in a cross sectional analysis they are apparently of less importance in determining dietary intervention outcome. This seems to be in contrast to another study in a T2D Saudi population showing that VDR genetic variants (i.e., TaqI and BsmI) affect intervention outcome (i.e., insulin sensitivity measured by HOMA-IR) following vitamin D supplementation (21).

In conclusion, our findings indicate that variants in VDR TaqI and ApaI are associated with markers of adiposity. In addition, the VDR FokI G-allele is associated with elevated circulating FFA and Adipo-IR in overweight/obese Caucasians. These VDR SNPs were not related changes in body weight and insulin sensitivity as result of dietary intervention. Nevertheless, these VDR SNPs had no effect on the transcriptional level, at least in abdominal SAT, indicating that the putative mechanisms of action remain to be determined.

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Supplemental Table S1. Minor allele frequencies (MAFs) and Hardy-Weinberg equilibrium (HWE) of VDR genotype prevalence in overweight/obese Caucasians.

SNP	Location	MAF	Genotype prevalence			HWE*
Study subjects (N=553)			Homozygote	Heterozygote	Homozygote	
TaqI (rs731236 A>G)	Exon 9	0.168	AA (37.8%)	AG (45.4%)	GG (16.8%)	0.25
ApaI (rs7975232 C>A)	Intron 8	0.175	CC (37.3%)	CA (45.2%)	AA (17.5%)	0.16
BsmI (rs1544410 C>T)	Intron 8	0.224	TT (30.0%)	TC (47.6%)	CC (22.4%)	0.31
FokI (rs10735810 A>G)	Exon 2 (start codon)	0.121	GG (41.0%)	GA (46.8%)	AA (12.1%)	0.64

HWE, Hardy-Weinberg equilibrium; MAF, Minor allele frequencies; SNP, single nucleotide polymorphisms; VDR, Vitamin D receptor. (*) P value of HWE equilibrium test.

Supplementary Table S2. Haplotype frequencies and association with BMI, WC, and FM.

				BMI	WC	FM
Haplotype	**TaqI**	**ApaI**	**Frequency (%)**	**P-value**	**P-value**	**P-value**
H1	A	C	59.58	-	-	-
H2	G	A	39.24	0.10	0.76	0.12
H3*	A	A	0.91	0.11	0.02	0.44
H4*	G	C	0.27	0.11	0.02	0.44
Haplotype	**TaqI**	**BsmI**	**Frequency (%)**	**P-value**	**P-value**	**P-value**
H1	A	C	46.10	-	-	-
H2	G	T	39.41	0.097	0.77	0.13
H3	A	T	14.39	0.79	0.43	0.51
H4*	G	C	0.10	0.83	0.98	0.023
Haplotype	**ApaI**	**BsmI**	**Frequency (%)**	**P-value**	**P-value**	**P-value**
H1	C	C	46.10	-	-	-
H2	A	T	40.04	0.16	0.90	0.26
H3	C	T	13.75	0.84	0.81	0.80
H4*	A	C	0.11	<0.001	0.42	0.037

Abbreviations: BMI, body mass index; FM, fat mass; WC, waist circumference. P-value is age, sex, and center adjusted. (*) rare haplotypes which are indicated by % frequency in this population close to zero.

Supplementary Table S3. The association between FokI VDR variants and circulating FFA.

		Circulating FFA			
FokI rs10735810	**Genotypes**	**N**	**Mean**	**SE**	***P* -value**
Co-dominant	GG	202	617.2	15.1	**0.039***
	GA	225	656.2	21.4	
	AA	58	547.9	24.7	
Dominant	GG	202	617.2	15.1	0.31
	GA+AA	283	634.0	17.9	
Recessive	GG+GA	427	637.8	13.4	**0.011***
	AA	58	547.9	24.7	
Over-dominant	GG+AA	260	601.8	13.0	0.51
	GA	225	656.2	21.4	

(*) *P*-value are age, sex, center, and fat mass adjusted.

Supplemental Table S4. *Cis* eQTL analysis for the association between VDR SNPs and abdominal SAT transcriptome

External Gene Name	Ensemble Gene ID	SNPs ID	Uncorrected p-value
AC004241.1	ENSG00000257433	rs731236	0.99469
AC004241.1	ENSG00000257433	rs1544410	0.88040
AC004466.1	ENSG00000268069	rs731236	0.93225
AC004466.1	ENSG00000268069	rs1544410	0.98856
AC004801.1	ENSG00000226138	rs731236	0.30670
AC004801.1	ENSG00000226138	rs1544410	0.72247
AC004801.2	ENSG00000240399	rs731236	0.42504
AC004801.2	ENSG00000240399	rs1544410	0.35540
AC008014.1	ENSG00000257261	rs731236	0.48353
AC008014.1	ENSG00000257261	rs1544410	0.93297
AC008083.2	ENSG00000257925	rs731236	0.20447
AC008083.2	ENSG00000257925	rs1544410	0.12114
AC008083.3	ENSG00000258181	rs731236	0.26733
AC008083.3	ENSG00000258181	rs1544410	0.20223
AC024257.3	ENSG00000269514	rs731236	0.25634
AC024257.3	ENSG00000269514	rs1544410	0.39731
AC090115.1	ENSG00000257735	rs731236	0.87932
AC090115.1	ENSG00000257735	rs1544410	0.83515
AC119044.1	ENSG00000258352	rs731236	0.44192
AC119044.1	ENSG00000258352	rs1544410	0.31780
ADCY6	ENSG00000174233	rs731236	0.39809
ADCY6	ENSG00000174233	rs1544410	0.28094
AMIGO2	ENSG00000139211	rs731236	0.37283
AMIGO2	ENSG00000139211	rs1544410	0.90822
ASB8	ENSG00000177981	rs731236	0.02566
ASB8	ENSG00000177981	rs1544410	0.03282
C12orf54	ENSG00000177627	rs731236	0.61907
C12orf54	ENSG00000177627	rs1544410	0.40474
CACNB3	ENSG00000167535	rs731236	0.91111
CACNB3	ENSG00000167535	rs1544410	0.76199
CCDC184	ENSG00000177875	rs731236	0.48553
CCDC184	ENSG00000177875	rs1544410	0.48287
CCNT1	ENSG00000129315	rs731236	0.52803
CCNT1	ENSG00000129315	rs1544410	0.38410
DDX23	ENSG00000174243	rs731236	0.70691
DDX23	ENSG00000174243	rs1544410	0.50571
ENDOU	ENSG00000111405	rs731236	0.09196
ENDOU	ENSG00000111405	rs1544410	0.23893
H1FNT	ENSG00000187166	rs731236	0.63911

H1FNT	ENSG00000187166	rs1544410	0.26691
HDAC7	ENSG00000061273	rs731236	0.60242
HDAC7	ENSG00000061273	rs1544410	0.84823
KANSL2	ENSG00000139620	rs731236	0.01986
KANSL2	ENSG00000139620	rs1544410	0.15509
LINC02156	ENSG00000257906	rs731236	0.74041
LINC02156	ENSG00000257906	rs1544410	0.67127
MIR4701	ENSG00000264201	rs731236	0.80517
MIR4701	ENSG00000264201	rs1544410	0.50309
OR10AD1	ENSG00000172640	rs731236	0.81170
OR10AD1	ENSG00000172640	rs1544410	0.66316
OR5BK1P	ENSG00000257763	rs731236	0.87842
OR5BK1P	ENSG00000257763	rs1544410	0.75369
OR5BT1P	ENSG00000258024	rs731236	0.80873
OR5BT1P	ENSG00000258024	rs1544410	0.91078
OR8T1P	ENSG00000226413	rs731236	0.11767
OR8T1P	ENSG00000226413	rs1544410	0.56028
PCED1B	ENSG00000179715	rs731236	0.11442
PCED1B	ENSG00000179715	rs1544410	0.53613
PCED1B-AS1	ENSG00000247774	rs731236	0.42487
PCED1B-AS1	ENSG00000247774	rs1544410	0.48036
PFKM	ENSG00000152556	rs731236	0.14653
PFKM	ENSG00000152556	rs1544410	0.16194
RAPGEF3	ENSG00000079337	rs731236	0.45454
RAPGEF3	ENSG00000079337	rs1544410	0.50054
RPAP3	ENSG00000005175	rs731236	0.17424
RPAP3	ENSG00000005175	rs1544410	0.11689
SENP1	ENSG00000079387	rs731236	0.32878
SENP1	ENSG00000079387	rs1544410	0.44986
SLC48A1	ENSG00000211584	rs731236	0.80739
SLC48A1	ENSG00000211584	rs1544410	0.73488
SNORA2A	ENSG00000206612	rs731236	0.81513
SNORA2A	ENSG00000206612	rs1544410	0.37916
SNORA2C	ENSG00000221491	rs731236	0.54230
SNORA2C	ENSG00000221491	rs1544410	0.16198
TEX49	ENSG00000257987	rs731236	0.94032
TEX49	ENSG00000257987	rs1544410	0.21053
TMEM106C	ENSG00000134291	rs731236	0.98853
TMEM106C	ENSG00000134291	rs1544410	0.79571
VDR	ENSG00000111424	rs731236	0.96896
VDR	ENSG00000111424	rs1544410	0.86894
ZNF641	ENSG00000167528	rs731236	0.65912
ZNF641	ENSG00000167528	rs1544410	0.44825

Abbreviation: SNPs, single-nucleotide polymorphisms

Supplemental Table S5. *Trans* eQTL analysis for the association between VDR SNPs and abdominal SAT transcriptome

Gene Symbol / Name	Ensemble Gene ID	SNPs ID	Uncorrected p-value
ADIPOQ	ENSG00000181092	rs731236	0.455771
ADIPOQ	ENSG00000181092	rs1544410	0.583178
ADIPOQ	ENSG00000181092	rs7975232	0.547899
ADIPOQ	ENSG00000181092	rs10735810	0.471824
ADRB2	ENSG00000169252	rs731236	0.554752
ADRB2	ENSG00000169252	rs1544410	0.758225
ADRB2	ENSG00000169252	rs7975232	0.568370
ADRB2	ENSG00000169252	rs10735810	0.291999
AGPAT2	ENSG00000169692	rs731236	0.709753
AGPAT2	ENSG00000169692	rs1544410	0.679197
AGPAT2	ENSG00000169692	rs7975232	0.666850
AGPAT2	ENSG00000169692	rs10735810	0.991221
AKT	ENSG00000142208	rs731236	0.745880
AKT	ENSG00000142208	rs1544410	0.702942
AKT	ENSG00000142208	rs7975232	0.903707
AKT	ENSG00000142208	rs10735810	0.161249
AMPK	ENSG00000132356	rs731236	0.842185
AMPK	ENSG00000132356	rs1544410	0.711332
AMPK	ENSG00000132356	rs7975232	0.664204
AMPK	ENSG00000132356	rs10735810	0.984760
ASAP2	ENSG00000151693	rs731236	0.321611
ASAP2	ENSG00000151693	rs1544410	0.367287
ASAP2	ENSG00000151693	rs7975232	0.389561
ASAP2	ENSG00000151693	rs10735810	0.655794
BCL6	ENSG00000113916	rs731236	0.325408
BCL6	ENSG00000113916	rs1544410	0.188299
BCL6	ENSG00000113916	rs7975232	0.383846
BCL6	ENSG00000113916	rs10735810	0.681122
CD14	ENSG00000170458	rs731236	0.564831
CD14	ENSG00000170458	rs1544410	0.949605
CD14	ENSG00000170458	rs7975232	0.552160
CD14	ENSG00000170458	rs10735810	0.764573

CD274	ENSG00000120217	rs731236	0.508695
CD274	ENSG00000120217	rs1544410	0.519024
CD274	ENSG00000120217	rs7975232	0.544318
CD274	ENSG00000120217	rs10735810	0.734286
CD36/GP4/FAT	ENSG00000135218	rs731236	0.564701
CD36/GP4/FAT	ENSG00000135218	rs1544410	0.768725
CD36/GP4/FAT	ENSG00000135218	rs7975232	0.670100
CD36/GP4/FAT	ENSG00000135218	rs10735810	0.179631
CEBP alpha	ENSG00000245848	rs731236	0.710341
CEBP alpha	ENSG00000245848	rs1544410	0.879930
CEBP alpha	ENSG00000245848	rs7975232	0.726116
CEBP alpha	ENSG00000245848	rs10735810	0.443595
CEBP beta	ENSG00000172216	rs731236	0.120219
CEBP beta	ENSG00000172216	rs1544410	0.863106
CEBP beta	ENSG00000172216	rs7975232	0.129213
CEBP beta	ENSG00000172216	rs10735810	0.944037
CGI58/ABHD5	ENSG00000011198	rs731236	0.480435
CGI58/ABHD5	ENSG00000011198	rs1544410	0.394122
CGI58/ABHD5	ENSG00000011198	rs7975232	0.572882
CGI58/ABHD5	ENSG00000011198	rs10735810	0.364466
cGMP	ENSG00000164430	rs731236	0.554868
cGMP	ENSG00000164430	rs1544410	0.534201
cGMP	ENSG00000164430	rs7975232	0.674139
cGMP	ENSG00000164430	rs10735810	0.289114
ChREBP	ENSG00000009950	rs731236	0.877966
ChREBP	ENSG00000009950	rs1544410	0.847874
ChREBP	ENSG00000009950	rs7975232	0.999319
ChREBP	ENSG00000009950	rs10735810	0.849390
CPT1 alpha	ENSG00000110090	rs731236	0.075466
CPT1 alpha	ENSG00000110090	rs1544410	0.367426
CPT1 alpha	ENSG00000110090	rs7975232	0.117869
CPT1 alpha	ENSG00000110090	rs10735810	0.586946
CYP27A1	ENSG00000135929	rs731236	0.888183
CYP27A1	ENSG00000135929	rs1544410	0.628180
CYP27A1	ENSG00000135929	rs7975232	0.797584
CYP27A1	ENSG00000135929	rs10735810	0.294358

CYP27B1	ENSG00000111012	rs731236	0.764641
CYP27B1	ENSG00000111012	rs1544410	0.482150
CYP27B1	ENSG00000111012	rs7975232	0.844958
CYP27B1	ENSG00000111012	rs10735810	0.864502
DUSP10	ENSG00000143507	rs731236	0.581689
DUSP10	ENSG00000143507	rs1544410	0.716124
DUSP10	ENSG00000143507	rs7975232	0.471904
DUSP10	ENSG00000143507	rs10735810	0.659463
ELOVL3	ENSG00000119915	rs731236	0.353104
ELOVL3	ENSG00000119915	rs1544410	0.128534
ELOVL3	ENSG00000119915	rs7975232	0.346517
ELOVL3	ENSG00000119915	rs10735810	0.565609
ELOVL5	ENSG00000012660	rs731236	0.764295
ELOVL5	ENSG00000012660	rs1544410	0.996432
ELOVL5	ENSG00000012660	rs7975232	0.676255
ELOVL5	ENSG00000012660	rs10735810	0.986764
ELOVL6	ENSG00000170522	rs731236	0.280761
ELOVL6	ENSG00000170522	rs1544410	0.642418
ELOVL6	ENSG00000170522	rs7975232	0.302517
ELOVL6	ENSG00000170522	rs10735810	0.239844
FABP4/aP2	ENSG00000170323	rs731236	0.252726
FABP4/aP2	ENSG00000170323	rs1544410	0.114612
FABP4/aP2	ENSG00000170323	rs7975232	0.311057
FABP4/aP2	ENSG00000170323	rs10735810	0.414575
FASN	ENSG00000169710	rs731236	0.643663
FASN	ENSG00000169710	rs1544410	0.904114
FASN	ENSG00000169710	rs7975232	0.564575
FASN	ENSG00000169710	rs10735810	0.474491
FBP1	ENSG00000165140	rs731236	0.988056
FBP1	ENSG00000165140	rs1544410	0.589106
FBP1	ENSG00000165140	rs7975232	0.851003
FBP1	ENSG00000165140	rs10735810	0.203295
FUCA1	ENSG00000179163	rs731236	0.761917
FUCA1	ENSG00000179163	rs1544410	0.902608
FUCA1	ENSG00000179163	rs7975232	0.811523
FUCA1	ENSG00000179163	rs10735810	0.921394

G0S2	ENSG00000123689	rs731236	0.400351
G0S2	ENSG00000123689	rs1544410	0.900682
G0S2	ENSG00000123689	rs7975232	0.378927
G0S2	ENSG00000123689	rs10735810	0.986559
GCR/NR3C1	ENSG00000113580	rs731236	0.899442
GCR/NR3C1	ENSG00000113580	rs1544410	0.709112
GCR/NR3C1	ENSG00000113580	rs7975232	0.974601
GCR/NR3C1	ENSG00000113580	rs10735810	0.328819
G6PDH	ENSG00000160211	rs731236	0.951515
G6PDH	ENSG00000160211	rs1544410	0.386809
G6PDH	ENSG00000160211	rs7975232	0.865034
G6PDH	ENSG00000160211	rs10735810	0.045936
GLUT4	ENSG00000181856	rs731236	0.490177
GLUT4	ENSG00000181856	rs1544410	0.937162
GLUT4	ENSG00000181856	rs7975232	0.436036
GLUT4	ENSG00000181856	rs10735810	0.821755
HIF-2	ENSG00000135245	rs731236	0.205890
HIF-2	ENSG00000135245	rs1544410	0.065505
HIF-2	ENSG00000135245	rs7975232	0.148536
HIF-2	ENSG00000135245	rs10735810	0.518143
IGFBP3	ENSG00000146674	rs731236	0.482403
IGFBP3	ENSG00000146674	rs1544410	0.637022
IGFBP3	ENSG00000146674	rs7975232	0.595182
IGFBP3	ENSG00000146674	rs10735810	0.903709
IL-6	ENSG00000136244	rs731236	0.140935
IL-6	ENSG00000136244	rs1544410	0.807286
IL-6	ENSG00000136244	rs7975232	0.094911
IL-6	ENSG00000136244	rs10735810	0.332016
IL10	ENSG00000136634	rs731236	0.715458
IL10	ENSG00000136634	rs1544410	0.403498
IL10	ENSG00000136634	rs7975232	0.717372
IL10	ENSG00000136634	rs10735810	0.116512
IL1b	ENSG00000125538	rs731236	0.881061
IL1b	ENSG00000125538	rs1544410	0.731165
IL1b	ENSG00000125538	rs7975232	0.766322
IL1b	ENSG00000125538	rs10735810	0.291848

IL4	ENSG00000113520	rs731236	0.432480
IL4	ENSG00000113520	rs1544410	0.535233
IL4	ENSG00000113520	rs7975232	0.412625
IL4	ENSG00000113520	rs10735810	0.235396
IRF/8	ENSG00000140968	rs731236	0.857759
IRF/8	ENSG00000140968	rs1544410	0.317458
IRF/8	ENSG00000140968	rs7975232	0.689645
IRF/8	ENSG00000140968	rs10735810	0.336422
IRS-1	ENSG00000169047	rs731236	0.237335
IRS-1	ENSG00000169047	rs1544410	0.708163
IRS-1	ENSG00000169047	rs7975232	0.203457
IRS-1	ENSG00000169047	rs10735810	0.468792
JAK	ENSG00000162434	rs731236	0.999467
JAK	ENSG00000162434	rs1544410	0.617411
JAK	ENSG00000162434	rs7975232	0.868002
JAK	ENSG00000162434	rs10735810	0.888699
KLF5	ENSG00000102554	rs731236	0.263342
KLF5	ENSG00000102554	rs1544410	0.498447
KLF5	ENSG00000102554	rs7975232	0.340295
KLF5	ENSG00000102554	rs10735810	0.986493
LEPTIN gene	ENSG00000174697	rs731236	0.455041
LEPTIN gene	ENSG00000174697	rs1544410	0.635911
LEPTIN gene	ENSG00000174697	rs7975232	0.571979
LEPTIN gene	ENSG00000174697	rs10735810	0.273339
Lipolysis Gene (HSL)	ENSG00000079435	rs731236	0.618543
Lipolysis Gene (HSL)	ENSG00000079435	rs1544410	0.721577
Lipolysis Gene (HSL)	ENSG00000079435	rs7975232	0.624253
Lipolysis Gene (HSL)	ENSG00000079435	rs10735810	0.629081
Lipolysis Gene (ATGL/PNPLA2)	ENSG00000177666	rs731236	0.225928
Lipolysis Gene (ATGL/PNPLA2)	ENSG00000177666	rs1544410	0.728314
Lipolysis Gene (ATGL/PNPLA2)	ENSG00000177666	rs7975232	0.282723
Lipolysis Gene (ATGL/PNPLA2)	ENSG00000177666	rs10735810	0.902261
LPGAT1	ENSG00000153395	rs731236	0.705962
LPGAT1	ENSG00000153395	rs1544410	0.742170
LPGAT1	ENSG00000153395	rs7975232	0.689354
LPGAT1	ENSG00000153395	rs10735810	0.285120

