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Vitamin D Immune Modulatory Effect on the Anti-inflammatory potential of HDL Associated Proteins

BY

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ABSTRACT

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Title: Vitamin D Immune Modulatory Effect on the Anti-inflammatory potential of HDL Associated Proteins

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Introduction: Vitamin D deficiency is highly prevalent in Qatar. Vitamin D exerts immune-modulatory effects leading to reduced inflammation. Therefore, vitamin D deficiency is associated with increased subclinical inflammation. vitamin D deficiency associated with dyslipidemia. HDL possesses anti-inflammatory properties, and neutralizes endotoxin (LPS). Therefore it is an anti-inflammatory modulator during sepsis and inflammation. We suggest that HDL and the associated proteins ApoM, ApoD, ApoA-1, and LL-37 are playing an important role in LPS detoxification process, by clearing the endotoxins and minimizing the cytokines released. The nature of the relationship between HDL and associated proteins and vitamin D and their role in endotoxins clearance is not fully understood.

Methodology: The study is designed to have two arms. **Translational arm** focusing on the nature of the association between vitamin D deficiency, dyslipidemia, inflammation, HDL, and HDL- associated proteins ApoM, ApoD, AopA-1, and LL-37 in healthy adults, and identifying the modifications of proteomic and metabolomic profile during vitamin D and dyslipidemia. **In vitro studies** investigated the anti-inflammatory potential of HDL-associated proteins, including identifying the relationship between vitamin D levels and HDL-associated proteins ApoM, ApoD, ApoA-1, and LL-37 expression in THP1 using PCR, and ELISA. Determining the role of ApoM in endotoxin neutralization using molecular docking simulations of ApoM -

E. coli LPS, isothermal titration calorimetry (ITC), and measuring the TNF-α, nitric oxide release in THP1 cells.

Rationale of the study: This research will enhance understanding of the mechanistic physiology associated proteins during inflammation and will illustrate the clinical correlation of HDL-associated proteins to VitD deficiency. Therefore, will facilitate the therapeutic design of engineered functional HDL particles for potential use in sepsis.

Result and conclusion Vitamin D deficiency is inversely associate with subclinical inflammation marker monocyte percentage to HDL ratio (MHR). Alterations in sphingomyelins are observed in participants with combined vitamin D deficiency and dyslipidemia. Also, enrichment of inflammation and cancer pathways is revealed by proteomics analysis. Vitamin D deficiency affected HDL-associated proteins expression and impacted the anti-inflammatory potential of HDL. ApoM binds endotoxin with high affinity and contributes to neutralization and clearance by HDL. Vitamin D modulated the expression of HDL-associated apolipoproteins ApoA-1 and ApoM in monocytes.

DEDICATION

To my Amazing supervisor Dr. Susu Zughaier and to my beloved ones Sarah, and Ahmad Najlaoui, to my friends especially Aisha Al- Saei

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1. Vitamin D

Vitamin D is a fat-soluble prohormone known as the key element in bone formation through maintaining the homeostasis of calcium and phosphorus. Vitamin D has two sources; either from dietary sources e.g. Salmon, Egg yolk, and mushrooms, or from skin exposure to the sun's UVB light. The native form of vitamin D converts in the liver to 25-hydroxyvitamin D [25(OH)D], a major circulatory vitamer. However, the active form of vitamin D takes a place in the kidney upon conversion of the 25(OH)D to 1,25 dihydroxyvitamin D (1,25(OH)2D) via 25-hydroxyvitamin D-1α-hydroxylase [2]. The native form of vitamin D has two analogs D2 and D3. D2 also known as Ergocalciferol is obtained from plants' dietary sources like soya beans. Whereas, D3 (cholecalciferol) is an animal source like fish. The differences in structures of both forms impacted the binding affinity to the other proteins including their carrier vitamin D binding protein (DBP), thereby affecting plasma half-life. D2 has a shorter plasma half-life and less affinity to binding DBP, and vitamin D receptor (VDR) [3].