LPIN1	ENSG00000134324	rs731236	0.983600
LPIN1	ENSG00000134324	rs1544410	0.742501
LPIN1	ENSG00000134324	rs7975232	0.890749
LPIN1	ENSG00000134324	rs10735810	0.805104
LPL	ENSG00000175445	rs731236	0.716027
LPL	ENSG00000175445	rs1544410	0.318743
LPL	ENSG00000175445	rs7975232	0.579031
LPL	ENSG00000175445	rs10735810	0.955493
LRRC25	ENSG00000175489	rs731236	0.652329
LRRC25	ENSG00000175489	rs1544410	0.993398
LRRC25	ENSG00000175489	rs7975232	0.733143
LRRC25	ENSG00000175489	rs10735810	0.133150
LRRC8A	ENSG00000136802	rs731236	0.718964
LRRC8A	ENSG00000136802	rs1544410	0.744592
LRRC8A	ENSG00000136802	rs7975232	0.622977
LRRC8A	ENSG00000136802	rs10735810	0.587621
MAPK1	ENSG00000100030	rs731236	0.416836
MAPK1	ENSG00000100030	rs1544410	0.408182
MAPK1	ENSG00000100030	rs7975232	0.328973
MAPK1	ENSG00000100030	rs10735810	0.334360
MARRS/PDIA3	ENSG00000167004	rs731236	0.529960
MARRS/PDIA3	ENSG00000167004	rs1544410	0.927959
MARRS/PDIA3	ENSG00000167004	rs7975232	0.527661
MARRS/PDIA3	ENSG00000167004	rs10735810	0.860993
MCP-1/CCL2	ENSG00000108691	rs731236	0.169003
MCP-1/CCL2	ENSG00000108691	rs1544410	0.184940
MCP-1/CCL2	ENSG00000108691	rs7975232	0.133740
MCP-1/CCL2	ENSG00000108691	rs10735810	0.920663
MEK	ENSG00000169032	rs731236	0.334461
MEK	ENSG00000169032	rs1544410	0.882005
MEK	ENSG00000169032	rs7975232	0.569745
MEK	ENSG00000169032	rs10735810	0.410889
MGLL	ENSG00000074416	rs731236	0.449126
MGLL	ENSG00000074416	rs1544410	0.765235
MGLL	ENSG00000074416	rs7975232	0.456461
MGLL	ENSG00000074416	rs10735810	0.582794

NFATC/2	ENSG00000101096	rs731236	0.790467
NFATC/2	ENSG00000101096	rs1544410	0.651108
NFATC/2	ENSG00000101096	rs7975232	0.674252
NFATC/2	ENSG00000101096	rs10735810	0.663359
NFKB1	ENSG00000109320	rs731236	0.600707
NFKB1	ENSG00000109320	rs1544410	0.689101
NFKB1	ENSG00000109320	rs7975232	0.570783
NFKB1	ENSG00000109320	rs10735810	0.236408
NFKB2	ENSG00000077150	rs731236	0.589044
NFKB2	ENSG00000077150	rs1544410	0.741047
NFKB2	ENSG00000077150	rs7975232	0.561895
NFKB2	ENSG00000077150	rs10735810	0.300123
NPR(A)	ENSG00000169418	rs731236	0.791393
NPR(A)	ENSG00000169418	rs1544410	0.894629
NPR(A)	ENSG00000169418	rs7975232	0.900030
NPR(A)	ENSG00000169418	rs10735810	0.838292
NPR(C)	ENSG00000113389	rs731236	0.357951
NPR(C)	ENSG00000113389	rs1544410	0.125813
NPR(C)	ENSG00000113389	rs7975232	0.359934
NPR(C)	ENSG00000113389	rs10735810	0.047163
NRIP1	ENSG00000180530	rs731236	0.484102
NRIP1	ENSG00000180530	rs1544410	0.792344
NRIP1	ENSG00000180530	rs7975232	0.531864
NRIP1	ENSG00000180530	rs10735810	0.331990
P38/MAPK14	ENSG00000112062	rs731236	0.972425
P38/MAPK14	ENSG00000112062	rs1544410	0.680897
P38/MAPK14	ENSG00000112062	rs7975232	0.893236
P38/MAPK14	ENSG00000112062	rs10735810	0.340913
p53	ENSG00000141510	rs731236	0.770322
p53	ENSG00000141510	rs1544410	0.701156
p53	ENSG00000141510	rs7975232	0.728798
p53	ENSG00000141510	rs10735810	0.198160
PDK4	ENSG00000004799	rs731236	0.356615
PDK4	ENSG00000004799	rs1544410	0.215324
PDK4	ENSG00000004799	rs7975232	0.294460
PDK4	ENSG00000004799	rs10735810	0.225928

Pi3K	ENSG00000121879	rs731236	0.319843
Pi3K	ENSG00000121879	rs1544410	0.411791
Pi3K	ENSG00000121879	rs7975232	0.240937
Pi3K	ENSG00000121879	rs10735810	0.322286
PKA-cAMP	ENSG00000072062	rs731236	0.080860
PKA-cAMP	ENSG00000072062	rs1544410	0.246549
PKA-cAMP	ENSG00000072062	rs7975232	0.077146
PKA-cAMP	ENSG00000072062	rs10735810	0.618250
PKG, PRKG1	ENSG00000185532	rs731236	0.792431
PKG, PRKG1	ENSG00000185532	rs1544410	0.861508
PKG, PRKG1	ENSG00000185532	rs7975232	0.881917
PKG, PRKG1	ENSG00000185532	rs10735810	0.885963
PLIN1	ENSG00000166819	rs731236	0.493276
PLIN1	ENSG00000166819	rs1544410	0.487446
PLIN1	ENSG00000166819	rs7975232	0.422464
PLIN1	ENSG00000166819	rs10735810	0.320787
PPARG	ENSG00000132170	rs731236	0.214866
PPARG	ENSG00000132170	rs1544410	0.164932
PPARG	ENSG00000132170	rs7975232	0.280887
PPARG	ENSG00000132170	rs10735810	0.570991
RAF	ENSG00000157764	rs731236	0.892289
RAF	ENSG00000157764	rs1544410	0.669877
RAF	ENSG00000157764	rs7975232	0.791940
RAF	ENSG00000157764	rs10735810	0.790475
RXR (also known as NR2B1)	ENSG00000186350	rs731236	0.497599
RXR (also known as NR2B1)	ENSG00000186350	rs1544410	0.706210
RXR (also known as NR2B1)	ENSG00000186350	rs7975232	0.416367
RXR (also known as NR2B1)	ENSG00000186350	rs10735810	0.766505
SIRT1	ENSG00000096717	rs731236	0.918472
SIRT1	ENSG00000096717	rs1544410	0.616911
SIRT1	ENSG00000096717	rs7975232	0.751828
SIRT1	ENSG00000096717	rs10735810	0.211152
SIRT2	ENSG00000068903	rs731236	0.916038
SIRT2	ENSG00000068903	rs1544410	0.601722
SIRT2	ENSG00000068903	rs7975232	0.853919
SIRT2	ENSG00000068903	rs10735810	0.996848

SIRT3	ENSG00000142082	rs731236	0.872985
SIRT3	ENSG00000142082	rs1544410	0.824033
SIRT3	ENSG00000142082	rs7975232	0.891138
SIRT3	ENSG00000142082	rs10735810	0.868598
SREBP1	ENSG00000072310	rs731236	0.699367
SREBP1	ENSG00000072310	rs1544410	0.890846
SREBP1	ENSG00000072310	rs7975232	0.621155
SREBP1	ENSG00000072310	rs10735810	0.492756
STAT5B	ENSG00000173757	rs731236	0.276236
STAT5B	ENSG00000173757	rs1544410	0.157144
STAT5B	ENSG00000173757	rs7975232	0.292999
STAT5B	ENSG00000173757	rs10735810	0.904678
STEAP4	ENSG00000127954	rs731236	0.714747
STEAP4	ENSG00000127954	rs1544410	0.986450
STEAP4	ENSG00000127954	rs7975232	0.776424
STEAP4	ENSG00000127954	rs10735810	0.498933
STS	ENSG00000101846	rs731236	0.237906
STS	ENSG00000101846	rs1544410	0.229482
STS	ENSG00000101846	rs7975232	0.277679
STS	ENSG00000101846	rs10735810	0.298352
TNF alpha	ENSG00000232810	rs731236	0.670245
TNF alpha	ENSG00000232810	rs1544410	0.352961
TNF alpha	ENSG00000232810	rs7975232	0.683853
TNF alpha	ENSG00000232810	rs10735810	0.945494
TLR4	ENSG00000136869	rs731236	0.435463
TLR4	ENSG00000136869	rs1544410	0.819043
TLR4	ENSG00000136869	rs7975232	0.471708
TLR4	ENSG00000136869	rs10735810	0.100343
TREM1	ENSG00000124731	rs731236	0.649246
TREM1	ENSG00000124731	rs1544410	0.566640
TREM1	ENSG00000124731	rs7975232	0.622751
TREM1	ENSG00000124731	rs10735810	0.672667
TRPV	ENSG00000196689	rs731236	0.114610
TRPV	ENSG00000196689	rs1544410	0.344408
TRPV	ENSG00000196689	rs7975232	0.126326
TRPV	ENSG00000196689	rs10735810	0.697008

UCP1	ENSG00000109424	rs731236	0.168400
UCP1	ENSG00000109424	rs1544410	0.498550
UCP1	ENSG00000109424	rs7975232	0.228401
UCP1	ENSG00000109424	rs10735810	0.591485
UCP2	ENSG00000175567	rs731236	0.699806
UCP2	ENSG00000175567	rs1544410	0.535526
UCP2	ENSG00000175567	rs7975232	0.770138
UCP2	ENSG00000175567	rs10735810	0.483619
UCP3	ENSG00000175564	rs731236	0.651331
UCP3	ENSG00000175564	rs1544410	0.811421
UCP3	ENSG00000175564	rs7975232	0.604212
UCP3	ENSG00000175564	rs10735810	0.388739
VDR	ENSG00000111424	rs731236	0.968960
VDR	ENSG00000111424	rs1544410	0.868942
VDR	ENSG00000111424	rs7975232	0.961647
VDR	ENSG00000111424	rs10735810	0.584302

Abbreviation: SNPs, single-nucleotide polymorphisms

Supplemental Table S6. The relationship between VDR polymorphisms and weight loss-weight maintenance (regain).

		Weight loss (kg)*				Weight maintenance/regain (kg)#			
TaqI rs731236	**Genotype**	**N**	**Mean**	**SE**	***P*-value****	**N**	**Mean**	**SE**	***P*-value##**
	AA	182	-10.6	0.4		141	0.7	0.4	
Co-dominant	AG	228	-11.3	0.2	0.97	156	0.1	0.5	0.68
	GG	81	-11.0	0.4		59	0.9	0.7	
Dominant	AA	182	-10.6	0.4	0.80	141	0.7	0.4	0.75
	AG+GG	309	-11.3	0.2		215	0.3	0.4	
Recessive	AA+AG	410	-11.0	0.2	0.89	297	0.4	0.3	0.51
	GG	81	-11.0	0.4		59	0.9	0.7	
Over-dominant	AA+GG	263	-10.7	0.3	0.89	200	0.8	0.4	0.42
	AG	228	-11.3	0.2		156	0.1	0.5	
ApaI rs7975232	**Genotype**	**N**	**Mean**	**SE**	***P*-value****	**N**	**Mean**	**SE**	***P*-value##**
	CC	179	-10.6	0.4		138	0.7	0.4	
Co-dominant	CA	228	-11.3	0.2	0.95	157	0.1	0.5	0.73
	AA	84	-11.0	0.4		61	0.9	0.7	
Dominant	CC	179	-10.6	0.4	0.76	138	0.7	0.4	0.82
	CA+AA	312	-11.2	0.2		218	0.4	0.4	
Recessive	CC+CA	407	-11.0	0.2	0.83	295	0.4	0.3	0.53
	AA	84	-11.0	0.4		61	0.9	0.7	
Over-dominant	CC+AA	263	-10.7	0.3	0.90	199	0.8	0.4	0.48
	CA	228	-11.3	0.2		157	0.1	0.5	
BsmI rs1544410	**Genotype**	**N**	**Mean**	**SE**	***P*-value****	**N**	**Mean**	**SE**	***P*-value##**
	TT	146	-11.2	0.3		104	0.9	0.5	
Co-dominant	TC	238	-10.9	0.3	0.48	172	0.4	0.4	0.48
	CC	107	-10.9	0.5		80	0.1	0.6	
Dominant	TT	146	-11.2	0.3	0.55	104	0.9	0.5	0.29
	TC+CC	345	-10.9	0.2		252	0.3	0.4	
Recessive	TT+TC	384	-11.0	0.2	0.43	276	0.6	0.3	0.36
	CC	107	-10.9	0.5		80	0.1	0.6	
Over-dominant	TT+CC	253	-11.1	0.3	0.23	184	0.6	0.4	0.84
	TC	238	-10.9	0.3		172	0.4	0.4	
FokI rs10735810	**Genotype**	**N**	**Mean**	**SE**	***P*-value****	**N**	**Mean**	**SE**	***P*-value##**
	GG	206	-11.1	0.3		147	-0.1	0.5	
Co-dominant	GA	225	-11.0	0.3	0.45	165	1.0	0.5	0.22
	AA	60	-10.8	0.6		44	0.4	0.6	
Dominant	GG	206	-11.1	0.3	0.34	147	-0.1	0.5	0.14
	GA+AA	285	-10.9	0.3		209	0.9	0.4	
Recessive	GG-GA	431	-11.0	0.2	0.62	312	0.5	0.3	0.67
	AA	60	-10.8	0.6		44	0.4	0.6	
Over-dominant	GG+AA	266	-11.1	0.3	0.20	191	0.1	0.4	0.09
	GA	225	-11.0	0.3		165	1.0	0.5	

*Weight loss is absolute change of body weight after weight loss intervention and initial body weight (baseline).
#Weight regain is absolute change of body weight after maintenance phase and body weight after weight loss intervention.
N weight loss= 491; N weight regain= 356. **P-value is adjusted with initial body weight,
P-value was corrected for weight loss during LCD and mean body weight pre and post LCD.

Supplemental Table S7. The association between VDR polymorphisms and change of tissue-specific insulin resistance index following weight loss.

		Change of HIRI†				Change of MISI‡				Change of Adipo-IR#			
TaqI rs731236	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
Co-dominant	AA	139	-7.7	1.0	0.16	135	0.00	0.01	0.73	143	-1.4	0.3	0.67
	GA	175	-6.0	1.0		167	0.00	0.0		189	-2.2	0.5	
	GG	61	-8.6	1.4		62	0.00	0.01		58	-2.8	0.9	
Dominant	AA	139	-7.6	1.0	0.26	135	0.00	0.01	0.55	143	-1.4	0.3	0.91
	GA+GG	236	-6.7	0.8		229	0.00	0.0		247	-2.3	0.4	
Recessive	AA+GA	314	-6.7	0.7	0.28	302	0.00	0.0	0.49	332	-1.8	0.3	0.42
	GG	61	-8.6	1.4		62	0.00	0.01		58	-2.8	0.9	
Over-dominant	AA+GG	200	-7.7	0.8	0.08	197	0.00	0.0	0.95	201	-1.8	0.3	0.50
	GA	175	-6.0	1.0		167	0.00	0.0		189	-2.2	0.5	
ApaI rs7975232	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
Co-dominant	CC	135	-7.7	1.1	0.31	131	0.00	0.01	0.80	141	-1.4	0.3	0.59
	CA	175	-6.2	1.0		168	0.00	0.0		188	-2.1	0.5	
	AA	65	-8.1	1.4		65	0.00	0.01		61	-2.9	0.9	
Dominant	CC	135	-7.7	1.1	0.29	131	0.00	0.01	0.56	141	-1.4	0.4	0.96
	CA+AA	240	-6.7	0.8		233	0.00	0.0		249	-2.3	0.4	
Recessive	CC+CA	310	-6.8	0.7	0.49	299	0.00	0.0	0.61	329	-1.8	0.3	0.34
	AA	65	-8.1	1.4		65	0.00	0.01		61	-2.9	0.9	
Over-dominant	CC+AA	200	-7.8	0.8	0.12	196	0.00	0.0	0.87	202	-1.9	0.3	0.45
	CA	175	-6.2	1.0		148	0.00	0.0		188	-2.1	0.5	
BsmI rs1544410	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
Co-dominant	TT	110	-7.6	1.2	0.97	110	0.00	0.01	0.11	109	-3.2	0.7	0.49
	TC	188	-6.8	0.9		181	0.00	0.0		202	-1.5	0.3	
	CC	77	-7.1	1.4		73	0.00	0.01		80	-1.7	0.3	
Dominant	TT	110	-7.6	1.2	0.81	110	0.00	0.01	0.09	109	-3.2	0.7	0.54
	TC+CC	265	-6.8	0.7		254	0.00	0.0		281	-1.5	0.2	
Recessive	TT+TC	298	-7.0	0.7	0.90	291	0.00	0.0	0.96	310	-2.1	0.3	0.43
	CC	77	-7.1	1.4		73	0.00	0.01		80	-1.7	0.3	
Over-dominant	TT+CC	187	-7.4	0.9	0.90	183	0.00	0.0	0.08	189	-2.5	0.5	0.24
	TC	188	-6.7	0.9		181	0.00	0.0		201	-1.5	0.3	
FokI rs10735810	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
Co-dominant	GG	163	-7.0	1.0	0.22	160	0.01	0.0	0.10	171	-1.9	0.4	0.45
	GA	161	-6.3	1.0		158	0.00	0.0		174	-2.2	0.5	
	AA	51	-9.6	1.7		46	0.00	0.01		45	-1.4	0.4	
Dominant	GG	163	-7.0	1.0	0.91	160	0.01	0.0	0.06	171	-1.9	0.4	0.29
	GA+AA	212	-7.1	0.8		204	0.01	0.0		219	-2.0	0.4	
Recessive	GG+GA	324	-6.7	0.7	0.10	318	0.00	0.0	0.30	345	-2.1	0.3	0.31
	AA	51	-9.6	1.7		46	0.00	0.01		45	-1.4	0.4	
Over-dominant	GG+AA	214	-7.6	0.8	0.30	206	0.01	0.0	0.13	216	-1.8	0.3	0.69
	GA	161	-6.3	1.0		158	0.00	0.0		174	-2.2	0.5	

Abbreviations: Adipo-IR, adipose tissue insulin resistance index; HIRI, hepatic insulin resistance index; MISI, muscle insulin sensitivity index. For each index, *P*-value was adjusted with baseline HIRI, MISI, and Adipo-IR, respectively. (†) N=375; (‡) N=364; (#) N=390.

Supplemental Table S8. The association between VDR polymorphisms and change of tissue-specific insulin resistance index following weight maintenance (regain).

		Change of HIRI†				Change of MISI‡				Change of Adipo-IR#			
TaqI rs731236	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	AA	99	6.0	1.4		76	0.00	0.0		51	-0.1	0.3	
Co-dominant	GA	110	7.2	1.5	0.30	85	0.01	0.0	0.21	55	-0.4	0.6	0.85
	GG	42	5.4	2.1		40	0.01	0.0		19	0.1	1.1	
Dominant	AA	99	6.0	1.4	0.38	76	0.00	0.0	0.09	51	-0.1	0.3	0.62
	GA+GG	152	6.7	1.3		125	0.01	0.0		74	-0.3	0.5	
Recessive	AA+GA	209	6.7	1.1	0.38	161	0.01	0.0	0.72	106	-0.3	0.4	0.65
	GG	42	5.4	2.1		40	0.02	0.0		19	0.1	1.1	
Over-dominant	AA+GG	141	5.8	1.2	0.13	116	0.00	0.0	0.16	70	-0.1	0.3	0.88
	GA	110	7.2	1.5		85	0.01	0.0		55	-0.4	0.6	
ApaI rs7975232	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	CC	97	6.1	1.4		76	0.00	0.0		51	-0.1	0.3	
Co-dominant	CA	109	7.3	1.5	0.32	84	0.01	0.0	0.15	54	-0.3	0.7	0.94
	AA	45	5.1	2.0		41	0.01	0.0		20	-0.2	1.0	
Dominant	CC	97	6.1	1.4	0.43	76	0.00	0.0	0.09	51	-0.1	0.3	0.83
	CA+AA	154	6.7	1.2		125	0.01	0.0		74	-0.3	0.6	
Recessive	CC+CA	206	6.7	1.1	0.37	160	0.00	0.0	0.91	105	-0.2	0.4	0.75
	AA	47	5.1	2.0		41	0.01	0.0		20	-0.2	1.0	
Over-dominant	CC+AA	142	5.8	1.2	0.14	117	0.00	0.0	0.08	71	-0.1	0.3	0.98
	CA	109	7.3	1.5		84	0.01	0.0		54	-0.3	0.7	
BsmI rs1544410	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	TT	75	6.7	1.8		65	0.01	0.0		36	-0.7	0.9	
Co-dominant	TC	121	6.4	1.4	0.96	93	0.00	0.0	0.67	62	0.2	0.4	0.60
	CC	55	6.1	1.7		43	0.00	0.0		27	-0.3	0.4	
Dominant	TT	78	6.7	1.8	0.88	65	0.01	0.0	0.38	36	-0.7	0.9	0.36
	TC+CC	179	6.3	1.1		136	0.00	0.0		89	0.0	0.3	
Recessive	TT+TC	201	6.5	1.1	0.78	158	0.00	0.0	0.63	98	-0.2	0.4	0.92
	CC	56	6.1	1.7		43	0.01	0.0		27	-0.3	0.4	
Over-dominant	TT+CC	130	6.4	1.3	0.93	108	0.01	0.0	0.67	63	-0.6	0.6	0.37
	TC	121	6.4	1.4		93	0.00	0.0		62	0.2	0.4	
FokI rs10735810	**Genotype**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**	**N**	**Mean**	**SE**	***P*-value**
	GG	109	7.8	1.4		92	0.00	0.01		53	-0.0	0.5	
Co-dominant	GA	109	5.3	1.4	0.33	86	0.01	0.01	0.12	61	-0.4	0.5	0.77
	AA	33	5.7	2.6		23	0.02	0.01		11	-0.2	1.1	
Dominant	GG	109	7.8	1.4	0.15	92	0.00	0.01	0.08	53	-0.0	0.5	0.47
	GA+AA	142	5.4	1.3		109	0.01	0.01		72	-0.3	0.5	
Recessive	GG+GA	218	6.6	1.0	0.38	178	0.01	0.0	0.14	114	-0.2	0.4	0.78
	AA	33	5.7	2.6		23	0.02	0.01		11	-0.2	1.1	
Over-dominant	GG+AA	142	7.3	1.2	0.40	115	0.00	0.0	0.38	64	-0.1	0.4	0.59
	GA	109	5.3	1.4		80	0.01	0.0		61	-0.4	0.5	

Abbreviations: Adipo-IR, adipose tissue insulin resistance index; HIRI, hepatic insulin resistance index; MISI, muscle insulin sensitivity index. For each index, *P*-value was corrected for the changes of HIRI, MISI, and Adipo-IR values during LCD, and mean of HIRI, MISI, Adipo-IR pre and post LCD, respectively. (†) N=251; (‡) N=192; (#) N=125.

CHAPTER 6

The effect of vitamin D supplementation on insulin sensitivity:

a systematic review and meta-analysis

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Abstract

Background: Vitamin D has been suggested to affect peripheral insulin sensitivity. Evidence regarding the effect of vitamin D supplementation on insulin sensitivity is still conflicting.

Purpose: This meta-analysis aimed to assess the effect of vitamin D supplementation on insulin sensitivity in humans with or at risk of insulin resistance.

Data source and study selection: PubMed, Web of Science, Embase, CINAHL, and Cochrane library were systematically searched for randomized controlled trials (RCTs) from 1980 until 31 December 2018, reporting treatment effects of vitamin D supplementation on insulin sensitivity.

Data extraction: The main outcome of interest was the change in insulin sensitivity, derived from the gold standard hyperinsulinemic euglycemic clamp or the Matsuda index derived from Oral Glucose Tolerance Test (OGTT) and insulin sensitivity index from Intravenous Glucose Tolerance Test (IVGTT). We extracted data on the standardized mean difference (SMD) between the vitamin D treatment and placebo groups in change from baseline of insulin sensitivity.

Data synthesis: Eighteen RCTs were included in this meta-analysis comparing vitamin D supplementation (n= 612) with placebo (n=608). Vitamin D supplementation had no effect on insulin sensitivity (SMD: -0.01; 95% CI: -0.12, 0.10; P= 0.87, I^2= 0%). Visual inspection of funnel plot symmetry did not suggest potential publication bias.

Limitations: The number of individuals who participated in the included studies was relatively small possibly due to the invasive character of the measurement (e.g. clamp).

Conclusions: This meta-analysis provides no evidence that vitamin D supplementation has a beneficial effect on peripheral insulin sensitivity in people with or at risk of insulin resistance.

Introduction

The International Diabetes Federation (1) has reported that the worldwide diabetes prevalence in the adult population reached 8.8% (424.9 million people) in 2017. The majority (87-91%) of these diabetes cases concerns type 2 diabetes. Type 2 diabetes has become a major public health concern due to its complications. It decreases quality of life and increases mortality risk (2). Additionally, about 7.3% of the adult population (352.1 million people) suffers from prediabetes (1). Prediabetes is defined by increased fasting glucose and/or impaired glucose tolerance (postprandial hyperglycemia), which are both strongly associated with obesity (3). Moreover, postprandial hyperglycemia is a major risk factor for developing type 2 diabetes (4).

Obesity, prediabetes, and type 2 diabetes are often characterized by low circulating levels of vitamin D (vitamin D deficiency) (5). Indeed, based on cross sectional studies, vitamin D deficiency has been linked with an impaired insulin sensitivity in humans (6). Furthermore, people with low vitamin D levels (7) may be at greater risk to develop type 2 diabetes. Of interest, the vitamin D receptor, which mediates the function of vitamin D, is also expressed in insulin-sensitive tissues (including adipose tissue, muscle, and pancreas) (8-10). Therefore, currently non-skeletal functions of vitamin D in insulin-sensitive organs are being investigated.

Several plausible mechanisms have been proposed to explain a potential role of vitamin D in improving insulin sensitivity (6, 11). In adipose tissue, vitamin D may affect lipid metabolism (12) and may reduce inflammation (13). Vitamin D may affect pancreatic insulin secretion via protection of beta-cells from local inflammation (14). Vitamin D may also affect insulin secretion, which is mediated by a calcium-dependent mechanism (15). In addition, it has been demonstrated that vitamin D affects skeletal muscle metabolism, insulin sensitivity and lipid composition (16, 17). Thus, increasing circulating vitamin D concentration might be expected to have beneficial effects on tissue energy and substrate metabolism thereby contributing to an improvement of whole-body insulin sensitivity.

In recent years, several meta-analyses (18-23) have been conducted to investigate the effect of vitamin D supplementation on whole-body insulin sensitivity. However, the findings from these reports are inconsistent. From the six meta-analyses published between 2015 and 2018, 4 studies (18-21) indicated insufficient evidence/no effect on glycemic control and insulin resistance whereas 2 studies (22, 23) indicated sufficient evidence for an improvement of glycemic control and insulin sensitivity. Moreover, these meta-analyses have been focusing only on glucose homeostasis under fasting conditions by studying parameters such as homeostatic model assessment of insulin resistance (HOMA-IR), fasting glucose, fasting insulin, and Glycated haemoglobin 1c (HbA1c) as main outcome measures. Of importance, the skeletal muscle is considered as an important organ in peripheral insulin sensitivity as it affects 70-90% of total glucose disposal under postprandial conditions (non-fasting conditions) (24, 25). Studies have shown that vitamin D may affect skeletal muscle substrate metabolism and insulin sensitivity (16, 17). Thus, increasing circulating vitamin D concentration by means of supplementation might be expected to have beneficial effects during postprandial conditions by improving skeletal muscle glucose handling/ insulin sensitivity. Therefore, we undertook a systematic review and meta-analysis of RCTs to delineate the impact of vitamin D supplementation on insulin sensitivity derived from the gold standard hyperinsulinemic euglycemic clamp or from a multi-sampled oral or

intravenous glucose tolerance tests (OGTT or IVGTT) as well as the postprandial glucose concentrations (OGTT) in humans with or at risk of insulin resistance.

Methods

Literature search strategy and study selection

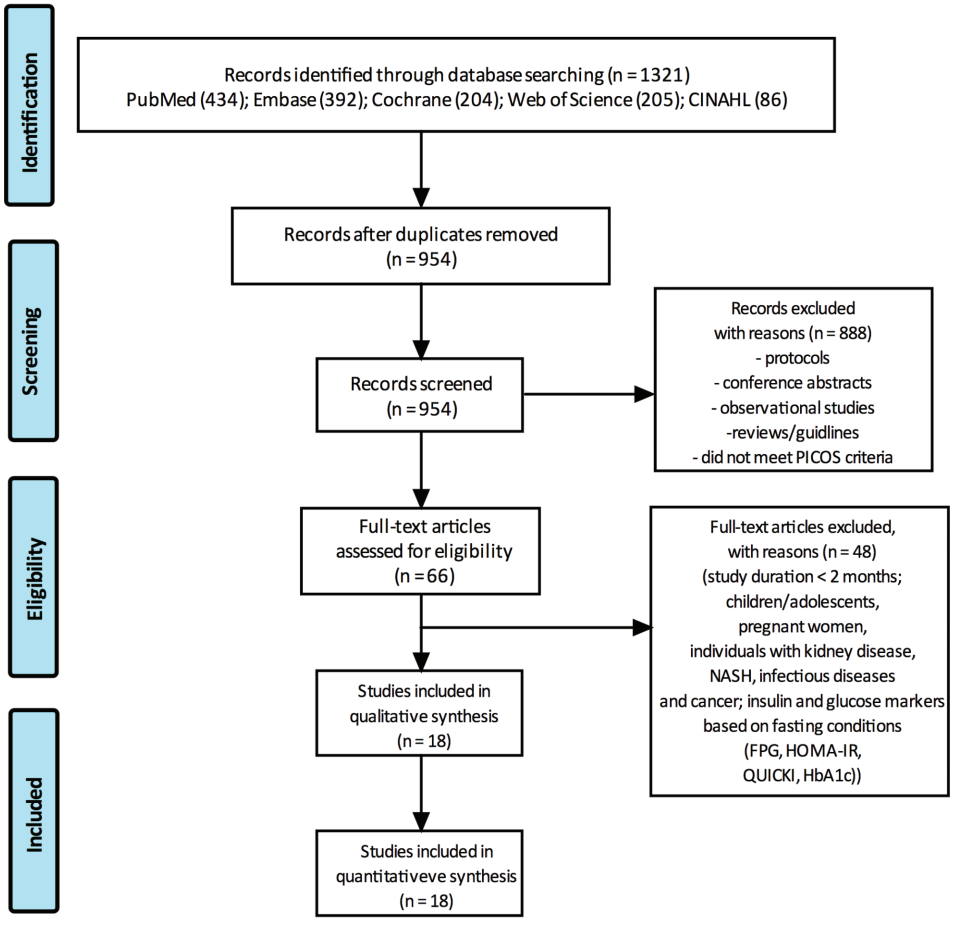


Figure 1. Flowchart diagram for study selection of systematic review (based on Preferred Reporting Items for Systematic Reviews and Meta-Analysis/PRISMA guideline)

We conducted our systematic review and meta-analysis according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (26). The protocol for our systematic review was registered at the International Prospective Register of Systematic Reviews, PROSPERO (registration CRD42018092961). The main outcome of interest was the change in insulin sensitivity, derived from a gold standard hyperinsulinemic euglycemic clamp or in the form of the Matsuda Index derived from OGTT and/or insulin

sensitivity index derived from intravenous glucose tolerance test (IVGTT), subsequent to vitamin D administration in individuals with or at risk of insulin resistance. The secondary outcome was the change in 2-hour postprandial glucose concentration and/or area under the curve (AUC) glucose during an OGTT.

A comprehensive literature search's [PubMed/Medline (Medical Literature Analyses and Retrieval System Online), Cochrane library, Web of Science, Embase database (OVID) and The Cumulative Index to Nursing and Allied Health Literature (CINAHL) was performed to identify articles from 1980 until 31 December 2018. The main keywords used were overweight, obesity, prediabetes, type 2 diabetes mellitus, vitamin D, insulin sensitivity, insulin resistance, blood glucose. These keywords were combined with Boolean operators (e.g. OR, AND, NOT), and all Fields or Medical subject subheading (MeSH) terms. This set of search terms was slightly modified when searching in every database due to a different system and technical limitations (**Supplemental Table S1**).

Eligible studies met the PICOS (Patients/participants, Intervention, Comparison/control group, Outcome, and Study Design) criteria: (1) study was a randomized controlled trial; (2) study population consisted of individuals with elevated (risk for) insulin resistance (overweight, obesity, prediabetes, polycystic ovary syndrome (PCOS), and type 2 diabetes without complications); (3) participants were ≥ 18 years; (4) interventions were vitamin D supplementation vs the appropriate placebo; (5) vitamin D supplementation dose was daily, weekly, or monthly; (6) trial length was ≥ 2 months; (7) serum 25(OH)D level was measured; (8) insulin sensitivity was measured by Matsuda index derived from an OGTT and/or insulin sensitivity index derived from IVGTT, or by a hyperinsulinemic euglycemic clamp at the beginning and at the end of the trial; and (9) study was published in English.

Following the search, duplicates were removed. A total of 18 studies were included in this systematic review (**Figure 1**). Exclusion criteria were as follows: (1) non clinical trial studies; (2) studies without outcome of insulin sensitivity derived from multi-sampled oral glucose tolerance test or hyperinsulinemic euglycemic clamp; (3) study populations with end-stage renal disease (kidney disease), cancers, gestational diabetes, Non-alcoholic steatohepatitis (NASH), cardiovascular diseases complications, infectious diseases; (4) intervention periods of < 2 months; (5) vitamin D supplementation provided as a single dose; and (6) study performed in children and/or adolescents (<18 years).

Titles and abstracts were screened by two authors (A.P. and M.A.v.B.). Final study selection, based on the inclusion criteria, was done by two authors (A.P. and M.A.v.B.) and approved by another author (E.E.B.). Any disagreements between the authors were resolved through discussion with the fourth author (J.W.E.J.).

Data extraction and management

Data were extracted by two authors (A.P., M.A.v.B.). Data extracted from each study included the following items: first author, reference, year of publication, country of study, study design, inclusion criteria, sample size, form of vitamin D, dose and frequency of vitamin D supplementation, any co-supplementation (e.g. diet or calcium), treatment control group, duration of supplementation, participants' characteristics [n, sex (% male), age, BMI, ethnicities], comorbidities, baseline and follow-up serum 25(OH)D levels and outcome measures (e.g. M value or glucose infusion rate (GIR) or insulin-mediated glucose uptake derived from clamp, Matsuda index, Insulin sensitivity index, 2h glucose or AUC

glucose from an OGTT)] **(Table 1)**. The corresponding author of an eligible publication was contacted by e-mail for additional data when relevant. Any further necessary calculations on study data, such as converting measurement units or calculating standard deviation (SD), was conducted by the first author (A.P.) and checked by another author (M.A.v.B.). Serum 25(OH)D levels were collated in nmol/L; a multiplication factor of 2.456 was used to convert 25(OH)D levels from ng/mL to nmol/L. 2-hour glucose tolerance test glucose concentrations (2h-glucose) were collated in mmol/L; we used a multiplication factor of 0.0555 to convert glucose levels from mg/dL to mmol/L, as appropriate (27).

Quality assessment

The quality of selected RCTs was assessed independently by two authors (A.P., M.A.v.B.) using the risk of bias checklist (RoB) from the Cochrane Collaboration (28). The quality assessments of the checklist included (1) bias from randomization process (random sequence generation and allocation concealment), (2) bias due to deviations from intended interventions (blinding of participants and personnel), (3) bias due to missing outcome data, (4) bias from measurements of the outcome, (5) bias from selection of reported result, and other sources of bias. Each criterion could be answered in three ways: "low risk" (adequate information), "unclear risk" (if there was unclear information), and "high risk" (if there was a high concern). Study quality and the risk of bias in the eligible RCTs was systematically assessed using software Review Manager 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark, 2014).

Data synthesis and statistical analysis

To calculate the effect size of each study, we used the mean change and SD of the outcome measures from baseline to the end of the intervention in the control and intervention groups (29). When the outcome measure was reported as mean and 95% confidence interval [CI] or mean and standard error of mean (SEM), values were estimated using Cochrane Review Manager 5.3 software (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark, 2014) (30). If the outcome measures were reported in median, range or 25^{th}–75^{th} percentiles (P25–P75), mean and standard SD values were estimated using formulas published by Wan et al (31). If the outcome measures were only reported in figures, we used software to estimate the value. SDs of the mean difference were estimated using the following formula: SD = square root [$(SD\ pre\text{-}treatment)^2 + (SD\ post\text{-}treatment)^2$) - (2R * SD pre-treatment * SD post-treatment)]. Because the pretest–posttest correlation coefficients (r) were not reported in studies, an r value of 0.5 was assumed throughout this meta-analysis (30). If a study included more than two intervention groups (e.g. two different doses of vitamin D), which were compared with one placebo (control) group, the number of subjects of the control group was divided by the number comparisons. If the outcome measurement was performed at multiple time-points after the intervention period, we only used first time-point after the intervention (29).

The effect size is reported as standardized mean difference with its 95% confidence interval (CI) (26, 32). Standardized mean difference is used as a summary statistic in meta-analysis when the studies all assess the same outcome but measure it in a variety of ways (in this study for example, all studies measured insulin sensitivity but use different methodology

i.e. clamp or Matsuda index or insulin sensitivity index). A random-effects model was used to estimate outcomes. Heterogeneity was assessed using the I^2 (I square) statistic, indicating what proportion of the variation in observed effects across studies is due to the variation in true effects, with values above 60% indicating substantial heterogeneity. A p-value < 0.05 is considered statistically significant (29). The analysis was performed and generated using Cochrane Review Manager 5.3 software (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark, 2014). The PRISMA checklist was used as a guide for checking the quality of our systematic review **(Supplemental Table S2)**.

Publication Bias

Publication bias was analyzed by visual inspection of the funnel plots.

Meta regression and sensitivity analysis

If the heterogeneity was >60%, additional analyses were conducted. Meta-regression analysis and sensitivity analysis using the leave-one-out method (removing one study each time and repeating the analysis) were applied to gain insight into the source of the heterogeneity (29).

Results

Study characteristics

In total, 18 RCTs (33-50) published between 2011 and 2018 with 1220 participants were included in the current meta-analysis. Characteristics of the studies are summarized in **Table 1**. The studies were conducted in the United States of America (n=5), Canada (n=1), Iran (n=1), India (n=1), Malaysia (n=1), Australia (n=2), The Netherlands (n=1), Denmark (n=1), Norway (n=1), Finland (n=1), Sweden (n=1), Austria (n=1), and Italy (n=1). Most studies included both men and women, except two studies that included only women (43, 45) and two other studies that included only men (44, 50). Among the 18 studies, 8 enrolled several ethnic populations (multi-ethnicity) (33, 36-38, 40, 46-48), two enrolled individuals from African-American ethnicity (34, 44) two enrolled in Asians (43, 45), one enrolled participants from Middle-Eastern countries (35), and five studies did not provide information about ethnicity (39, 41, 42, 49, 50). Sixteen trials were in participants with overweight, obesity, prediabetes, type 2 diabetes, or metabolic syndrome, two of these trials also included apparently healthy participants (40, 50), while participants in the two other trials had PCOS (Polycystic Ovary Syndrome) (45) or a history of GDM (Gestational Diabetes Mellitus) (43). Studies used different cut-offs for baseline vitamin D levels: nine studies (34, 37-40, 45-47) recruited individuals with vitamin D levels <50 nmol/L, and the others (33, 35, 36, 41, 42, 44, 48-50) included individuals with vitamin D levels <75 nmol/L at the beginning of trial **(Table 1)**. Mean serum vitamin D 25(OH)D concentrations at baseline varied from 19.2 nmol/L (45) to 59.9 nmol/L (33) in vitamin D-supplemented groups and 16.9 nmol/L (45) to 61.4 (33) in placebo groups. The mean oral dose of vitamin D was 4,608 IU per day (range 1,200 IU (37) to 12,695 IU (36) per day), with the majority of studies providing daily doses ≤ 4000 IU (33, 34, 37-39, 41, 43, 45, 46, 48-50). The duration of vitamin D supplementation ranged from 2 (42) to 12 months (44).