VDR is a member of a superfamily of nuclear receptors working as a transcription regulator. VDR gene is located on chromosome 12q13.11[4]. VDR is usually allocated in the cytoplasm. VDR is expressed in the intestine, immune cells, and bone and a lesser amount in bone marrow, colon, brain, and breast [5]. Upon the interaction of VDR and 1,25(OH)2D3, conformational changes occur. Later, VDR dimers with retinoid X receptor (RXR) and transfers to the nucleus. In the nucleus, the complex is binding the vitamin D response elements (VDRE) in the vitamin D responsive genes. Subsequently, this process ends up activating or suppressing some genes according to the target genes [6, 7]. For example, Takeuchi et. ai. study showed that 1,25(OH)2D3 is inhibiting the cytokine genes in human T cells may, through

suppression of the transcription factor NFAT activity, NFAT is an IL-2 promoter [8]. . In an independent matter, studies indicate that RXR could bound to several genes in the absence of its ligand VDR, spotting the light on a regulatory role of 1,25(OH)2D3 [9].

1.1. Vitamin D in physiological conditions

In Normal conditions vitamin D involving in the regulation of many biological processes. The 1,25(OH)2D classical function is promoting calcium transfer from the kidney, gut, and bone to the blood. VDR-knockout mice were found to have an interruption in calcium influx from intistine[10]. The parathyroid hormone (PTH) works as a regulator of 1,25(OH)2D synthesis. PTH is suppressed directly by 1,25(OH)2D and through the increase of calcium [11]. Add to that, vitamin D upregulates a set of genes like renal osteocalcin, osteopontin, calbindin, 24-hydroxylase Ca, and Mg-adenosine triphosphatase, which in turn control bone mineralization [12, 13].

From an immunological perspective, VDR is notably highly expressed in T lymphocytes and macrophages. However, studies show that vitamin D modulates the immune system on several levels. Vitamin D decreases T lymphocyte activity by stimulating the transcription of growth factor TGFβ-1 and interleukin 4 (IL-4)[14]. Moreover, 1,25(OH)2D3 was found to suppress the release of the cytokine in Th1, e.g. interleukin (IL)-2 through its effect on transcription factor NF-AT complex formation. The VDR-retinoid X receptor heterodimer blocks NFATp/AP-1 complex formation by binding directly to the distal part of NF-AT in the human IL-2 promoter [15]. Interferon (IFN)-γ is another example; the formed complex of the ligand-bound VDR and VDRE is attached to the (IFN)-γ promoter region, casing a repressive effect. At the same time, increases IL-10 production, IL-10 is an anti-inflammatory cytokine [16].

NF-κB is one of 1,25(OH)2D3 targets. Upon the activation of NF-κB; a downstream target nucleotide-binding oligomerization domain 2 (NOD2) will be stimulated. NOD2 is a pathogen-recognizing protein. Subsequently, human β-defensin 2 DEFB4 (skin-antimicrobial peptide 1 (SAP1)) will be activated by NOD2; this process is downregulated by the presence of 1,25(OH)2D3. Another antimicrobial peptide cathelicidin AMP (CAMP)(LL-37 is another name for the human cathelicidin) is affected by vitamin D, where it has been noticed to be highly induced by 1,25(OH)2D3. The activity of 1,25(OH)2D3 in stimulating the antimicrobial peptides was seen against some pathogens such as *Pseudomonas aeruginosa*. This effect has been reported in human immune cells, keratinocytes, monocytes, and neutrophils [17-19]. This upregulation of antimicrobial peptides might have a role in controlling and containing inflammatory responses and the sequels of overstimulating of immune systems such as in sever cases of COVID-19 [20].

Additionally, 1,25(OH)2D3 has been demonstrated to activate macrophage autophagy, and autophagy-associated proteins Atg-5 and Beclin-1. Those proteins are also induced through the upregulation of cathelicidin. Cathelicidin or LL-37 activation leads to the induction of p38, ERK, and C/EBPβ [21]. Autophagy is a catabolic biological process mediated by a lysosome aiming to remove unwanted or dysfunctional components [22].

T cells express both the VDR and CYP27B1, the enzyme which metabolizes vitamin D into its active. However naive T cells don't express VDR and CYP27B1 as much as mature T cells. This is pointing to the crucial role of 1,25(OH)2D3 in regulating the different subclasses of T cells. The active hormone 1,25(OH)2D3 suppresses Th1-type differentiation and the secretion of inflammatory cytokines (IL-2, IFNγ, and TNF-α), and promotes Th2-type differentiation and the secretion of anti-inflammatory cytokines (IL-4, IL-5, and IL-10). More, (IL-17, IFNγ, IL-21, and IL-22) the Th17-related cytokines are inhibited by 1,25(OH)2D3 as

well. Vitamin D also modulates the immune system through inducing the FoxP3 transcription factor, which promotes the differentiation of regulatory T cells [23-25].