Table 1. Characteristics of randomized-controlled trial studies included in the meta analysis (continued)

Author (Year)	Study location	N subjects (Tx/Px)	Sex	Ethnicity	BMI (kg/m^2)	Health status	Vitamin D levels at Baseline (nmol/L)	Vitamin D levels after intervention (nmol/L)	Type of vitamin D; mode of delivery; and dose (IU/day)	Analytical measures of vitamin D	Duration (month)	Outcome measured
Mitri et al. (2011)	USA	23/24	M/F	Multi-ethnic	≥ 25 (≥23 if Asian)	Prediabetes (IGT, IFG, HbA1c≥5.8%)	T: 59.9±2.7[§] P: 60.4±2.7[§]	T: +15.7±3.7[§] (Change from baseline) P: −20.5±3.5[§]	Vitamin D3; oral; 2000	LC-MS	4	Insulin sensitivity index (FIVGTT), 2h-glucose (OGTT)
Harris et al. (2012)	USA	43/46	M/F	African-American	25-39.9	Prediabetes or early T2D (HbA1c 6.7-7%); baseline vitamin D 25(OH)D <50 nmol/L	T: 39.6±12.9; P: 38.2±15.5	T: 41.6±3.1[§] (Change from baseline) P: 0.9±2.9[§]	Vitamin D3; oral; 4000	RIA	3	Matsuda index, 2h-glucose (OGTT)
Iraj et al. (2012)	Iran	20/20	M/F	Middle-Eastern	≥ 25	First degree relatives of T2D; Vitamin D 25(OH)D <75 nmol/L; Prediabetes	T: 27.5±15.0 P: 28.2±15.0	T: 87.4±49.9 P: 38.7±54.9	Vitamin D3; injection; 10000	CLIA	2	Matsuda index, AUC Glucose (OGTT)
Davidson MB et al. (2013)	USA	56/53	M/F	Multi-ethnic (Hispanic and African-American)	≥ 25	Prediabetes; Vitamin D 25(OH)D <75 nmol/L	T: 54.9±12.5 P: 54.9±12.5	T: 169.6±37.4 P: 54.9±17.4	Vitamin D3; oral; 12695	LC-MS	3	Matsuda index, 2h-glucose
Oosterwerff et al. (2014)	Netherlands	53/57	M/F	Multi-ethnic (Moroccan, Suriname, Turkey)	≥ 25	IFG or IGT; Vitamin D 25(OH)D <50 nmol/L	T: 25.0±10.8 P: 22.0±11.0	T: 60.0±16.0 P: 23.0±15.0	Vitamin D3; oral; 1200	LC-MS	4	Insulin sensitivity index, 2h-glucose
Gagnon et al. (2014)	Australia	35/45	M/F	Multi-ethnic	25-40	Vitamin D 25(OH)D <50 nmol/L, Prediabetes	T: 47.0±13.0 P: 43.2±13.0	T: 89.1±16.5 P: 41.4±16.3	Vitamin D3; oral; 2000	CLIA	6	Matsuda index

CHAPTER 6

Table 1. Characteristics of randomized-controlled trial studies included in the meta analysis (continued)

Author (Year)	Study location	N subjects (Tx/Px)	Sex	Ethnicity	BMI (kg/m^2)	Health status	Vitamin D levels at Baseline (nmol/L)	Vitamin D levels after intervention (nmol/L)	Type of vitamin D; mode of delivery; and dose (IU/day)	Analytical measures of vitamin D	Duration (month)	Outcome measured
Kampmann et al. (2014)	Denmark	08-Jul	M/F	NR	≥ 25	T2D; Vitamin D 25(OH)D <50 nmol/L	T: 31.0±4.9§ P: 34.8±3.8§	T: 104.9±19.0§ P: 32.1±3.8§	Vitamin D3; oral; 6400	ELISA	3	M-value derived from clamp
Mitchell et al. (2015)	USA	40/50	M/F	Multi-ethnic	Median 25	Vitamin D 25(OH)D <50 nmol/L	T: 44.9±17.5 P: 44.9±17.5	T: 107.3±29.9 P: 49.9±24.9	Vitamin D2; oral; 7142	LC-MS	3	Insulin sensitivity index (FIVGTT)
Tuomainen et al. (2015)	Finland	24/21/21	M/F	NR	≥ 25	Prediabetes; Vitamin D 25(OH)D <75 nmol/L	Mean T+P baseline: 57.0 ± 11.0	T1: +27.7±17.2 (Change from baseline) T2: +45.0±23.4 (Change from baseline)	T1 Vitamin D3; oral; 1600 T2 Vitamin D3; oral; 3200	HPLC-CEAD HPLC-CEAD	5 5	Matsuda index Matsuda index
Wagner et al. (2016)	Sweden	21/22	M/F	NR	≤ 32	Prediabetes or drug naive diabetes; HbA1c ≤ 7.9%; FPG <9 mmol/L; Vitamin D 25(OH)D <75 nmol/L	Median (IQR) T: 43.0 (36.0–50.0);	P: +4.1±17.3 Median change (IQR) T: +42.0 (32.0–50.0)	Vitamin D3; oral; 4285	CLIA	2	GIR derived from clamp
Yeow et al. (2015)	Malaysia	13/13	F	Asian	23 - 31	History of GDM during last pregnancy6-48 months postpartum; hypovitaminosis (Vitamin D 25(OH)D 15-50 nmol/L)	P: 43.0(37.0–54.0) Median (IQR) T: 35.6 (25.6-43.9)	P: 0.0(-7.0–11.0) Median change (IQR) T: +51.1 (39.9- 76.1)	Vitamin D3; oral; 4000	ECLIA	6	Insulin sensitivity index, 2h-glucose
Barengolts et al. (2015)	USA	87/86	M	African-American	28-39	Fasting glucose 5.3-6.9 mmol/L; HbA1c 5.7-6.4%; Vitamin D 25(OH)D 12.5-75 nmol/L	P: 35.1 (21.6- 40.7) T: 36.7±11.7	P: –0.2 (-10.18–11.8) T: 120.1±45.9	Vitamin D2; oral; 7142	CLIA	12	Matsuda index

Table 1. Characteristics of randomized-controlled trial studies included in the meta analysis

Author (Year)	Study location	N subjects (Tx/Px)	Sex	Ethnicity	BMI (kg/m^2)	Health status	Vitamin D levels at Baseline (nmol/L)	Vitamin D levels after intervention (nmol/L)	Type of vitamin D; mode of delivery; and dose (IU/day)	Analytical measures of vitamin D	Duration (month)	Outcome measured
Garg et al. (2015)	India	15/17	F	Asian	≥ 23	PCOS, vitamin D 25(OH)D <50 nmol/L	T: 19.2±15.1 P: 16.9±6.1	T: 78.6±34.6 P: 16.7±5.8	Vitamin D3; oral; 4000	CLIA	6	Matsuda index, AUC Glucose
Mousa et al. (2017)	Australia	28/26	M/F	Multi-ethnic	≥ 25	Vitamin D 25(OH)D <50 nmol/L	T: 31.4±12.6 P: 34.2±10.0	T: 88.4±21.0 P: 36.1±15.3	Vitamin D3; oral; 4000	CLIA	4	M-value derived from clamp
Gulseth et al. (2017)	Norway	33/29	M/F	Multi-ethnic (Nordic and South-Asians)	< 45	Type 2 diabetes; Vitamin D 25(OH)D <50 nmol/L	T: 38.0±11.9 P: 36.8±12.6	T: 53.7±9.2 P: 38.2±12.9	Vitamin D3; injection & oral; 3333	RIA	6	GIR derived from clamp
Lerchbaum et al. (2017)	Austria	49/49	M	NR	Median 25	Vitamin D 25(OH)D <75 nmol/L	Median (IQR) T: 52.0 (42.0 – 65.0) P: 51.0 (43.0 – 68.0)	Median (IQR) T: 107.0 (89.0 – 119.0) P: 69.0 (46.0 – 79.0)	Vitamin D3; oral; 2857	LC-MS	3	Matsuda index, AUC Glucose
Moreira-Lucas et al. (2017)	Canada	36/35	M/F	Multi-ethnic	< 40	Vitamin D 25(OH)D ≤ 65 nmol/L and HbA1c of 5.4% to 6.4%	T: 48.1±14.3 P: 47.6±14.3	Mean change (95%CI) T: 50.6 (36.7, 64.6) P: -2.11 (-6.11, 1.89)	Vitamin D3; oral; 4000	LC-MS	6	Matsuda index, AUC Glucose
Cefalo et al. (2018)	Italia	44083	M/F	NR	> 25	BMI≥30 kg/m2; Vitamin 25(OH)D <75 nmol/L	T: 36.7±13.2 P: 34.7±21.1	T: 74.8±18.7 P: 41.7±7.7	Vitamin D3; oral; 3571	NR	3	Insulin mediated glucose uptake, AUC Glucose (from OGTT)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; AUC, Area Under Curve; BMI, body mass index; CLIA, chemiluminescence immunoassay; ECLIA, electro-chemiluminescence immunoassay; FPG, Fasting Plasma Glucose; GIR, Glucose Infusion Rate; HbA1c, Glycated haemoglobin; IFG, Impaired Fasting Glucose; IGT, Impaired Glucose Tolerance; LC-MS, Liquid Chromatography-Mass Spectrophotometry; M, Male; F, Female; NR, Not Reported; RIA, Radioimmunoassay; Tx/Px, Treatment/Placebo group; T, Treatment; P, Placebo. §mean±SE

CHAPTER 6

The effect of vitamin D supplementation on insulin sensitivity

There were 18 studies with sufficient data to be included in the meta-analysis to estimate the overall effect of vitamin D supplementation on insulin sensitivity derived from a clamp (39, 42, 46, 47, 49) or indices of insulin sensitivity derived from an oral or intravenous glucose tolerance test (33-38, 40, 41, 43-45, 48, 50). The total number of included individuals was 1220; of these, 612 received vitamin D supplementation, and 608 patients received placebo. From the 13 studies that used the Matsuda index derived from OGTT and/or insulin sensitivity index from IVGTT (33-38, 40, 41, 43-45, 48, 50), none reported a significant change (increase or decrease) in insulin sensitivity after vitamin D supplementation compared with placebo. Furthermore, among those five studies in which the outcome of insulin sensitivity was derived from a clamp (39, 42, 46, 47, 49), only one study (Cefalo et al (49)) reported a statistically significant improvement of insulin sensitivity.

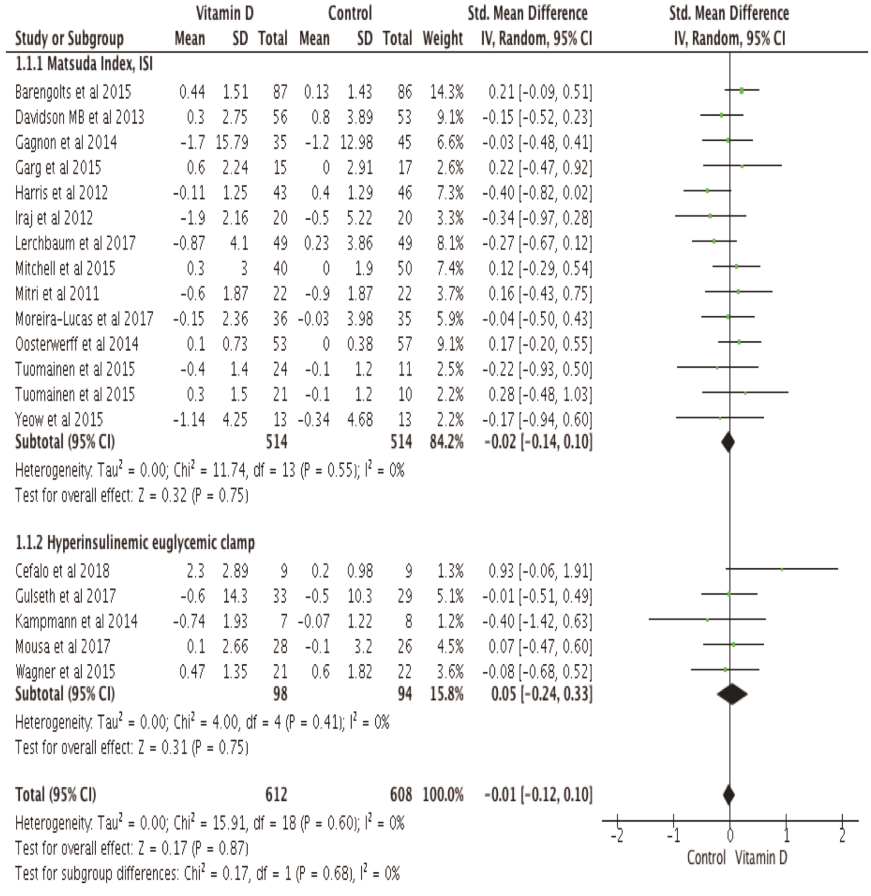
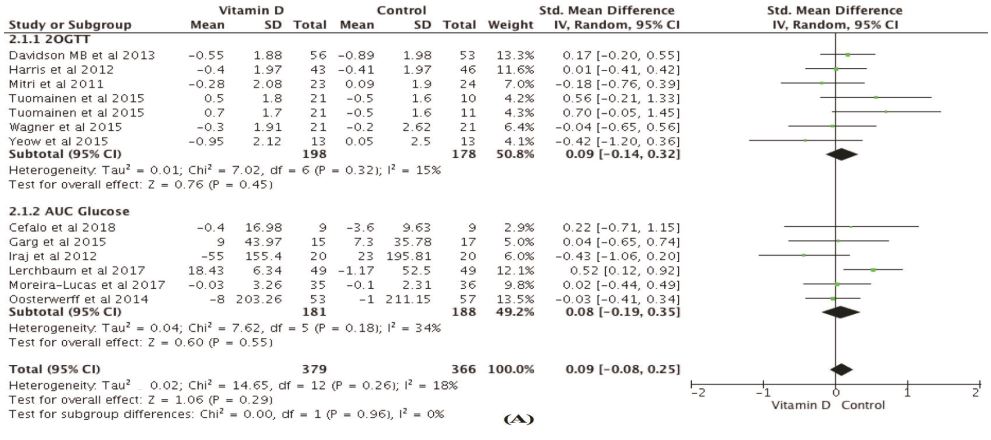


Figure 2. Forest plot of the effect of vitamin D supplementation on insulin sensitivity. Horizontal lines span individual study 95% confidence intervals (CI). Diamonds represent the combined study standardized mean value and the corresponding 95% CI values.

Based on a random-effect meta-analysis, comparing the standardized mean difference of the change in insulin sensitivity from baseline between the vitamin D–supplemented and placebo groups, the overall effect on insulin sensitivity was not significant after vitamin D supplementation (standardized mean difference: -0.01, 95% CI: -0.12 to 0.10, P= 0.87; I^2= 0%, P=0.60) (**Figure 2**).

The effect of vitamin D supplementation on postprandial glucose concentrations

There were 12 studies that reported 2h-glucose concentrations (6 studies) (33, 34, 36, 41-43) or AUC Glucose (6 studies) (35, 37, 45, 48-50) as an outcome measure. A meta-analysis of these data including 745 individuals (n = 379 treated with vitamin D and n = 366 with placebo) was performed to compare the mean change in 2h-glucose and/or AUC Glucose between the beginning and the end of study. Vitamin D supplementation had no effect on post-prandial glucose with a standardized mean difference of 0.09 (95% CI: -0.08 to 0.25, P = 0.29; I^2 = 18%, P=0.26) (**Figure 3A)**.

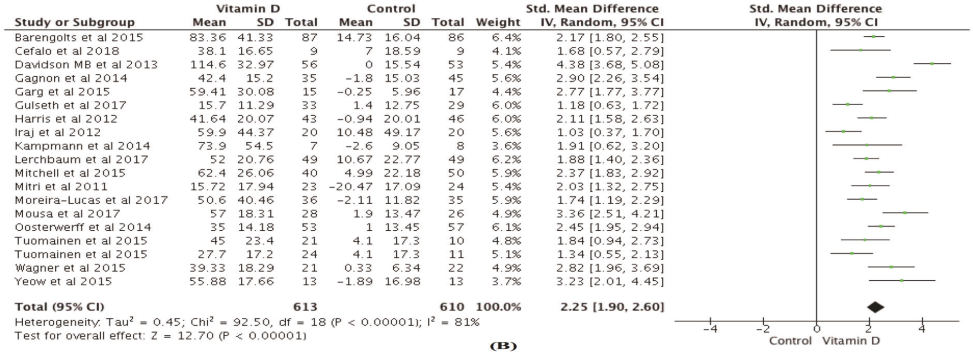


(3A)

Figure 3A. Forest plots of the effect of vitamin D supplementation on postprandial glucose, Horizontal lines span individual study 95% confidence intervals (CI). Diamonds represent the combined study standardized mean value and the corresponding 95% CI values.

The effect of vitamin D supplementation on serum vitamin D levels

All eighteen trials (n = 1223; 613 individuals treated with vitamin D and 610 individuals with placebo) reported the change in serum vitamin D level after the intervention. There was a significant increase in the serum vitamin D level in the vitamin D supplementation groups compared with the control groups (standardized mean difference = 2.25; 95%CI: 1.90–2.60, p < 0.001) (**Figure 3B**) with considerable heterogeneity (I^2 = 81%; p<0.001).



(3B)

Figure 3B. Forest plots of the effect of vitamin D supplementation on serum vitamin D levels, Horizontal lines span individual study 95% confidence intervals (CI). Diamonds represent the combined study standardized mean value and the corresponding 95% CI values.

Risk of bias

Most included studies had a low risk of bias according to randomization process (random sequence generation and allocation concealment), blinding participants (comparability of intervention groups and placebo (control) groups), incomplete outcome data (clear description of dropout/withdrawal/attrition rate). The blinding of outcome assessment was not clearly mentioned in some of studies (34-36, 41, 44, 45, 48, 49), resulted in unclear risk of detection bias. The overall quality assessment of the included trials in this meta-analysis is shown in **Supplemental figures S1A-B**.

Publication bias

The visual inspection of funnel plots of changes in insulin sensitivity, postprandial glucose and serum vitamin D changes in this meta-analysis following vitamin D supplementation did not indicate publication bias **(Supplemental figures S2A-C)**.

Meta regression and sensitivity analysis

Regarding the change in serum vitamin D levels following supplementation, there was a considerable heterogeneity (I^2=81%). A meta-regression analysis of dose or duration with standardized mean difference of serum vitamin D levels showed that the dose of vitamin D supplementation may partly explain the heterogeneity (**Supplemental Table S3**). Further sensitivity analysis by leave-one-out analysis, shows that no single study is responsible for the heterogeneity of changes in serum vitamin D levels.

Discussion

We conducted a systematic review and meta-analysis of 18 RCTs to determine the effect of vitamin D supplementation on insulin sensitivity in individuals with or at risk of

insulin resistance. In this meta-analysis, vitamin D supplementation had no significant effect on insulin sensitivity derived from a hyperinsulinemic euglycemic clamp or the Matsuda Index derived from an OGTT and/or Insulin sensitivity index from IVGTT. Additionally, no significant changes in 2h glucose or AUC glucose during an OGTT were observed following vitamin D supplementation.

In our analysis, study populations were often characterized by the presence of overweight or obesity (33-39, 41-44, 46-49), accompanied with baseline serum concentrations of 25(OH)D <50 nmol/L (33-44, 46-50) or <75 mmol (35, 36, 41, 42, 44, 49, 50). Indeed, it has been described that human obesity often coincides with low circulating vitamin D 25(OH) D levels (5). Several mechanisms, such as uptake/sequestration of vitamin D within adipose tissue (51) and volumetric dilution due to increased body volume (52) may explain the link between obesity and low vitamin D levels. Furthermore, a comprehensive systematic review by Autier and colleagues suggests that the low serum vitamin D levels could also be due to the chronic low grade inflammation which is an important characteristic of individuals with obesity and T2D (53). Thus, vitamin D supplementation in human overweight/obesity could be expected to increase serum vitamin D 25(OH)D levels. In this meta-analysis we showed that vitamin D supplementation increased the mean vitamin D 25(OH)D level of treatment group in all studies, although with considerable heterogeneity. Additional meta-regression analysis showed that some of the heterogeneity is explained by dose, but not by treatment duration. Nevertheless, in our meta-analysis, improving vitamin D 25(OH)D concentrations did not translate into an improvement in insulin sensitivity and glucose metabolism. In the present meta-analysis, we included only studies using standardized methodologies (non fasting measures of insulin sensitivity), either by multi-sampled OGTTs or IVGTTs (33-38, 40, 41, 43-45, 48, 50) or the gold standard hyperinsulinemic euglycemic clamp (39, 42, 46, 47, 49). Our meta-analysis, which shows no effect of vitamin D supplementation on peripheral insulin sensitivity and postprandial glucose handling, supports several other findings which used fasting indices of insulin sensitivity (e.g. HOMA-IR) (18, 19).

It has been shown that postprandial glycaemia is strongly associated with obesity (3), and predicts cardiovascular events among insulin resistant individuals (4, 54). Wood et al. (55) and Manson et al. (56). have shown that vitamin D supplementation did not result in the prevention of cardiovascular events. The most recent work by Pittas et al. (57) showed that vitamin D supplementation (4000 IU) did not result in a significantly lower risk of diabetes, assessed by 2h OGTT in 2423 individuals. The skeletal muscle is a key organ for peripheral insulin sensitivity as it is responsible for the majority of glucose disposal during postprandial conditions (24, 58). Although effects of vitamin D on glucose responsiveness, insulin receptor substrate (59) and insulin sensitivity (17), muscle mitochondria biogenesis (16) as well as lipid metabolism (17) have been documented at the transcriptional and post-translational level *in vitro*, our analysis shows no effect on postprandial glucose concentrations suggests that these beneficial effects do not translate into beneficial effects at the functional level. Studies have shown that vitamin D may affect insulin secretion (15, 33) rather than insulin sensitivity per se. Vitamin D may contribute to normalization of extracellular calcium, ensuring normal calcium flux through cell membranes and regulate the beta-cell calcium pool (15). Nevertheless, whether there is sufficient evidence that vitamin D supplementation may improve insulin secretion in postprandial condition (e.g. disposition index) needs further investigation. Furthermore, there is only one trial included in this meta analysis, by Cefalo et al. (49), which reported a significant increase of insulin

sensitivity derived from a clamp after vitamin D supplementation. Of note, in that study vitamin D supplementation was combined with a low-calorie diet intervention, yet the sample size of this study was relatively small. Therefore, whether combining vitamin D supplementation with diet restriction may improve insulin sensitivity in humans with or at risk of insulin resistance needs further investigation in a well-controlled study.

When interpreting the results, several factors should be taken into consideration. The optimal time of intervention and dose that are necessary to evaluate the effects of vitamin D supplementation on parameters related to glucose metabolism and insulin insulin sensitivity is not well established. We showed that neither dose nor treatment duration were associated with changes in insulin sensitivity and postprandial glucose. It also should be noted that the results could be affected by other factors such variation in age, season (19) and ethnicity (23) as well as tissue-specific metabolism and insulin resistance (60). A recent meta-analysis in type 2 diabetes populations by Li et al. (23) has suggested that vitamin D supplementation may have more beneficial effects in individuals from Middle eastern ethnicity as compared to other ethnicities, but some of the included studies on which this suggestion was based were of questionable quality. In this meta-analysis, we could not perform subgroup analysis based on ethnicity because 8 out of 18 trials (33, 36-38, 40, 46-48) were conducted in multi-ethnic populations, whereas 5 other studies (39, 41, 42, 49, 50) did not report any information about ethnicity. In addition, the heterogeneity regarding the change in serum vitamin D levels may also be partly explained by compliance that was achieved during intervention, which we were unable to ascertain in more detail. Another limitation might be that the number of individuals who participated in the included studies was relatively small possibly due to the invasive and expensive character of the measurement (e.g. clamp). Despite the fact that the number of studies using clamps, the gold standard for the measurement of insulin sensitivity, was relatively small (only 5 studies), the outcome on insulin sensitivity derived from clamps was similar to the outcome based on the results from OGTTs and IVGTTs (13 studies). In addition, 3 studies that met our inclusion criteria and used a clamp (61-63) were published in 2019, after the window for being included in the meta analysis. After repeating the meta-analysis with in total 21 studies (3 studies in 2019 and the original 18 studies), the outcome did not change (**Supplemental figures S3A-C**). In conclusion, this systematic review provides no evidence that supplementation with vitamin D has a beneficial effect on peripheral insulin sensitivity, as determined by hyperinsulinemic euglycemic clamp, the Matsuda or insulin sensitivity index, and postprandial glucose concentrations after an OGTT in people with, or at risk for insulin resistance.

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Author disclosure

All authors declare that they have no conflict of interest.

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Supplemental Tables

Supplemental Table S1. The search terms for each database

No	Database	Search terms
1	PubMed	("overweight"[MeSH Terms] OR "overweight"[All Fields]) OR ("obesity"[MeSH Terms] OR "obesity"[All Fields]) OR Obes[All Fields] OR "prediabetic state"[MeSH Terms] OR ("prediabetic"[All Fields] AND "state"[All Fields]) OR "prediabetic state"[All Fields] OR "prediabetes"[All Fields] OR ("diabetes mellitus, type 2"[MeSH Terms] OR "type 2 diabetes mellitus"[All Fields] OR "diabetes mellitus, type 2"[All Fields]) OR ("insulin resistance"[MeSH Terms] OR ("insulin"[All Fields] AND "resistance"[All Fields]) OR "insulin resistance"[All Fields]) OR ("insulin resistance"[MeSH Terms] OR ("insulin"[All Fields] AND "resistance"[All Fields]) OR "insulin resistance"[All Fields] OR ("insulin"[All Fields] AND "sensitivity"[All Fields]) OR "insulin sensitivity"[All Fields]) OR ("blood glucose"[MeSH Terms] OR ("plasma blood"[All Fields] AND "glucose"[All Fields]) OR "blood glucose"[All Fields] OR ("glucose"[All Fields] AND "blood"[All Fields]) OR "glucose, blood"[All Fields]) AND ("vitamin d"[MeSH Terms] OR "vitamin d"[All Fields] OR "cholecalciferol"[All Fields]) AND (Randomized Controlled Trial[ptyp] AND "humans"[MeSH Terms])
2	Cochrane	(overweight OR obesity OR obese OR prediabetes OR type 2 diabetes mellitus) AND (vitamin D OR vitamin D3 OR vitamin D2) AND (insulin sensitivity OR insulin resistance) AND (glucose control OR blood glucose)
3	Embase	(blood glucose, insulin resistance, insulin sensitivity, non insulin dependent diabetes mellitus, obesity, prediabetes, supplementation, vitamin d, cholecalciferol) using a combination of multi-field search in all fields and EMTREE
4	Web of Science	(overweight OR obesity OR prediabetes OR type 2 diabetes mellitus) AND (vitamin D OR vitamin D3 OR vitamin D2) AND (insulin sensitivity OR insulin resistance) AND (plasma glucose OR OGTT)
5	CINAHL	(overweight OR obesity OR obese OR prediabetes OR type 2 diabetes mellitus) AND (vitamin D OR 25 hydroxyvitamin D OR vitamin D3 OR vitamin D2) AND (insulin sensitivity OR insulin resistance) AND (blood glucose OR glucose control)

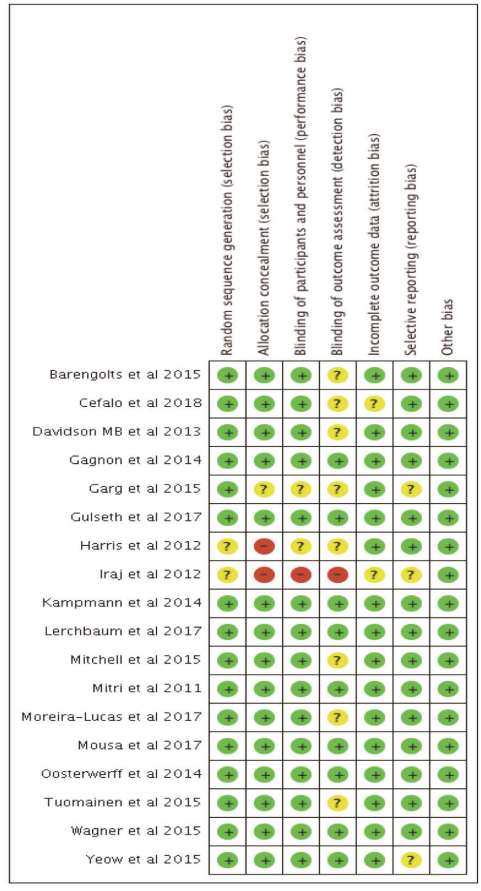
Supplemental Table S2. The PRISMA (2009) Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	4
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5,6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	7
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7,8
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	7,8
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	8
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	8,9
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	9
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	9
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	9
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	9 (and Figure 1)
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	9 to 11
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	12
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	10,11
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	10,11
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	12
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	12
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12 to 14
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	15
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	16

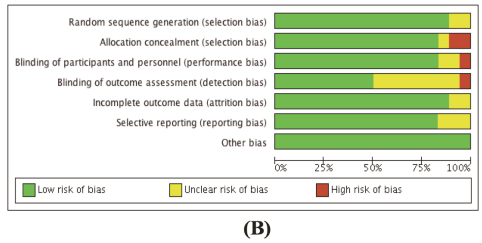
From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097. For more information, visit: **www.prisma-statement.org**

Supplemental Table S3. Results of meta-regression analyses with dose, duration and change in vitamin D level as independent variables and vitamin D level, insulin sensitivity, and postprandial glucose response as dependent variables.

	Standardized Mean Difference of serum vitamin 25OHD				Standardized Mean Difference of Insulin Sensitivity				Standardized Mean Difference of Postprandial Glucose (12 studies)			
	B (SE)	95% CI	P value	R^2	B (SE)	95% CI	P value	R^2	B (SE)	95% CI	P value	R^2
Model 1												
Dose (IU/day)												
< 4000 (Reference)	-	-	-		-	-	-		-	-	-	-
≥ 4000	0.690 (0.374)	-0.099 – 1.480	0,083	0,124	–0.070 (0.126)	-0.336 – 0.195	0,585	-0,171	–0.281 (0.168)	–0.652 – 0.090	0,124	0,096
Model 2												
Duration (months)												
≤ 4 months	-	-	-	-	-	-	-	-	-	-	-	-
> 4 months	-0.192 (0.404)	-1.045 – 0.660	0,641	0,005	0.133 (0.117)	-0.114 – 0.380	0,272	0,9	0.095 (0.199)	–0.344 – 0.535	0,642	-0,582
Model 3												
Dose (IU/day)	0.679 (0.387)	-0.142 – 1.489	0,099	0,065	–0.059 (0.120)	-0.316 – 0.197	0,629	0,193	–0.307 (0.178)	–0.704 – 0.888	0,114	-0,429
Duration (months)	–0.102 (0.389)	-0.927 – 0.722	0,795		0.129 (0.119)	-0.125 – 0.382	0,298		0.143 (0.196)	–0.293 – 0.581	0,481	
Model 4												
Change serum vitamin D levels												
< 50 nmol/L	-	-	-	-	-	-	-	-	-	-	-	-
≥ 50 nmol/L					0.113 (0.115)	-0.130 – 0.357	0,341	0,9	-0.094 (0.193)	-0.519 – 0.331	0,636	-0,686



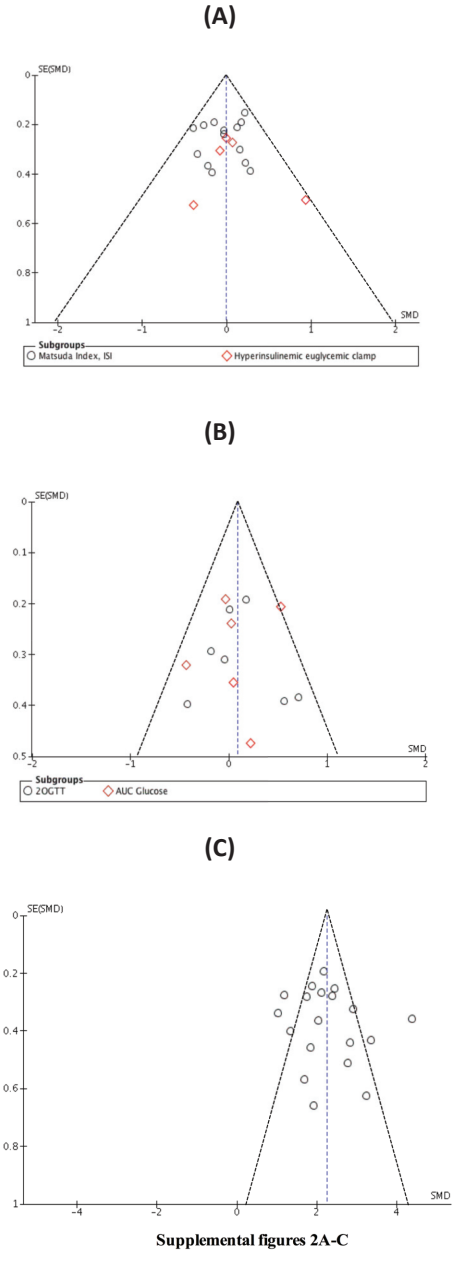
(A)



(B)

Supplemental figures 1A-B

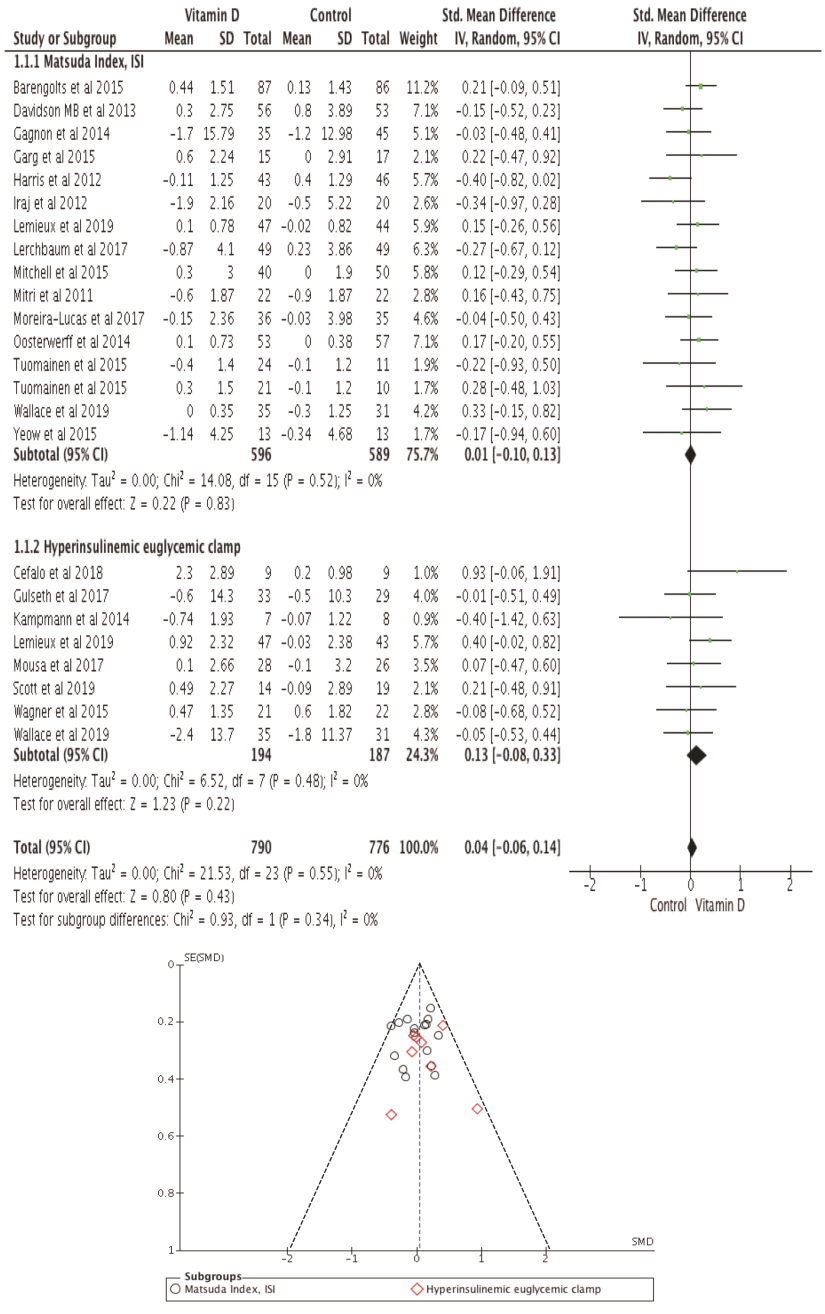
Supplemental figures S1A-B. Risk of bias analysis **(A)** The analysis of the individual studies included in the systematic review and meta-analysis. Green dot = low risk of bias; yellow dot = unclear risk of bias; red dot = high risk of bias **(B)** The summary of the risk of bias analysis.



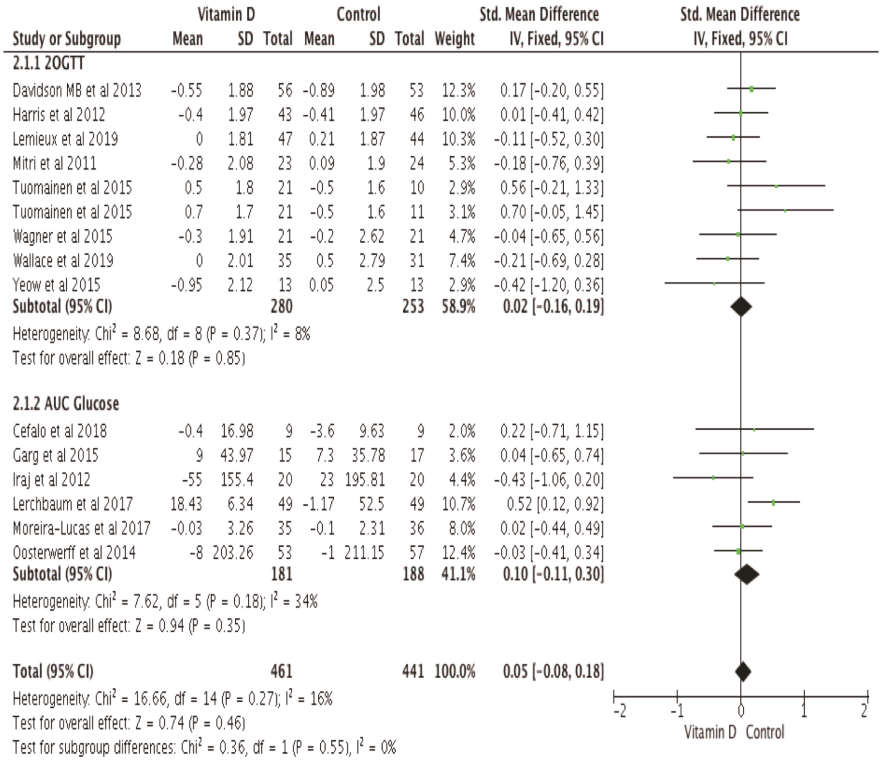
Supplemental figures 2A-C

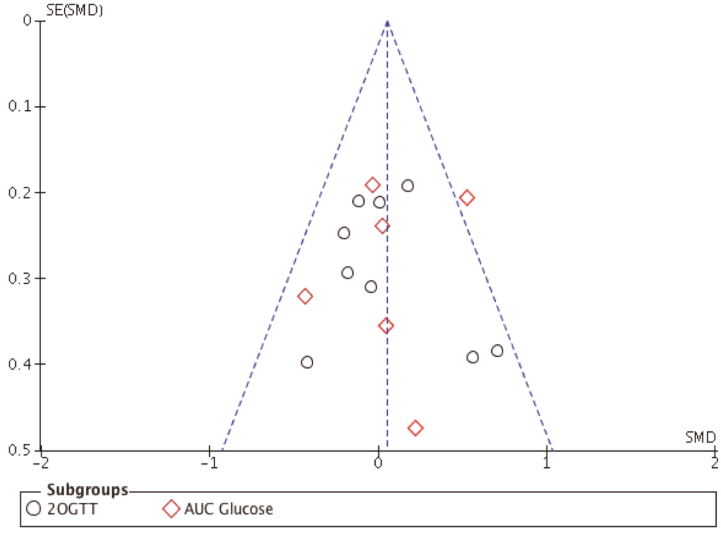
Supplemental figures S2A-C. Funnel plot of RCTs the effect of vitamin D supplementation on (A) insulin sensitivity; (B) postprandial glucose; (C) serum vitamin D levels. Symmetrical funnel plots suggest publication bias is unlikely. RCTs, randomized controlled trials; SE(SMD) Standard Error of Standardized Mean Difference.

Supplemental figures S3A-C. Forest plots and funnel plots after repeating the meta-analysis with in total 21 studies (additional 3 studies in 2019 and the original 18 studies) of the effect of vitamin D on: (S3A) insulin sensitivity; (S3B) postprandial glucose; (S3C) serum vitamin D levels.