1.2. Vitamin D deficiency

Vitamin D deficiency is a global health concern. Reports are pointing to 1 billion people suffering from various degrees of vitamin D deficiency and insufficiency. The highest rates of vitamin D deficiency are found in the elderly, obese people, residents of nursing homes, and hospitalized patients. [26]. In Qatar, reports revealed that around 64% of the population was vitamin D deficient [27]. The Institute of Medicine defined vitamin D deficiency by serum 25(OH)D concentrations is <12 ng/mL, while insufficiency is 12-<20 ng/mL, taking into consideration that the normal level of serum 25(OH)D above 20 ng/ml [28].

Significant associations are reported between vitamin D deficiency and chronic inflammatory diseases including metabolic syndrome, obesity, dyslipidemia, and chronic cardiovascular diseases. Vitamin D exerts immune-modulatory effects leading to reduced inflammation [1] Therefore, vitamin D deficiency is associated with increased subclinical inflammation.

Vitamin D deficiency was noticed to be correlated to some conditions like familial hypertension. This is due to polymorphisms of the VDR gene rs3847987 which were found to be associated with all vitamin D deficiency, hypertension, diabetes, and insulin resistance [29-31]. In addition, VDR polymorphism was linked to developing hypertriglyceridemia and predisposition to metabolomic Syndrom development [32]. On the other hand, patients suffering from vitamin D deficiency were observed to increase their predisposing to diseases e.g COPD [33]. Vitamin deficiency is not only associated with certain diseases but also associated with resistance to treatment, for example, renal denervation (RDN) is a technique

used by the clinician in the case of hypertension that didn't respond to the classical interventions to lower the blood pressure. Costa.let.al. study reported that hypertensive patients with low vitamin D concentrations were not responsive to this intervention [34]

1.3. Vitamin D deficiency and cardiovascular diseases and metabolomic syndrome

The risk for cardiovascular diseases is known to be impacted by a number of lifestyle factors, such as smoking, drinking alcohol, and malnutrition. In addition to metabolic abnormalities, such as diabetes, insulin resistance, and obesity. Those factors are cross-linked with risk factors of vitamin D deficiency [35, 36]. Epidemiological studies such as Giovannucci et.al. that followed 18,000 male US citizens working as health professionals over a decade found a doubling in coronary heart disease events associated with a low level of 25(OH)D(≤15 ng/mL) in comparison with 25(OH)D sufficient individuals (≥30 ng/mL)[37]. Other studies such as Dobnig et.al. concluded similar results. The study was conducted on coronary angiography patients who were followed for 7.7 years. The investigations recorded an increase in cardiovascular mortality linked to the drop in the level of both 25-hydroxyvitamin D and 1,25-dihydroxy vitamin D as an independent factor [38]. Add to that, the risk of heart failure and sudden cardiac death was very high in patients with low 25(OH)D [39].

Vitamin D in general protects against cardiovascular diseases through multi-layers mechanisms. The first mechanism vitamin D help in relaxing vessel is by stimulating nitric oxide (NO) production from endothelial cells [40]. Moreover, vitamin D suppresses the NFkappaB and p38 which are responsible for the activation of the inflammatory pathways leading to exacerbating the atherosclerotic disorder [41].

1.4. Vitamin D supplementation

Restoring the optimal level of serum 25(OH)D above 20ng/mL (50nmol/L)- 30ng/mL (75nmol/L), preferably between 40-60 ng/mL (100-150 nmol/L) has documented health benefits including improving the outcomes of chronic disease and reducing the risks of getting the disease in the first place. Recent studies on COVID-19 patients provided evidence that vitamin D supplementation has a great role in minimizing the severity of the infection and modulating both

innate and adaptive immune systems[42]. Therefore, improving the clinical manifestations and outcomes. Studies also showed that vitamin D supplementation decreased hospitalization, ICU admission, and mortality rate. Although clinical studies didn't show any decrease in the risk of COVID-19 infection [43].

An interesting clinical trial on 90 healthy monozygotic twins supplemented with 2000 IU for two months showed modifications in body fat and lean mass besides elevating vitamin D level by 65% and VDR gene expression sixty times [44]. In the same context, the supplementation showed efficacy in enhancing lipid metabolism in general and decreasing insulin resistance and hyperandrogenism, in polycystic ovary syndrome patients [45].