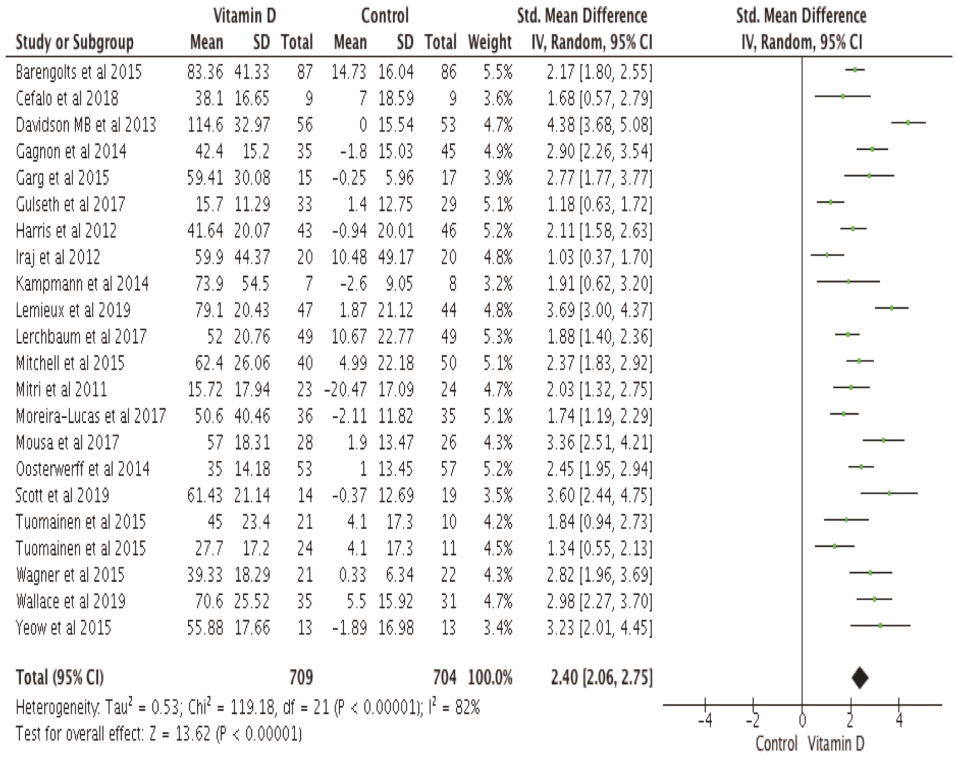


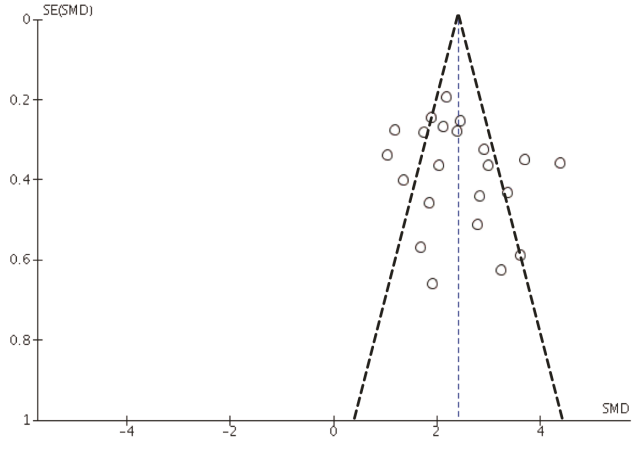
Supplemental figure S3A





Supplemental figure S3B





Supplemental figure S3C

CHAPTER 7

General Discussion

General Discussion

The increasing prevalence of obesity substantially leads to an increased risk for the development of insulin resistance, type 2 diabetes mellitus, cardiovascular diseases (hypertension and stroke), and certain types of cancer. In addition, obesity and obesity-associated health complications might contribute to a reduced quality of life (1). Obesity is often characterized by low vitamin D 25(OH)D_3 concentrations (also known as vitamin D deficiency *or hypovitaminosis D*) as indicated by a recent population-based study reporting 42.5% vitamin D deficiency among individuals with BMI $\geq$ 30 kg/m^2 (2). The underlying mechanism linking vitamin D 25(OH)D_3 and obesity is still unclear (3), whereas reports on the link between vitamin 1,25$(OH)_2D_3$ concentrations and obesity are inconsistent (4, 5). Additionally, in the past decades, insulin resistance and type 2 diabetes mellitus (T2D) have been linked with vitamin D deficiency (6). The US National Health Survey (NHNES) 2001-2006 showed that obese individuals with vitamin D deficiency had higher risk for the development and maintenance of insulin resistance as compared to obese individuals with sufficient vitamin D levels (7).

Therefore, in the present thesis we investigated whether and how vitamin D is related to insulin sensitivity in human obesity. At first, we investigated whether uptake and release of vitamin D by abdominal SAT is impaired in obese individuals and may thereby possibly contribute to the reduced circulating vitamin D levels *in vivo* in human obesity **(chapter 3)**. Next, we aimed to gather (mechanistic) insight into the association between circulating vitamin D metabolites [25(OH)D_3 and 1.25$(OH)_2D_3$] and gene-expression of vitamin D-related metabolism within SAT and tissue-specific insulin sensitivity in overweight/obese humans **(chapter 4)**. Furthermore, we examined whether genetic variation in the vitamin D receptor is related to tissue-specific insulin sensitivity in overweight/obesity individuals in the large *Pan-European* dietary intervention trial (*DiOGenes*) **(chapter 5)**. Finally, we conducted a meta-analysis of human randomized controlled-trials to investigate whether there is sufficient evidence that vitamin D may affect insulin sensitivity derived from the gold standard hyperinsulinemic euglycemic clamp or from the Matsuda or insulin sensitivity indices derived from OGTT or IVGTT respectively **(chapter 6)**.

Association of circulating vitamin D, obesity and insulin resistance

The prevalence of low circulating vitamin D 25(OH)D_3 levels (<50 nmol/L) has been reported to be about 40.7% in individuals with obesity and insulin resistance (2). In line with these data, about 45.7% of obese-insulin resistant individuals in our study (**chapter 4)** were classified as vitamin D deficient. The above data suggest that vitamin D 25(OH)D_3 deficiency is a commonly observed characteristic in obese insulin resistant individuals. However, it is unclear whether vitamin D deficiency is associated with obesity (BMI) or insulin resistance per se.

In chapter 4, we conducted a cross sectional analysis in overweight and obese individuals with either normal or impaired glucose metabolism to investigate whether obesity (BMI) is related to plasma vitamin D metabolites [25(OH)D_3 and 1.25$(OH)_2D_3$ or the ratio between both in overweight/obese individuals. We demonstrated that BMI was negatively associated with circulating vitamin D 25(OH)D_3 but not with the active metabolite of vitamin D [1.25$(OH)_2D_3$]. The association between BMI and vitamin D 25(OH)D_3 remained

significant after adjustment for age and sex. This finding is consistent with other findings, indicating an inverse correlation between plasma 25(OH)D_3 concentration and BMI across age, different ethnicities and in a range of geographic locations (8-11).

In contrast to our findings, a previous study in individuals with a wide range of BMI (mean±SD= 32.0 ± 6.8; estimated range from 18.4 to 45.6 kg/m^2) (12) and a recent study in middle-aged sedentary adults demonstrated a negative correlation between BMI and active metabolite of vitamin D [1.25$(OH)_2D_3$] independent of age and sex (5). These discrepancies could possibly be explained by the different BMI ranges of these studies (i.e., our cohort mean±SD= 30.6±2.9; range 25.5 – 38.6 kg/m^2 vs. The FIT-AGEING study (5) mean±SD= 26.7±3.8; estimated range 19.1 – 34.3 kg/m^2).

In chapter 4, we extended our cross sectional analysis, to determine whether vitamin D metabolites relate to tissue-specific insulin sensitivity and whether these relationships are independent of BMI, age, and sex. We observed that none of vitamin D metabolites [25(OH)D_3 nor 1,25(OH)D_3 nor its ratio] were associated with hepatic, muscle nor adipose tissue insulin sensitivity derived from the gold standard two-steps hyperinsulinemic euglycemic clamp technique. Further correction for BMI did not change the outcome. In line, Ter Horst et al (4), also observed no association between vitamin D 25(OH)D_3 and insulin sensitivity determined by a gold standard two-steps hyperinsulinemic euglycemic clamp in human obesity.

As mentioned above there is a large amount of observational studies that investigated the link between vitamin D deficiency and insulin resistance, but the outcomes are inconsistent **(chapter 2)**. Analyses of the National Health and Nutrition Examination Survey 1989–1994 (NHANES III) disclosed that serum 25(OH)D_3 was inversely associated with diabetes risk and measures of insulin resistance in general population (13). Further cross-sectional analysis from population-based study (NHANES 2001-2006), has shown that the interaction between low serum 25(OH)D_3 and high BMI may explain 47% of insulin resistance cases as assessed by HOMA-IR (7). Significant associations have been reported between circulating 25(OH)D_3 and hyperglycemic clamp-induced insulin response in normal glucose tolerant (obese/overweight) individuals of various ethnic backgrounds (14). In contrast, a study conducted in a European populations with metabolic syndrome, showed that serum vitamin D 25(OH)D_3 levels were not associated with insulin action and glucose metabolism derived by intravenous glucose tolerance test (IVGTT) after correction for BMI (15). In addition, other recent studies have shown no association between vitamin D 25(OH)D_3 and HOMA-IR and Matsuda index after adjustment for BMI (15-18).

Of note, vitamin D 25(OH)D_3 still needs to be hydroxylated to produce its hormonally active metabolite 1,25$(OH)_2D_3$ (19). Mechanistically, vitamin D 1,25$(OH)_2D_3$ may directly affect insulin signaling (20) and/or glucose stimulated pancreatic insulin secretion (21). However, as mentioned above, We observed no association between 1,25(OH)D_3 markers of tissue insulin sensitivity in chapter 4. In contrast, a recent observational study suggested 1,25$(OH)_2D_3$ is independent of age, sex, and BMI for features of metabolic syndrome (MetS) (22). However, data on the association of 1,25$(OH)_2D_3$ and metabolic markers is limited, possibly due to technical issues and vitamin D 1,25$(OH)_2D_3$ stability over longer period of time (23, 24).

Taken together, the majority data suggests that vitamin D 25(OH)D_3 deficiency is merely associated with BMI but not with indices of insulin resistance. Notably, studies using hyperinsulinemic euglycemic clamps, IVGTT as well as the Matsuda index do not report

an association between vitamin D 25(OH)D_3 deficiency and insulin resistance in line with our data in chapter 4. Nevertheless, whether vitamin D deficiency is associated with insulin sensitivity in subgroups based on ethnicity, sex and metabolic health status needs to be studied further. Data on the association between1,25(OH)$_2D_3$, BMI and insulin resistance is still inconsistent and warrant further investigation.

Adipose tissue mass and metabolism in relation to vitamin D deficiency (inactive and active metabolite)

As reviewed in the **chapter 2**, an altered vitamin D metabolism within adipose tissue (25) and an increased sequestration of vitamin D in adipose tissue (26) may partly explained vitamin D deficiency in obesity. Furthermore, the dilution of vitamin D 25(OH)D_3 due to a higher volume of distribution in obese individuals has been proposed as underlying mechanism for low serum 25(OH)D_3 concentrations in obesity (27).

In **chapter 2,** we described that there is evidence showing that vitamin D-related metabolism is altered in obese SAT (25). Wamberg et al. observed a decreased RNA expression of 25-hydroxylase enzymes in SAT of obese compared with lean women (25). Interestingly, a recent study using the murine 3T3-L1 adipocyte model demonstrated that adipocytes are capable of converting vitamin D_3 precursor into vitamin D 25(OH)D_3, but this conversion occurs to a lesser extent in insulin resistant adipocytes due to a reduced expression of the 25-hydroxylation enzyme (28). Together, these data may suggest that large quantities of vitamin D_3 are stored as the native compound, presumably in body fat, and are only slowly released as vitamin D 25(OH)D_3 due to less efficient 25-hydroxylation in SAT in the obese insulin resistant state (29).

However, in **chapter 4,** further analysis revealed that the association between BMI and plasma vitamin D 25(OH)D_3 levels in overweight and obese individuals did not change after adjustment for the SAT gene expression of the 25-hydroxylases CYP2J2, CYP27A1; and the 1-alpha-hydroxylase enzyme CYP27B1. Our study also did not find any associations between vitamin D 1.25(OH)$_2D_3$ levels and the expression of 25- or 1-alpha-hydroxylase enzymes within SAT. Thus, our results may suggest that BMI and adiposity and not an altered adipose tissue metabolism is the main determinant for circulating vitamin D 25(OH)D_3 and D 1.25(OH)$_2D_3$ levels in overweight/obese individuals. However, we cannot exclude that other hydroxylase enzymes might be involved in vitamins D metabolism in human SAT, which needs to be investigated in more detail in future research.

Of note, **in chapter 4** we only investigated expression of vitamin D enzymes in the abdominal SAT. A study in obese women showed that the expression of the 25-hydroxylation enzymes (CYP27A1, CYP27B1) was higher in VAT compared to SAT and positively correlated with circulating vitamin D 25(OH)D_3 concentration (25). Therefore, whether vitamin D 25(OH)D_3 hydroxylation may be regulated differently depending on AT depot as well as the relative contribution of AT vitamin D precursor (stored as native compound) to circulating 25(OH)D_3 concentration in obese-insulin resistant state needs further investigation.

It was two decades ago, that Wortsman et al documented that vitamin D_3 (a precursor for inactive vitamin D metabolite/25(OH)D_3) is sequestered in adipose tissue of obese individuals (26). Furthermore, not only the precursor vitamin D_3 (30) but also the metabolite vitamin D 25(OH)D_3 has been shown to accumulate in abdominal SAT (31). Of

interest, an impaired release of vitamin D_3 (pre-vitamin D) and vitamin D 25(OH)D_3 has been documented from subcutaneous adipocytes of obese donor compared lean *ex vivo* (28). This impairment of vitamin D release was co-existing with an impaired lipolytic response following beta-adrenergic stimulation in adipocytes from obese donors (28).

To investigate the contribution of vitamin D metabolites from abdominal adipose tissue, we measured vitamin D metabolites using arterio-venous blood sampling across abdominal SAT in combination with AT blood flow measurements in chapter 3. We did not observe significant *in vivo* vitamin D 25(OH)D_3 release across SAT of lean or obese men following an overnight fast and during acute ß-adrenergic stimulation. However, we observed a blunted release of active vitamin D metabolite 1.25$(OH)_2D_3$ following ß-adrenergic stimulation in obese but not in lean men. Of interest, 1,25$(OH)_2D_3$ is a ligand for vitamin D receptor (VDR), and it has been shown that VDR expression is increased in abdominal SAT of individuals with obesity (32). In line, our data (**chapter 4)** showed a positive association between BMI and VDR expression in abdominal SAT. Taken together, it could be speculated that vitamin D 1,25$(OH)_2D_3$ binds to a higher extent to VDR within SAT in individuals with obesity, resulting in less spillover/release of vitamin D 1,25$(OH)_2D_3$ in the circulation in obese but not in lean individuals, which still needs further investigation.

The exact association between the impaired ß-adrenergic stimulated lipolysis and vitamin D release and to what extent they are interconnected remains to be largely determined. Among the physiological factors stimulating release of free fatty acids (FFAs) from adipose tissue, exercise contributes to the greatest proportion (33). During exercise, lipolytic hormones (such as catecholamines and atrial natriuretic peptide (ANP)) (34, 35) and ATBF are increased (36). Several cross-sectional studies report a positive correlation between serum 25(OH)D_3 concentrations and higher physical activity independent of BMI (37, 38). Interestingly, a recent RCT (39) has observed an elevated serum 25(OH)D_3 concentrations in lean individuals in response to 30 minutes cycling exercise. This increased circulating 25(OH) D_3 was observed immediately post-exercise and even persisted for 24 hours. Therefore, whether exercise has an effect on 25(OH)D_3 and 1.25$(OH)_2D_3$ concentrations – potentially via improved intracellular metabolism (e.g. hydroxylation) and mobilization of adipose tissue-derived vitamin D warrants further investigation.

Another type of intervention that induces lipolysis is weight loss induced via caloric restriction in lifestyle interventions (40). Recently, a 1-year lifestyle intervention in obese individuals resulted in a 26% increase in circulating 25(OH)D_3 along with a 26% decrease in VAT and 18% decrease in SAT volume (37). This increased 25(OH)D level correlated inversely with changes in both SAT and VAT (37). Furthermore, an increased expression of the catabolizing enzyme CYP24A1 was observed in SAT of obese individuals following weight loss (25). As CYP24A1 degrades both 25(OH)D_3 and 1.25$(OH)_2D_3$ (41), this may suggest a higher turnover of both 25OHD_3 and 1.25$(OH)_2D_3$ following weight loss. Further research is needed to understand vitamin D dynamics (uptake and release) across different adipose tissue depots and under physiological challenges like weight loss and exercise.

Vitamin D and insulin sensitivity

Data from literatures regarding the effect of vitamin D supplementation on insulin sensitivity and glucose homeostasis are still inconsistent as reviewed in **chapter 2.** The majority of these meta-analyses have used a surrogate marker of insulin sensitivity such us

HOMA-IR. In **chapter 6,** we conducted a systematic review and meta-analysis to determine the effect of vitamin D supplementation on insulin sensitivity in individuals with or at risk of insulin resistance.

As reviewed in **chapter 2**, mainly *in vitro* and animal data suggest a potential role of vitamin D in insulin-sensitive organs. In adipose tissue, vitamin D may affect lipid metabolism (42) and may reduce inflammation (43). Vitamin D may affect pancreatic insulin secretion via an immunometabolic protection of beta-cells (44) as well as calcium-dependent mechanisms (45). In addition, it has been demonstrated that vitamin D affects skeletal muscle substrate and energy metabolism, insulin sensitivity and lipid composition (46, 47). More importantly, skeletal muscle affects 70-90% of postprandial glucose disposal and is therefore a key organ in in the regulation of glucose homeostasis and whole body insulin sensitivity.

Thus, increasing circulating vitamin D concentration by means of supplementation might be expected to have beneficial effects during fasting and postprandial conditions by improving skeletal muscle glucose handling/insulin sensitivity. However, in **chapter 6,** despite the fact that in all supplementation studies increased concentrations of serum vitamin D 25(OH)D were observed, our meta-analysis indicated no evidence that supplementation with vitamin D has a beneficial effect on peripheral insulin sensitivity, as determined by hyperinsulinemic euglycemic clamp, the Matsuda or insulin sensitivity index nor on postprandial glucose levels. Furthermore, when we also take into account the 3 papers that were published since we complete our meta–analysis in 2019 (48-50) (that met our inclusion criteria and used a clamp), the results do not change (**chapter 6**). Thus, although mechanistic evidence of vitamin D on glucose responsiveness, insulin receptor substrate (20) and insulin sensitivity (47), muscle mitochondria biogenesis (46) as well as lipid metabolism (47) have been documented at the transcriptional and post-translational level *in vitro*, our analysis indicate that these do not translate into beneficial functional *in vivo* effect on peripheral insulin sensitivity and postprandial glucose concentrations.

It might be argued that the participants from included studies (**chapter 6**) were not deficient enough to observe any major effects of vitamin D supplementation. In **chapter 6**, the mean serum vitamin D 25(OH)D concentrations at baseline varied from 19.2 nmol/L to 59.9 nmol/L in vitamin D-supplemented groups and 16.9 nmol/L to 61.4 in placebo groups. Currently, there is a major discussion on the cut-off point for vitamin D deficiency. Circulating 25(OH)D_3 concentration < 25/30 nmol/L or 25–50 nmol/L are defined as severe deficient or deficient (51-53), whereas circulating 25(OH)D_3 concentration between 50 to 75 nmol/L and > 75 nmol/L are considered as sufficient (52-55). However, the Endocrine Society defines vitamin D sufficient when circulating 25(OH)D_3 levels > 75 nmol/L (51).

When we re-analyzed 9 out of 18 studies with mean baseline serum vitamin D levels ≤30 nmol/L, the outcome remains unchanged, suggesting no evidence that vitamin D supplementation improves peripheral insulin sensitivity in individuals with a low vitamin D status. Most (but not all) RCTs included in our meta-analysis recruited participants with BMI ≥ 25 kg/m^2 and almost all RCTs administrated vitamin D_3 as supplementation. It has been suggested that large quantities of vitamin D_3 may potentially be stored in body fat, and are only slowly released as vitamin D 25(OH)D_3 (29). In these particular individuals, weight loss has been shown to increase 25(OH)D_3 levels (25, 37), and potentially increases the availability of vitamin D 25(OH)D_3 for muscle tissue. Therefore, combining vitamin D_3 supplementation and weight loss in relation to effects on peripheral insulin sensitivity may

warrant further investigation in more detail.

The VDR gene expression, genetic variants and obesity-insulin resistance

In chapter 2, we reviewed that there is strong evidence that vitamin D affects adipose tissue function via its transcription factor VDR, although the effects may vary between species and cell models used (56, 57). **In chapter 4,** we observed that higher abdominal SAT VDR gene expression was associated with a reduced AT insulin sensitivity independent of BMI, age, sex, and explained 14% of the variance in AT insulin sensitivity. Recent cross sectional data, showed that BMI (32, 58) and HOMA-IR (58, 59) are positively associated with subcutaneous adipose tissue VDR gene expression. This relationship of higher VDR in AT and obesity might be a primary (genetic) defect or more secondary to the obese insulin resistant state and mediated by several factors, including vitamin D status and low-grade inflammation. Vitamin D 25(OH)D_3 deficiency/insufficiency might lead to an inflammatory process (60, 61) which could subsequently promote VDR expression. Of interest, *in vitro* data reported that the inflammatory factor TNF-alpha can increase expression and activity of VDR in keratinocytes (62), indicating that VDR levels in abdominal SAT may be elevated in response to obesity-associated adipose tissue inflammation. However this needs to be investigated in more detail in human adipocytes.

Polymorphisms in the VDR gene, namely, TaqI, BsmI, ApaI, and FokI, have been identified and may be associated with VDR activity and human obesity (63). In **chapter 5,** we investigated whether genetic variants of VDR are associated with obesity phenotypes in the DiOGenes study, a Pan-European multicenter, randomized, controlled dietary intervention study (64). In **chapter 5**, we showed that variants in VDR TaqI and ApaI are associated with elevated BMI, (contributing 0.9 kg/m2 per risk allele), WC (3 cm per risk allele) or FM (2 kg per risk allele) at baseline. In concordance with these results, the VDR TaqI was associated with a higher risk of obesity in Greek individuals with BMI <30 and ≥30 kg/m^2 (contributing 3 kg/m^2 per risk allele) (65). Our results are in line with some (65-67), but not all studies (68, 69) and discrepancies between studies may be partly explained by individuals' characteristics (including differences in the range in BMI, WC, FM, sex, ethnicity).

In **chapter 5**, we also investigated whether VDR genetic variants are associated with tissue-specific IR and abdominal SAT gene expression (VDR gene and genes related AT remodeling, lipid metabolism, and inflammation). VDR polymorphisms were not associated with hepatic nor muscle insulin sensitivity index at baseline. However, the FokI VDR polymorphism was associated with adipose tissue insulin resistance (Adipo-IR) as well as elevated circulating FFA (contributing 79 µmol/L per risk allele) at baseline (chapter 5). In line a recent meta-analysis showed that VDR polymorphisms may be associated with insulin resistance (i.e. HOMA-IR) only in Asian and dark-pigmented Caucasian but not in white Caucasian (70), which may suggest ethnic-differences. Currently, it is unclear whether the association between FokI variants and Adipo-IR or FFA is related to circulating vitamin D concentration or vitamin D status, which warrants further investigation.

Furthermore, our eQTL analysis observed no major effect of VDR SNP variants on abdominal SAT VDR or VDR target gene transcription (**chapter 5)**. This may suggest that VDR genetic variants are not a major determinant of VDR gene expression within abdominal SAT of overweight/obese individuals as observed **in chapter 4**. Finally, in **chapter 5** we also did not find any effects of VDR variants on body weight change, and tissue-specific IR indices

following weight loss and weight maintenance, suggesting that the VDR genetic variants may not relate to dietary intervention outcome. Of interest, the FokI polymorphism is located on the exon in the coding region of the VDR gene, resulting in different translation initiation sites (TIS) and giving rise to a full-length VDR protein or a three amino acid shorter VDR protein variant (71), having higher transcriptional activity (72, 73). Therefore, future studies need to investigate how these VDR variants affect functional VDR activity in human adipose tissue and other insulin sensitive tissues such as gut, liver and muscle.

Interestingly, a recent GWAS study in two independent cohorts has shown that genetic variation in VDR (rs7974353) is associated with overall microbial ß diversity (74). Further analysis indicated that *Parabacteroides* (phylum *Bacteroidetes*) and unclassified *Enterococcaceae* (phylum *Firmicutes*) were significantly correlated with the VDR *gene expression* in the gut enterocytes (74). The fact that VDR is highly expressed in intestinal tissue (mainly in enterocytes) (75), and that gut microbiota do not express VDR may suggest an indirect relationship between vitamin D, VDR expression and gut microbiota composition, which warrants further investigation.

Main outcomes of this thesis and future perspectives

The following gives an overview of the main outcomes of this thesis as well as several perspectives for future research,

1. We showed that BMI was inversely associated with vitamin D 25(OH)D3 concentrations which could not be explained by alterations in abdominal SAT vitamin D-metabolizing enzymes. In the present study, plasma vitamin D metabolites [25(OH)D3 and 1,25(OH)2D3] were not related to hepatic, muscle, and adipose tissue insulin sensitivity. These data suggest that BMI but not insulin resistance is the main determinant of vitamin D 25(OH)D3 concentrations in obesity (chapter 4).
2. A blunted catecholamine-mediated lipolysis was accompanied with a decreased 1,25(OH)2D3 but not vitamin D 25(OH)D3 release across abdominal SAT in obese men, but did not affect circulating 1,25(OH)2D3 concentration in obese men (chapter 3). Additionally, VDR expression in abdominal SAT was positively associated with BMI indicating an increased VDR expression in obesity (chapter 4). Whether a blunted 1,25(OH)2D3 release might be linked with an increased VDR expression in obese SAT still needs further investigation. Of interest, we showed that abdominal SAT VDR expression is negatively associated with AT insulin sensitivity (chapter 4). Whether this is a primary defect or more a secondary phenomenon of the obese insulin resistant state (e.g. low-grade inflammation) needs to be investigated in future research.
3. The VDR polymorphisms are unlikely to play a primary role in tissue-specific insulin resistance (chapter 5). However, VDR polymorphisms were associated with markers of adiposity including BMI, WC and FM. Of interest, a recent study from an Asian population (76) demonstrated that VDR polymorphisms, the fat mass and the obesity-associated FTO gene affected weight-loss following 6-months dietary and exercise intervention, indicating that VDR polymorphisms may interact with other gene polymorphisms in determining

intervention-induced weight loss. Therefore, whether and how VDR genetic variants in combination with other risk alleles affect lifestyle intervention-induced weight loss needs further investigation.

4. Our systematic review and meta-analysis from RCTs (conducted between 1980 - 2019 year) using Matsuda Index, Insulin sensitivity Index, and gold standard hyperinsulinemic euglycemic clamp showed no effect of vitamin D supplementation on insulin sensitivity or postprandial glucose metabolism in individuals with or at risk of insulin resistance (**chapter 6)**.

However, it is possible that vitamin D supplementation may have a beneficial effect on other cardiometabolic risk factor like low-grade inflammation. Recent systematic reviews and meta-analyses (77, 78) reported small, but significant, decreases in high-sensitivity C-reactive protein (hs-CRP) and Tumor Necrosis Factor alpha (TNF-α) concentrations following vitamin D supplementation. It has been shown recently from a Mendelian Randomization study, that hs-CRP is one of 4 inflammatory biomarkers in 3 independent clusters (ILs, adhesion molecules, acute-phase proteins) which was inversely associated with serum 25(OH)D_3 concentration (79). Further studies are warranted to investigate whether vitamin D supplementation may have beneficial effects on obesity-related inflammatory markers or might be beneficial in the prevention and treatment of immune-related disease like cancer and inflammatory bowel disease (IBD) (80, 81). In line, It has been recently suggested that vitamin D may also link to the gut metabolism relating to local gut inflammation and gut microbiota composition (82). A recent RCT suggested that vitamin D plays a role in maintaining the intestinal permeability and reducing systemic CRP concentrations (83). Further, there appears to be a bi-directional interplay between the gut microbiota, vitamin D and intestinal inflammation (84). The available evidence suggests that the gut microbiome is responsive to both vitamin D deficiency and/or supplementation. However, the precise nature of the mechanisms in the context of obesity related metabolic disorders is unclear and further investigations are warranted.

Finally, recent findings suggest that the effect of vitamin D supplementation on metabolic health may be affected by VDR genetic variants (85, 86). Several studies also indicate beneficial effects on metabolic health by combining vitamin D supplementation and other modes of intervention such as lifestyle-induced weight loss (i.e. diet and exercise) (87-89). Therefore, further studies with more personalized (sub-group, i.e. carrier and non-carriers of VDR SNPs) approaches and combining vitamin D supplementation with other modes of intervention might provide new strategies towards a more personalized approach for treatment and prevention of insulin resistance in humans.

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APPENDIX

Summary/Ringkasan, Valorization, Acknowledgements, About the author, PhD portofolio

SUMMARY

Currently, the global prevalence of obesity has doubled over decades across socio-economic and demographic status. At the same time, vitamin D insufficiency and deficiency are major public health issue worldwide, where the prevalence of vitamin D deficiency was estimated high in obesity regardless of ethnicity, age and sex. Evidence from observational studies suggests a negative correlation between body mass index (BMI) and circulating vitamin D 25(OH)D_3 levels. Up to now, the vitamin D status in the general population is based on circulating vitamin D 25(OH)D_3 (inactive metabolite) concentrations. Furthermore, vitamin D deficiency has also been reported to relate to whole-body insulin resistance. Of note, the development of insulin resistance is caused by a complex inter-organ crosstalk, including several insulin sensitive tissues such as the liver, the skeletal muscle, and the adipose tissue (AT). In this thesis, we aimed to investigate the link between vitamin D status and (tissue-specific) insulin sensitivity in human obesity.

Chapter 2 provides an extensive literature review of studies that have examined the effects of vitamin D on glucose and lipid metabolism, as well as inflammation in insulin sensitive tissues such as the liver, skeletal muscle, AT, pancreas, and the gastro-intestinal tract. The majority of the data about the effects of vitamin D in these tissues were derived from animal or *in vitro* studies, with often inconsistent findings. From human observational studies, more evidence supports the association between vitamin D and obesity-related insulin resistance. However, from human randomized clinical trials (RCTs), evidence for a causal role is debatable. Most of RCTs used surrogate markers such as Homeostatic model assessment of insulin resistance (HOMA-IR) to define insulin resistance and did not take into account ethnic differences. Based on our extensive literature review we concluded that vitamin D deficiency may be associated with obesity through several mechanisms including sequestration in the AT, an impaired vitamin D related metabolism within AT, and a blunted release of vitamin D from AT. However, the link between vitamin D with (tissue-specific) insulin sensitivity warrants further investigation, using more standardized (state-of-the-art) methodologies such as hyperinsulinemic-euglycemic clamps or insulin sensitivity indices derived from an OGTT.

Sequestration of vitamin D metabolites in the excessive amount of AT in conditions of increased adiposity is one of the mechanisms that may explain the low circulating vitamin D 25(OH)D_3 levels in human obesity. Vitamin D is a fat-soluble vitamin, and *ex vivo evidence* from obese AT donors suggests that vitamin D release from the AT obese is blunted following lipolytic stimulation with adrenaline. Therefore, we hypothesized that in human obesity the often observed blunted catecholamine-mediated lipolysis coincides with a blunted release of vitamin D metabolites *in vivo*. Therefore, in **chapter 3**, we analyzed vitamin D (inactive) 25(OH)D_3 and (active)1.25(OH)$_2D_3$ fluxes across the abdominal subcutaneous adipose tissue (SAT) of obese compared to lean men, in relation to changes in circulating vitamin D levels and local AT lipolysis. We observed that both net glycerol release (marker of lipolysis) and net release of the active vitamin D metabolite 1,25(OH)$_2D_3$ across abdominal SAT during ß-adrenergic stimulation were significantly reduced in obese as compared to lean men, suggesting a blunted vitamin D 1,25(OH)$_2D_3$ release and an impaired lipolysis across abdominal SAT in obese men *in vivo*. In contrast, no significant release of the inactive vitamin D metabolite 25(OH)D_3 across SAT was observed in lean or obese men following an

overnight fast or during ß-adrenergic stimulation, possibly indicating an impaired AT vitamin D metabolism (hydroxylation of 25(OH)D_3) in human obesity. Total adipose tissue mass may be an important determinant of circulating vitamin D levels as it has been proposed that a sequestration of vitamin D in the expanded adipose tissue mass may be a responsible factor for the vitamin D deficiency in obesity. Additionally, an altered vitamin D metabolism in AT may also contribute to vitamin D deficiency and insulin resistance.

In **chapter 4**, we investigated (1) whether obesity (BMI) is related to circulating vitamin D levels (active, inactive and ratio), and whether alterations in plasma vitamin D metabolites may be mediated by an altered AT expression of VDR or vitamin D-metabolizing (hydroxylation) enzymes; (2) whether plasma vitamin D and AT expression of VDR relates to tissue-specific (adipose tissue, liver and skeletal muscle) insulin sensitivity determined by a 2–step hyperinsulinemic-euglycemic clamp with a [6,6-$^2H_2$]-glucose tracer; (3) Finally, we investigated whether an altered AT vitamin D metabolism may relate to AT insulin sensitivity. We demonstrated that (1) BMI was negatively associated with of plasma 25(OH)D_3 but not with plasma 1,25$(OH)_2D_3$, nor its ratio; (2) Plasma vitamin D 25(OH)D_3 deficiency was neither related to changes in SAT vitamin D-metabolizing enzymes nor SAT VDR gene expression; (3) plasma 25(OH)D_3, 1,25$(OH)_2D_3$, and the active/inactive metabolite ratio were not significantly associated with hepatic, peripheral or AT insulin sensitivity; (4) Interestingly, SAT VDR gene expression was negatively associated with AT insulin sensitivity (as indicated by % suppression of systemic FFA). Therefore, future studies are needed to unravel the molecular mechanisms by which nuclear and membrane-bound VDR interacts with insulin action and lipolysis in abdominal SAT.

Twin and familial studies from past decades have demonstrated a nontrivial heritability of both obesity and circulating vitamin D concentrations. Moreover, several large-scale genome-wide association studies (GWAS) have discovered associations with key vitamin D enzymes and serum levels of vitamin D. Therefore, we hypothesized that VDR genetic variants may be associated with adiposity, whole body insulin resistance and the development of T2D. However, whether these VDR variants may also affect AT vitamin D metabolism and human SAT at the transcriptional level is unknown. In **chapter 5**, we tested the hypothesis that VDR genetic variants are associated with obesity phenotypes, tissue-specific insulin resistance and changes in the SAT transcriptome. Therefore, we included 553 overweight/obesity men and women from DiOGenes study, a multi-center, randomized double-blind controlled dietary intervention trial in 8 European countries. We estimated hepatic insulin resistance (HIRI) and muscle insulin sensitivity (MISI) using insulin and glucose concentrations from a 5 time-points oral glucose tolerance test (OGTT) and adipose tissue insulin resistance (Adipo-IR) from fasting insulin and free fatty acids levels. We found that TaqI and ApaI genetic variants were associated with markers of adiposity including BMI, WC, and Fat Mass. However, the VDR genetic variants were not associated with HIRI or MISI. Variants in FokI VDR were associated with Adipo-IR as well as elevated circulating FFA. However, cis and trans eQTL analysis demonstrated no major effects of these VDR polymorphisms on the SAT transcriptome, indicating that the putative mechanisms of action remain to be determined. The VDR polymorphisms did not relate to changes in body weight and insulin resistance as result of the dietary intervention.

Several plausible mechanisms to explain a potential role of vitamin D in improving insulin sensitivity have been described **in chapter 2,** including its effects on skeletal muscle substrate metabolism, insulin sensitivity and lipid composition. Importantly, the skeletal

muscle is recognized as key organ in peripheral insulin sensitivity as it affects 70-90% of total glucose disposal under postprandial conditions (non-fasting conditions). Based on the conclusions derived from our review in **chapter 2**, we conducted a systematic review and meta-analysis on the effect of vitamin D supplementation on insulin sensitivity in individuals with or at risk of insulin resistance (Chapter 6). We systematically searched and performed a meta-analysis from studies (published between 1980 and 2018) that met the PICOS (Patients/participants, Intervention, Comparison/control group, Outcome, and Study Design) criteria: (1) study was a randomized controlled trial; (2) study population consisted of individuals with elevated (risk for) insulin resistance (overweight, obesity, prediabetes, polycystic ovary syndrome (PCOS), and type 2 diabetes without complications); (3) participants were ≥ 18 years; (4) interventions were vitamin D supplementation vs the appropriate placebo; (5) vitamin D supplementation dose was daily, weekly, or monthly; (6) trial length was ≥ 2 months; (7) serum 25(OH)D level was measured; (8) insulin sensitivity was measured by Matsuda index derived from an OGTT and/or insulin sensitivity index derived from IVGTT, or by a hyperinsulinemic-euglycemic clamp at the beginning and at the end of the trial. This systematic review (**chapter 6**) provides no evidence that supplementation with vitamin D has a beneficial effect on peripheral insulin sensitivity, as determined by the hyperinsulinemic-euglycemic clamp, the Matsuda or insulin sensitivity index, and postprandial glucose concentrations after an OGTT in people with, or at risk for insulin resistance.

In conclusion, we found that BMI but not insulin sensitivity (in the liver, muscle, or adipose tissue) is the main determinant of circulating vitamin D 25(OH)D_3 concentration. However, the VDR gene expression in abdominal SAT is associated with adipose tissue insulin sensitivity, Whether this is a primary defect or more a secondary phenomenon of the obese insulin resistant state (e.g. chronic low-grade inflammation) needs to be investigated in future research. Furthermore, a blunted vitamin D 1,25$(OH)_2D_3$ release and an impaired lipolysis across abdominal SAT in obese men was observed *in vivo* following ß-adrenergic stimulation. Further studies are still needed to investigate whether this impaired release of vitamin D 1,25$(OH)_2D_3$ might be linked with changes in the VDR expression in abdominal SAT of obese individuals or possibly due to an impaired AT vitamin D metabolism (i.e. hydroxylation) in human obesity. In addition, The VDR polymorphisms are unlikely to play a primary role in tissue-specific insulin resistance. Finally, our meta-analysis showed no effect of vitamin D supplementation on insulin sensitivity or postprandial glucose metabolism in individuals with or at risk of insulin resistance. Nevertheless, it is possible that vitamin D supplementation may have a beneficial effect on other cardio-metabolic risk factor like chronic low-grade inflammation and may have beneficial effects on gut microbiota composition/diversity and intestinal health. Further studies with more specific approaches by taking into account genetic variations of the VDR and combining vitamin D supplementation with other modes of intervention might provide new strategies towards a more personalized approaches for treatment and prevention of insulin resistance in humans.

RINGKASAN

Prevalensi obesitas di dunia telah berlipat ganda tanpa memandang status sosial-ekonomi dan demografis. Pada saat yang sama, kekurangan vitamin D juga merupakan masalah kesehatan masyarakat. Prevalensi kekurangan vitamin D diperkirakan tinggi pada orang dengan obesitas tanpa memandang etnis, usia dan jenis kelamin. Bukti dari studi observasional menunjukkan korelasi negatif antara indeks massa tubuh (IMT) dan kadar vitamin D 25(OH)D_3 darah. Kekurangan vitamin D juga dilaporkan berhubungan dengan resistensi insulin. Perkembangan resistensi insulin dapat terjadi secara spesifik di beberapa organ yang sensitif terhadap insulin seperti hati, otot, dan jaringan adiposa. Dalam tesis ini, kami bertujuan untuk menyelidiki hubungan antara status vitamin D dan sensitivitas insulin yang spesifik (di otot, hati, dan adiposa) pada orang yang obesitas.

Bab 2 berisi tinjauan literatur yang luas mengenai berbagai studi yang telah meneliti efek vitamin D pada metabolisme glukosa dan lemak, serta inflamasi pada organ yang sensitif terhadap insulin seperti hati, otot, adiposa, pankreas, dan saluran pencernaan. Sebagian besar data-data tersebut berasal dari studi pada hewan coba atau studi *in vitro* (menggunakan sel model), dengan temuan yang tidak konsisten. Dari studi pengamatan di manusia, terdapat cukup bukti yang mendukung hubungan antara vitamin D dan resistensi insulin terkait obesitas. Akan tetapi, dari uji klinis acak pada manusia (*randomized control trial*/RCT), peran vitamin D terhadap resistensi insulin masih diperdebatkan. Sebagian besar RCT menggunakan biomarker tidak langsung seperti penilaian model homeostasis resistensi insulin (HOMA-IR) untuk menentukan resistensi insulin. Berdasarkan tinjauan literatur kami yang luas, kami menyimpulkan bahwa kekurangan vitamin D mungkin dapat dikaitkan dengan obesitas melalui beberapa mekanisme termasuk sekuestrasi vitamin D di jaringan adiposa, gangguan metabolisme vitamin D di jaringan adiposa, dan gangguan/hambatan pelepasan vitamin D dari jaringan adiposa. Namun, hubungan antara vitamin D dengan sensitivitas insulin yang spesifik (di jaringan adiposa, otot, dan hati) memerlukan investigasi lebih lanjut, menggunakan metodologi yang lebih terstandarisasi (mutakhir) seperti teknik *hyperinsulinemic-euglycemic clamp* atau indeks sensitivitas insulin yang berasal dari beberapa tahap pengambilan glukosa darah setelah tes oral cairan glukosa (*oral glucose tolerance test*).

Sekuestrasi vitamin D metabolit dalam jumlah berlebihan di adiposa yang terjadi seiring peningkatan adipositas (penumpukan jaringan lemak/adiposa) diduga menjadi salah satu mekanisme yang dapat menjelaskan rendahnya sirkulasi vitamin D 25(OH)D_3 pada obesitas. Vitamin D adalah vitamin yang larut dalam lemak, dan studi *ex vivo* dari jaringan adiposa laki-laki pendonor yang obesitas menunjukkan bahwa terjadi hambatan pelepasan vitamin D setelah distimulasi dengan adrenalin. Kami menduga bahwa pada secara *in vivo*, orang yang obesitas mengalami gangguan pelepasan lemak yang dimediasi katekolamin, bersamaan dengan itu juga terjadi hambatan pelepasan vitamin D metabolit dari jaringan adiposa. Dalam **bab 3**, kami menganalisis vitamin D 25(OH)D_3 dan 1,25$(OH)_2D_3$ fluks dari jaringan adiposa subkutan di bagian perut laki-laki yang obesitas dibandingkan dengan laki-laki normal. Kami mengamati bahwa pelepasan gliserol (salah satu penanda proses pelepasan lemak/lipolisis) dan pelepasan vitamin D 1,25$(OH)_2D_3$ dari jaringan adiposa subktan selama stimulasi beta agonis isoprenaline secara signifikan lebih rendah pada laki-laki yang obesitas dibandingkan dengan laki-laki normal, mengindikasikan terjadi

hambatan pelepasan lemak dan vitamin D 1,25$(OH)_2D_3$ pada obesitas. Sebaliknya, tidak ada pelepasan signifikan dari metabolit vitamin D 25$(OH)D_3$ dari jaringan adiposa subkutan di bagian perut pada laki-laki obesitas atau normal, setelah puasa semalam atau selama stimulasi beta agonis isoprenaline. Hal ini mungkin mengindikasikan gangguan metabolisme vitamin D (hidroksilasi 25$(OH)D_3$) di jaringan adiposa pada orang yang obesitas. Total massa jaringan adiposa mungkin merupakan determinan penting dari kadar vitamin D darah, dan mungkin merupakan faktor yang bertanggung jawab atas defisiensi vitamin D pada obesitas. Gangguan metabolisme vitamin D pada obesitas mungkin juga berkontribusi terhadap defisiensi vitamin D dan resistensi insulin.