In in vitro studies as well, vitamin D supplementation expressed an enhancement of immune response toward infection, including increasing the phagocytes, suppressing the inflammatory cytokine production, and inducing antimicrobial peptides such as cathelicidin expression [46]. A meta-analysis reported that vitamin D supplementation might help with infertility and improve chemical pregnancy outcomes and showing an increase in the success rate of in vitro fertilization [47]. The guidelines suggested that the daily recommended dose of vitamin D is 400-800IU, while the therapeutic dose is either an intramuscular injection of

600.000 IU/month, or oral tablet of 200.000 IU/ month, or 50.000 IU/ week. The period of treatment doses is 8 weeks, followed by a maintenance dose of 50.000 IU/ month or every two months [48].

Some age categories benefit more from vitamin D supplementation such elderly. The elderly are prone to suffering from vitamin D deficiency, secondary hyperparathyroidism, and cortical bone loss, which could decrease bone density and increase hip fractures. Clinical trials proved that Vitamin D supplementation could increase the vitamin D level and overcome the overactivation of parathyroid function, therefore, preventing more bone loss [49].

2. Dyslipidemia

Dyslipidemia, or dyslipoproteinemia, involves a range of abnormalities in the metabolism of plasma lipids and lipoprotein resulting in augmenting or reducing the concentrations of lipoprotein, or changing the particle configuration [50]. Dyslipidemia triggers can be related to genetic factors causing familial dyslipidemia, also called primary dyslipidemia. Conversely, environmental factors such as diseases, diet, and general life regime cause acquired dyslipidemia, also named secondary dyslipidemia [51]. Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), or high-density lipoprotein cholesterol (HDL-c) concentrations deviate when suffering from dyslipidemia [52]. Guidelines defined the disturbance in lipoprotein according to the following values: high total cholesterol (>6.2 mmol/L), high LDL-c (>4.1 mmol/L), and high TG (>2.3 mmol/L) [176]. Variation in lipid metabolites marks dyslipidemia as a major risk factor in several diseases, for instance, cardiovascular disease, diabetes, and stroke [53]. Dyslipidemia prevalence is unique to each geographical area, however, an estimated of 50% of the global adult population experience dyslipidemia [54]. In Qatar, the prevalence of dyslipidemia is higher than diabetes according

to the QBB database. Qatar's Stepwise survey disclosed the key risk factors for Atherosclerotic cardiovascular disease (ASCVD) in the population as follows: raised total cholesterol (19.1% men, 24.6% women), low HDL-c levels (49.2% men, 37.3% women) and high LDL-c levels (8.2% men, 9.9% women) signifying the prevalence of dyslipidemia in correlation to incidence within Qatar population (The Assessment and Management of Dyslipidemia) [55]. Studies of dyslipidemia and associated macromolecules have linked vitamin D deficiency as a potential risk factor, as vitamin D receptors were found in multiple cell types, including vascular endothelial cells and cardiomyocytes [50]. Dyslipidemia is a major element in metabolic syndrome which is characterized by a combination of several pathological conditions, mainly central obesity, hypertension, low levels of HDL, elevated LDL and TG, endothelial dysfunction, and diabetes [56]. Studies reported a strong correlation between metabolic syndrome and vitamin D deficiency, but the bidirectional nature of this association is still not clear [57, 58].

2.1. Lipoproteins

Plasma lipoproteins are complexes of lipids and proteins that have a high affinity to bind lipids. Their main function is transporting cholesterol from the intestine to the liver, and from the liver to tissues. The lipoproteins are classified into four main groups: chylomicrons (CM), low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), and high-density lipoproteins (HDL) [59]. Structurally, lipoproteins are emulsion particles consisting of a hydrophobic core of triglycerides lipids (TGA) and cholesterol ester (CE). The lipoproteins surface is made of hydrophilic layers containing apolipoproteins, free cholesterol, and a monolayer of amphipathic (hydrophilic and hydrophobic) phospholipids (PL) [60].

2.1.1. LDL particle

LDL is the main transporter of cholesterol from the liver to peripheral tissues. It plays a fundamental role in atherosclerosis plaque formation and cardiovascular disease [61]. LDL is mainly produced in the liver from the conversion of VLDL into intermediate-density lipoproteins (IDL) by the lipoprotein lipase enzyme. Later, the hepatic triglyceride lipase is converting the IDL into LDL [62]. This lipoprotein is relatively big, it varies from 22 nm to 27.5 nm according to lipid content and has a mass of about 3 million daltons [63]. Structurally, LDL is composed of a polyunsaturated fatty acid called linoleate, triglycerides, esterified and unesterified cholesterol molecules. In addition to the proteomic contents, that include large apolipoprotein; Apo B-100(550 kDa) [64].