Dalam **bab 4**, kami menyelidiki (1) apakah obesitas (dilihat dari Indeks Massa Tubuh/IMT) terkait dengan sirkulasi kadar vitamin D (metabolit yang aktif, tidak aktif dan rasionya), dan apakah perubahan kadar vitamin D dimediasi oleh ekspresi gen enzim-enzim dalam metabolism vitamin D serta reseptor vitamin D; (2) apakah kadar vitamin D tubuh dan ekspresi gen enzim-enzim vitamin D serta gen reseptor vitamin D di jaringan adipose berhubungan dengan sensitivitas insulin di berbagai jaringan (jaringan adiposa, hati, dan otot). Sensitivitas insulin di jaringan spesifik tersebut diukur dengan teknik *gold standard hyperinsulinemic-euglycemic clamp* dengan pelacak radio-isotop glukosa yang stabil [6,6-$^2H_2$]-glukosa; (3) Kami juga menyelidiki apakah enzim-enzim metabolisme vitamin D di jaringan adiposa juga berhubungan dengan sensitivitas insulin yang spesifik di hati, otot, dan jaringan adiposa. Kami menunjukkan bahwa (1) IMT berhubungan negatif dengan kadar 25$(OH)D_3$ tetapi tidak dengan vitamin D 1,25$(OH)_2D_3$, atau rasionya; (2) Kekurangan vitamin D 25$(OH)D_3$ tidak berhubungan dengan ekspresi gen enzim-enzim metabolisme vitamin D atau ekspresi gen reseptor vitamin D; (3) Kadar vitamin D 25$(OH)D_3$, 1, 25$(OH)_2D_3$, dan rasionya tidak berhubungan dengan sensitivitas insulin yang spesifik di hati, otot, atau jaringan adiposa; (4) Menariknya, ekspresi gen reseptor vitamin D berhubungan negatif dengan sensitivitas insulin di jaringan adiposa. Oleh karena itu, studi yang lebih detail diperlukan untuk mengungkap mekanisme molekuler bagaimana reseptor vitamin D berinteraksi dengan aksi insulin, serta pemecahan lemak khususnya di jaringan adiposa.

Studi genetik, dari beberapa dekade terakhir telah menunjukkan heritabilitas nontrivial dari obesitas dan konsentrasi vitamin D. Selain itu, beberapa studi asosiasi genomik skala besar (GWAS) menunjukkan hubungan dengan variasi genetic dari enzim-enzim vitamin D dan kadar vitamin D darah. Oleh karena itu, kami berhipotesis bahwa variasi genetik reseptor vitamin D mungkin juga berhubungan dengan adipositas, resistensi insulin seluruh tubuh serta diabetes tipe 2. Namun, apakah variasi reseptor vitamin D ini mempengaruhi transkripsi berbagai gen di jaringan adiposa pada manusia belum diketahui. Dalam **bab 5**, kami menguji hipotesis bahwa varian genetik reseptor vitamin D terkait dengan obesitas, resistensi insulin yang spesifik di hati, otot, dan jaringan adiposa, serta mempengaruhi transkripsi genetik di jaringan adiposa dari bagian abdomen (perut). Kami menganalisis 553 pria dan wanita yang kelebihan berat badan / obesitas dari studi DiOGenes, sebuah studi klinis intervensi diet di 8 negara Eropa. Kami menganalisis resistensi insulin di hati (*hepatic insulin resistance index/*HIRI) dan sensitivitas insulin di otot (*muscle insulin sensitivity index/* MISI) berdasarkan konsentrasi insulin dan glukosa dari uji toleransi glukosa oral (OGTT) 5 titik waktu (0, 30, 60, 90, 120 menit) serta resistensi insulin jaringan adiposa (*adipose tissue insulin resistance index/*Adipo-IR) berdasarkan kadar insulin puasa dan asam lemak bebas dalam darah puasa. Kami menemukan bahwa varian genetik TaqI dan ApaI berhubungan dengan marker adipositas termasuk IMT, lingkar pinggang, dan massa lemak. Tidak ada

varian genetik reseptor vitamin D yang berhubungan dengan HIRI atau MISI. Varian genetik FokI resptor vitamin D berhubungan dengan Adipo-IR serta peningkatan kadar asam lemak bebas. Namun, analisis *cis*- dan *trans*- eQTL tidak menunjukkan ada pengaruh dari variasi genetik reseptor vitamin D ini pada transkripsi genetik di jaringan adiposa, menunjukkan bahwa mekanisme keterkaitan varian genetik reseptor vitamin D dengan resistensi insulin di jaringan adiposa masih harus diteliti lebih lanjut. Pada studi kami, variasi genetik reseptor vitamin D juga tidak berhubungan dengan perubahan berat badan dan resistensi insulin setelah di intervensi diet.

Beberapa mekanisme mungkin dapat menjelaskan potensi vitamin D dalam meningkatkan sensitivitas insulin telah dijelaskan pada **Bab 2**, termasuk efeknya pada aksi reseptor insulin, komposisi lemak, dan metabolisme di jaringan otot. Lebih utamanya adalah otot merupakan organ kunci dalam sensitivitas insulin di jaringan tepi (perifer) karena mempengaruhi 70-90% dari total penggunaan glukosa di bawah kondisi *postprandial* (kondisi non-puasa). Berdasarkan ulasan kami di **Bab 2**, kami melakukan tinjauan sistematis dan meta-analisis tentang efek suplementasi vitamin D pada sensitivitas insulin pada individu dengan atau berisiko resistansi insulin (**Bab 6**). Kami secara sistematis mencari dan melakukan meta-analisis dari studi (diterbitkan antara 1980 dan 2018) yang memenuhi kriteria PICOS (**P***atients/* pasien/peserta penelitian, **I***ntervention/*intervensi yang diberikan, **C***ontrol/*kelompok pembanding/plasebo, **O***utcomes/*hasil/luaran yang diukur, dan **S***tudy design/*desain penelitian) kriteria: (1) desain studi adalah RCT; (2) populasi penelitian terdiri dari individu dengan/berisiko resistensi insulin (obesitas, prediabetes, sindrom ovarium polikistik (PCOS), dan diabetes tipe 2 tanpa komplikasi); (3) pasien berusia ≥ 18 tahun; (4) intervensi adalah suplementasi vitamin D vs plasebo yang sesuai; (5) dosis suplemen vitamin D adalah harian, mingguan, atau bulanan; (6) lama studi adalah ≥ 2 bulan; (7) kadar vitamin D 25(OH)D diukur; (8) sensitivitas insulin diukur dengan *indeks Matsuda* yang berasal dari OGTT atau indeks sensitivitas insulin yang berasal dari tes toleransi glukosa intravenous (IVGTT), atau *hyperinsulinemic-euglycemic clamp* di awal dan di akhir percobaan. Tinjauan sistematis dan meta analisis ini (**bab 6**) menunjukkan tidak ada efek suplementasi vitamin D terhadap sensitivitas insulin, yang diukur oleh metode *gold standard hyperinsulinemic-euglycemic clamp*, *indeks Matsuda* atau indeks sensitivitas insulin, serta tidak berefek menurunkan konsentrasi glukosa *postprandial* setelah OGTT pada orang dengan/berisiko mengalami resistensi insulin.

Sebagai kesimpulan, kami menemukan bahwa IMT (bukan sensitivitas insulin di hati, otot, atau jaringan adiposa) merupakan determinan utama kadar vitamin D 25(OH)D_3. Selanjutnya, ekspresi gen reseptor vitamin D pada jaringan adipose subkutan berhubungan dengan sensitivitas insulin di jaringan adiposa, Perlu diteliti lebih lanjut apakah kaitan tersebut merupakan gangguan primer atau lebih merupakan fenomena sekunder dari keadaan resisten insulin pada orang obesitas (misalnya sebagai akibat peradangan kronis tingkat rendah). Terjadi hambatan pelepasan vitamin D 1,25$(OH)_2D_3$ dan gangguan lipolisis pada jaringan adipose subkutan pada laki-laki obesitas secara *in vivo* setelah stimulasi katekolamin. Penelitian lebih lanjut masih diperlukan untuk menyelidiki apakah hambatan pelepasan vitamin D 1,25$(OH)_2D_3$ ini mungkin terkait dengan perubahan ekspresi reseptor vitamin D pada jaringan adiposa subkutan pada obesitas atau mungkin karena gangguan metabolisme vitamin D pada obesitas. Kemudian, variasi genetik reseptor vitamin D tidak berperan utama dalam resistensi insulin di jaringan hati, adiposa dan otot. Pada meta-analisis, tidak ada efek suplementasi vitamin D terhadap sensitivitas insulin atau penurunan

kadar glukosa *postprandial* pada individu dengan atau berisiko resistansi insulin. Namun, suplementasi vitamin D mungkin memiliki efek terhadap faktor risiko kardio-metabolik lainnya seperti kemungkinan menurunkan peradangan kronis dan mungkin memiliki efek terhadap keanekaragaman mikrobiota usus serta kesehatan intestinal (usus). Studi lebih lanjut dengan pendekatan yang lebih spesifik dengan mempertimbangkan variasi genetik metabolisme vitamin D dan kombinasi berbagai intervensi mungkin memberikan strategi baru menuju pendekatan yang lebih personal untuk pencegahan resistensi insulin pada manusia.

VALORIZATION

Obesity prevalence is increasing worldwide and often coincides with circulating vitamin D deficiency and whole-body insulin resistance. The present thesis describes how obesity-related phenotypes, vitamin D concentrations as well as vitamin D-related genes link to insulin resistance. The valorization potential of the work described in this thesis will be discussed in terms of societal and economic relevance, the implications for the scientific community and health care professionals, activities/products, including future planning and realization.

Societal and Economic Relevance

Obesity is associated with type 2 diabetes mellitus, cardiovascular diseases (mainly heart disease and stroke), diabetes, musculoskeletal disorders (especially osteoarthritis – a highly disabling degenerative disease of the joints), some types of cancers, depression and a reduced quality of life (1), and more recently it was identified as a major risk factor for fatal COVID-19 (2). Obesity is a chronic metabolic disorder resulting from an energy imbalance, by which a long-term positive energy balance leads to the storage of excess energy as body fat (3). Furthermore, obesity is often characterized with vitamin D deficiency. Putative mechanisms, linking obesity, vitamin D, and insulin resistance are described in this thesis, including obesity, vitamin D deficiency, adipose tissue dysfunction, and tissue-specific insulin sensitivity.

Our study showed that the prevalence of vitamin D 25(OH)D_3 deficiency [based on Endocrine Society cut-off value < 50 nmol/L] in our study was about 45.7%, and BMI is the main determinant of vitamin D 25(OH)D_3 concentration in individuals with overweight/obesity (**chapter 4)**, indicating that obesity-associated vitamin D deficiency could be recognized as an important public health concern. Ensuring sufficient circulating vitamin D 25(OH)D_3 level is essential to maintain general health but also may be of importance in the management of obesity, and the prevention of insulin resistance and T2D.

Oral and intravenous vitamin D supplementation have been suggested to effectively increase circulating vitamin D 25(OH)D_3 level. Our meta-analysis showed that vitamin D supplementation is an effective means to increase circulating vitamin D 25(OH)D_3 level, despite substantial heterogeneity (**chapter 6**). However, more research is needed to study the efficacy and bioavailability of different types (and routes of administration) of vitamin D supplementation to more effectively increase serum vitamin D levels in overweight/obesity.

We did not find any associations between circulating vitamin D levels with hepatic, muscle, and adipose tissue insulin sensitivity assessed by hyperinsulinemic-euglycemic clamp (**chapter 4**). Furthermore, our meta-analysis showed no beneficial effect of vitamin D supplementation on the improvement of insulin sensitivity (**chapter 6**), suggesting no direct causality between vitamin D deficiency and whole body and tissue specific insulin resistance in human obesity.

Our study using arterio-venous difference techniques across abdominal subcutaneous adipose tissue (SAT) showed that there was an impaired release of active vitamin D metabolite in obese men (**chapter 3**). In addition, we observed that VDR expression in abdominal SAT is increased in obesity and associated with adipose tissue insulin sensitivity

(chapter 4). This may suggest that adipose tissue may be a key target organ to improve vitamin D metabolism in the context of human obesity. Furthermore, currently a project on exercise-mediated Vitamin D mobilization sequestered in the human adipose tissue (Vita-DEx project) is ongoing (4). If exercise might be beneficial to mobilize vitamin D from adipose, then this may have implications regarding the management/treatment of a low vitamin D status in obesity.

Scientific Community

The content of the chapters in this thesis have been presented at national scientific meetings (i.e. NASO spring meeting and ADDRM meeting) and international conferences (The 25th and 26th European Congress on Obesity). The results have also been discussed to health professionals and colleagues inside and outside the scientific community with the purpose to discuss the medical and societal consequences of obesity and vitamin D deficiency. Moreover, the studies described in this thesis have been published or will become available to the scientific community through publication in peer-reviewed journals.

The findings presented in this thesis, may be of value for health care professionals (e.g. physicians and dieticians). Given that fact that the incidence of vitamin D deficiency is considerable high (>40%) among obese individuals, this may possibly have consequences for recommended nutritional intake. However, a recent RCT reported 750 g/week of salmon was not sufficient to prevent a decrease in serum 25-hydroxyvitamin D [25(OH)D_3] in autumn in South-Western Norway in adults with overweight/obesity (5). These data suggest that increasing vitamin D intake from diet only, may not be adequate to improve circulating vitamin D 25(OH)D_3 level in obese individuals. Furthermore, although Vitamin D supplementation increased circulating vitamin D levels 25(OH)D_3 levels, nevertheless, this did not translate to improved whole-body insulin sensitivity (**chapter 6**). Therefore, a combination mode of interventions (e.g. exercise and nutrition) and more personalized strategies should be explored in future research.

Activities/Products and Innovation

In this thesis, we combined state-of-the-art methodology including: hyper-insulinemic-euglycemic clamp, adipose tissue gene expression using qRT-PCR , plasma vitamin D and vitamin D fluxes analysis using gold standard LC/MS-MS measurement, adipose tissue blood flow measurements (i.e. xenon washout technique), arterio-venous balance technique, PCR-based genetic variant analysis in combination with subcutaneous adipose tissue transcriptomic, which together gave important insights in obesity related vitamin D metabolism. Furthermore, we have also conducted a systematic review and meta-analysis in this thesis, providing one of the highest levels of evidence in human clinical research to date.

Planning and Realization

The causal relationship between vitamin D and obesity related metabolic health is still under debate. Although, from studies described in this thesis, the direct link between vitamin D

and overall metabolic health (non-skeletal function) may not be mediated by tissue-specific insulin signaling/sensitivity pathways. However, the link between Vitamin D and metabolic health may be partly mediated via its effects on gut microbiota composition/diversity and gut health (6). This is supported by the presence of VDR expression in human enterocytes and from a recent genome wide association study that suggests a potential link between VDR variants and gut microbiota diversity (7). However, future studies are needed to investigate in more detail the relationship between vitamin D, gut microbiota, gut health, and its effects on host metabolic health.

Results from this thesis should encourage future Vitamin D research, for instance, how genetic variants (**chapter 5**) in vitamin D metabolisms (VDR and CYP) may influence the metabolic outcome of vitamin D supplementation. This will pave the way for studies with more personalized (sub-group, i.e. carrier and non-carriers of VDR SNPs) approaches. In addition, combining vitamin D supplementation with other modes of intervention (i.e. exercise / dietary intervention induced weight loss) might provide new strategies towards a more personalized approach for obesity related vitamin D deficiency treatment.

Last but not least, while obesity has been suggested as one of the major comorbidities of covid-19 (2), vitamin D deficiency is a common feature in obesity, and may also be a determinant of covid-19 outcome. More studies warrant to explore the link between vitamin D deficiency, obesity, and covid-19 which is highly relevant given the current pandemic our society and scientific community is dealing with.

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ABOUT THE AUTHOR

Adriyan Pramono was born on July 4, 1985, in Semarang. He did his bachelor of nutrition in the Faculty of Medicine, Diponegoro University, Semarang, Indonesia in 2003 and graduated in 2007. In 2008 while working as a research assistant, he continued his master degree in nutrition science and graduated in 2010. He then continued with several short courses which were mainly conducted in SEAMEO-RECFON (Southeast Asian Ministry Education Organization – Regional Center Food and Nutrition), University of Indonesia, Jakarta where he had the opportunity to meet the expert in nutrition such as Prof. Rosalind S Gibson, and a leader such as the CEO of General Electric Indonesia, Handry Santriago.

Since 2012, he has been appointed as junior faculty in the Department of Nutrition Science, Faculty of Medicine, Diponegoro University. Between 2012 and 2016, he was actively involved in the educational duties, university research, and community empowerment.

In September 2016, he started his PhD program at the Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center+, The Netherlands under the supervision of Prof. Ellen Blaak and Dr. Johan Jocken. Here in the NIMO-Lab research group, he studied about vitamin D and its relation to insulin sensitivity in human obesity, using humans cohort with overweight/obesity, state-of-the-art methodology including a 2 step hyperinsulinemic-euglycemic clamp, LC/MS-MS, arterio-venous difference, and gene expression analysis of abdominal adipose tissue.

During his PhD, he received awards; Travel Grant from LPDP (Indonesia Endowment Fund for Education) in 2018 and EASO (European Association for the Study of Obesity) in 2019 for presenting in The 25th and 26th European Congress on Obesity.

After his PhD, Adriyan will continue his carrier in Indonesia. He would like to continue his research focusing on vitamin D related metabolism, whole dietary pattern and metabolic health in overweight and obesity. He would like to do both fundamental and translational research as well as systematic reviews and meta-analysis.

PHD PORTFOLIO

LIST OF PUBLICATION

- **Pramono, A.**, Jocken, J. W. E., Essers, Y. P. G., Goossens, G. H., & Blaak, E. E. (2019). Vitamin D and Tissue-Specific Insulin Sensitivity in Humans With Overweight/Obesity. The Journal of clinical endocrinology and metabolism, 104(1), 49. https://doi.org/10.1210/jc.2018-00995
- **Pramono, A.,** Jocken, J. W. E., & Blaak, E. E. (2019). Vitamin D deficiency in the etiology of obesity related insulin resistance. Diabetes/metabolism research and reviews, e3146: https://doi.org/10.1002/dmrr.3146
- **Pramono, A.,** Jocken, J. W. E., Goossens, G. H., & Blaak, E. E. (2019). Vitamin D release across abdominal adipose tissue in lean and obese men: The effect of ß-adrenergic stimulation. Physiological Reports 7(24): e14308: DOI: 10.14814/phy2.14308
- **Adriyan Pramono,** Johan W.E. Jocken, Marleen A. van Baak, Ellen E. Blaak. The Effect of vitamin D supplementation on insulin sensitivity: a systematic review and meta-analysis. Diabetes Care 2020 Jul; 43(7): 1659 – 1669 (https://doi.org/10.2337/dc19-2265)
- **Adriyan Pramono**, Johan W.E. Jocken, M.A. Adriaens, M.F. Hjorth, A. Astrup, W.M. Saris, E.E. Blaak. The association between vitamin D receptor polymorphisms and tissue-specific insulin resistance in human obesity. **Submitted**

SELECTED ORAL/POSTER PRESENTATION

2018:

- The Netherlands Association for the Study of Obesity (NASO) Spring meeting, Utrecht 19 April 2018
- The 25th European Congress on Obesity (ECO), Vienna, Austria 23-26 May 2018
- Annual NUTRIM Symposium, Maastricht 21 November 2018

2019:

- The Netherlands Association for the Study of Obesity (NASO) Spring meeting, Utrecht 3 April 2019
- The 26th European Congress on Obesity (ECO), Glasgow, Scotland 28 April- 1 May 2019
- Annual NUTRIM Symposium, Maastricht, 27 November 2019

AWARD

- Travel Grant for ECO 2018 in Vienna, Austria from LPDP (Indonesia Endowment Fund for Education)
- Travel Grant for ECO 2019 in Glasgow, Scotland from EASO (European Association for the Study of Obesity)

COURSES

- Research Writing for PhD I – II (Biomedical Sciences)
- Presentation Course for PhD
- Biostatistics Part II – Regression Analysis

TEACHING

- Involved in the supervision of bachelor thesis