The raised level of LDL in the blood has a very bad influence on cardiovascular systems. LDL is the backbone of atherosclerotic plaque formation. When LDL enters the endothelial layer towards the intima layer in blood vessels, the free radicals oxidize the LDL particle, generating ox-LDL [65, 66]. The presence of ox-LDL stimulates endothelial to produce Monocyte Chemoattractant Protein-1 (MCP-1). As consequence, the monocytes will invade the intima and transform into macrophages. Those macrophages will try to eliminate the ox-LDL particles by ingesting them. Eventually, those macrophages will be over-saturated with ox-LDL and converted to foam cells. Foam cells release lots of chemokines to attract more macrophages to the site [67]. The accumulation of foam cells will work as a lipid storage vehicle and will lose the ability to migrate, therefore causing a release of more pro-inflammatory mediators in the site of plaque [68]. The high inflammatory status in the atherosclerotic plaque will produce reactive oxygen species and causes macrophage death leading to the form a necrotic core in the plaque [69]. In this regard, researchers recommended the normal level of LDL to be Less than 100 mg/dL [70].

2.1.2. VLDL particle

These triglyceride-rich particles are produced in liver. Their size varies also 30-80 nm according to their contents of triglyceride. The size of the released VLDL particles increases with increased triglyceride synthesis in the liver [71]. The condition which affects lipid metabolism such as in the case of insulin resistance also impacts VLDL production. In insulin resistance, an accumulation of free fatty acids in liver occurs therefore, the VLDL production and release to the blood increase, which will exacerbate insulin resistance [72]. VLDL particles, on the other hand, are smaller than chylomicrons and larger than the LDL. Apolipoproteins C-I, C-II, C-III, and E are associated with the VLDL particle, but B-100 is the primary apolipoprotein found in each VLDL particle [73]. High concentrations of VLDL-TG were linked to an increased risk of cardiovascular disease [74].

2.2. Chylomicrons

Chylomicrons are different from other lipoproteins in that they originate only from the intestine from dietary sources. More than 75% of chylomicrons are composed of triglycerides. For maturation of the chylomicrons particles, it acquires ApoC2 from circulating HDL [75]. The main protein associated with chylomicrons is ApoB-48. Chylomicrons also bind other apolipoproteins e.g. ApoA-1, ApoA-2, ApoA-3, ApoA-5, ApoC3, and ApoE. The main function of chylomicrons is to transport lipids from the intestine to peripheral tissues i.e. skeletal muscle, cardiovascular system, and adipose tissue [76]., The lipoprotein lipase enzyme in peripheral tissues is hydrolyzing the triglycerides inside chylomicron particles to release the free fatty acids, where it is utilized by those tissues. Upon transferring all triglycerides to the liver the ApoE facilitates the removal of chylomicron remnants from circulation and endocytosis by hepatic tissue [75]. Chylomicrons have the largest size and the least dense below 0.94 g/ml among all lipoproteins [76].

2.3. **HDL particles**

HDL “the good cholesterol” gain popularity over the years due to its important and protective role in the cardiovascular system against atherosclerosis. Recently, researchers focus on other properties of HDL such as anti-inflammatory, anti-apoptotic and antioxidant which play an important part in modifying the progression of several diseases [77]. HDL-cholesterol (HDL-c) is a good indicator of the functionality of HDL and the ability to carry cholesterol from tissues more than the HDL-P (particle), although HDL-P was more studied in clinical studies in cardiovascular disorders in isolation of HDL-c [78]. Low HDL-c has a major weight whether on disease prognosis or survival rate. For instance, in cancer patients, low HDL is considered a bad prognostic factor [79-81]. Patients with a serum level of HDL-c level <40 mg/dL were noticed to have a bad response to chemo-immunotherapy in follicular lymphoma patients [82]. Furthermore, in severe sepsis, it has been observed that patients who have low values of HDL upon the beginning of sepsis were deteriorating very rapidly, and have the worst mortality and clinical outcomes [83]. High values of HDL-c in ejection fraction reduced heart failure in patients and were associated with better prognosis and survival. In the same studies, patients with high HDL-c recorded a decline in inflammation biomarker C-reactive protein (CRP) values, and liver biomarker alanine aminotransferase (ALT). On the other hand, an increase in albumin levels was observed [75].

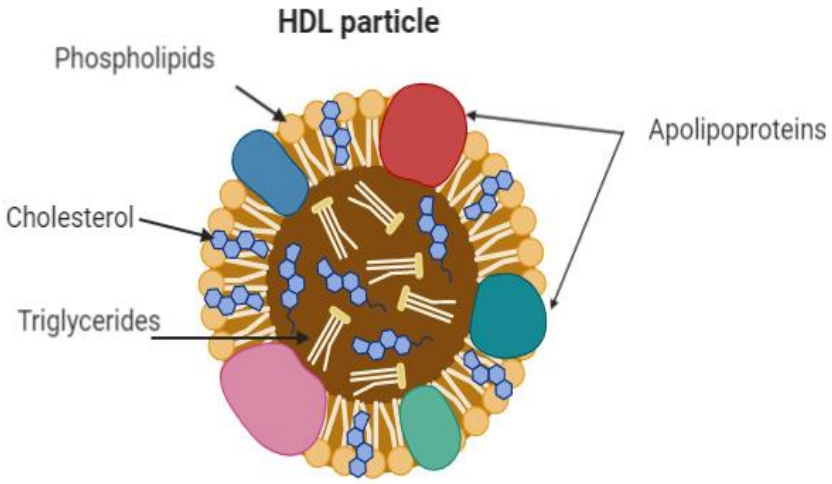

Figure 1. 1. Schematic illustration of mature HDL particle structure. Created by Hanaa Mousa.

The physiological value of HDL in serum is preferably to be above 60 mg/dL (1.6 mmol/L) in both men and women [84]. A slight difference has been found between men and women regarding HDL-c levels. Women have higher levels of HDL-c than men. Studies referred to estrogen's protective role [85]. HDL biogenesis HDL is a very condensed heterogeneous complex in comparison to other lipoproteins. HDL size is ranging from 1.063 to 1.210 g/mL. It contains a high composition of proteins in comparison with other lipoproteins [86]. Proteins form up to 60% of HDL mass, and ApoA-I is the main component of those proteins [87]. The biogenesis of HDL starts mainly in the liver upon the interaction between ApoA-I and the cell membrane protein ATP-binding cassette transporter A1 (ABCA1) [88]. ABCA1 is found in the basolateral surface of the hepatocytes [89]. The mutation in ABCA1 proteins could result in severe malfunctioning in HDL, as a result, a numerous amount of cholesterol is deposited in tissue macrophages leading to atherosclerotic formation [90]. Later,

this lipidated ApoA-I is catalyzed by an enzyme called lecithin/cholesterol acyltransferase (LCAT) [91]. The first step of transforming the immature HDL into mature occurs when scavenger receptor class B, type I (SR-BI) is mediating the cholesterol efflux from macrophages, and tissues into discoidal HDL [92].

The second step in transforming the discoidal hydrophobic core of ABCA1-derived HDL into spherical lipid-rich HDL is the interaction with LCAT for the second time [93]. A mutation in LCAT is also associated with cardiovascular diseases. Two disorders are related to LCAT mutation familial LCAT deficiency (FLD) which affects the esterification of cholesterol on HDL and LDL leading to the accumulation of discoidal HDL in the plasma. Fish eye disease (FED) affects the esterification of cholesterol on HDL only [94].

The cholesteryl ester transfer protein (CETP) pathway is following the RCT process. In this step, CETP facilitates the exchange of cholesteryl esters from HDL particles with the triglycerides in low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and Chylomicrons. Here, the liver uptakes the cholesterol via the LDL receptor, which is present in VLDL and LDL [95].

HDL is a dynamic particle, that undergoes continuous remodeling due to the instant interaction of cell receptors and other proteins and cofactors particularly serum amyloid A (SAA), hepatic lipase (HL), endothelial lipase (EL), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), scavenger receptor class B type I (SR-BI), ATP-binding cassette transporter G1 (ABCG1), the F1 subunit of ATPase (Ecto F1-ATPase), and apolipoprotein M (ApoM) [96, 97]. Furthermore, The HDL-associated apolipoproteins, which are bound to HDL interact with numerous cellular receptors e.g. ATP-binding cassette A1 or G1. This interaction is enabling the cholesterol efflux from cells to the HDL particle [98].

Therefore, HDL particles undergo continuous remodeling in physical structure and metabolites [99]. Importantly, inflammation disrupts the RCT pathway by reducing cholesterol trafficking from macrophage foam cells to the liver leading to dysfunctional HDL particles [100].

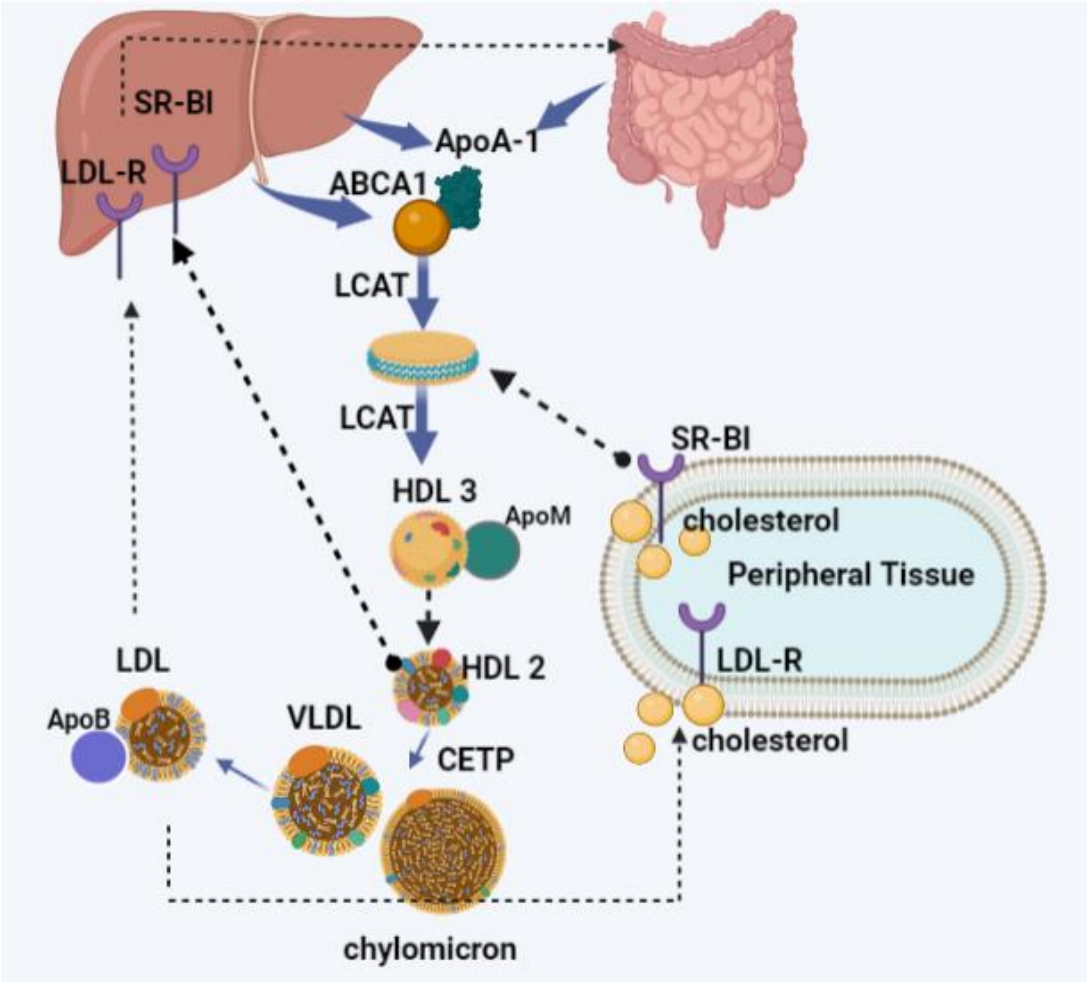

Figure 1. 2. The biogenesis of HDL and RCT. created by Hanaa Mousa**:** Upon the interaction of ApoA-1 and ABCA1, the resulting complex is catalyzed by the LCAT enzyme, forming a discoidal HDL. SR-B1 is mediating cholesterol efflux from macrophages, and tissues into discoidal HDL. Another interaction with LCAT formed the mature HDL. CETP facilitates the

exchange of cholesteryl esters from HDL particles with the triglycerides in LDL, VLDL, and Chylomicrons. The liver uptakes the cholesterol via the LDL receptor.

2.3.1 HDL anti-inflammatory properties

The apolipoproteins on the HDL surface such as ApoM and ApoD which are mainly associated with HDL particles, more than the other lipoproteins [101, 102], enable the HDL to possess other functions like anti-inflammatory, or anti-oxidative properties [87]. HDL suppresses the sphingosine kinase in the endothelial cell, an enzyme derived from sphingomyelins and considered essential in the activation of nuclear factor κB (NF-κB), which induces adhesion molecules [86]. In addition, HDL can suppress those adhesion molecules directly. An in vitro study in human umbilical vein endothelial cells (HUVECs), showed that HDL suppressed the cytokine-induced adhesion molecules in endothelial VCAM-1, ICAM-1, and E-selectin in a concertation-dependent manner [103]. Therefore HDL suppresses the adhesion molecules produced by endothelial, which is very crucial for cellular adhesion of circulating leukocytes during atherogenesis formation [104].

The anti-inflammatory effect was also seen in M2 macrophages where HDL3 induced markers such as reduced IL-6 and iNos expression. However, the scenario was opposed in van der Vorst et. al. study. In vitro experiments using murine and human primary macrophages illustrated that HDL provokes a pro-inflammatory reaction through passive cholesterol depletion. The result was confirmed using peritoneal macrophages from ApoA-1 transgenic mice, which have elevated HDL levels. *Pseudomonas aeruginosa* bacteria was used to induce infectious inflammatory conditions. HDL activated the TLR in general particularly TLR4 through PKC-NF-kB/STAT1-IRF1pathway followed by an elevation of inflammatory cytokine

expression IL-12 and TNF-α and down-regulation in the anti-inflammatory cytokines IL-10 [105].

HDL possesses some Antioxidant properties due to its association with antioxidant molecules e.g. paraoxonase/arylesterase 1 (PON1). PON1 enables the HDL to prevent LDL glycation and lipid peroxidation, thus protecting the cardiovascular system from the bad effect of glycated LDL particles [106].

2.3.2. Clinical aspects of HDL

A low serum concentration of HDL Less than 40 mg/dL (1.0 mmol/L) in men and Less than 50 mg/dL (1.3 mmol/L) in women is associated with a high risk of developing cardiovascular diseases [95]. Hyperalphalipoproteinemia is a genetic disease that causes a deficiency in HDL levels in the blood associated with a mild elevation in triglycerides. Hyperalphalipoproteinemia affects components responsible for HDL biogenesis ApoA-1, ABCA1, and LCAT. The Functional mutation in ABCA1 (Tangier disease) impairs the ABCA1's ability to lapidate the newly released ApoA-1 from the liver and intestines. That causes fast catabolism and clearance of ApoA-1, consequently low HDL levels. In in vivo trials, ABCA1 knockout in liver induced an 80% reduction in HDL concentration while ABCA1 knockout in the intestines causes a 30% reduction in HDL. When HDL cholesterol and ApoA-1 levels are low, as is the case in Fish Eye Disease, more small HDL particles are produced, and the overall HDL levels are decreased. The opposite scenario occurs in CETP deficiency where high levels of HDL cholesterol are associated with large HDL particles. Here, the deficient CETP will be unable to exchange triglycerides for cholesterol between HDL, VLDL, and LDL leading to the accumulation of large, malformed particles of HDL [107, 108].

Other than the hereditary causes of low HDL, factors like smoking, obesity particularly central obesity, alcohol, poor diet habits, lack of physical activity, metabolic disorders e.g., insulin resistance, and medications e.g. steroids, niacin, statins, etc. can lead to a decreased level of HDL [109, 110]. Low HDL was linked to several illnesses. A recent study of stable angina patients revealed that low HDL-c and high triglyceride levels were correlated with a higher prevalence of coronary atherosclerotic disease events. Accordingly, the ratio of HDL-c and triglycerides could predict the coronary atherosclerotic disease outcome independently of LDL-c [111]. Moreover, the risk of thyroid cancer was higher among the population with low HDL-c levels and disturbance of metabolomics status [112].

Nevertheless, A meta-analysis study of 108 randomized trials including 299 310 participants demonstrated that raising HDL alone was not able to modify the progression of cardiovascular diseases or the resulting death. Though the study suggested that lowering the LDL as a primary goal could be a good alternative [113]. The LDL-c/HDL-c ratio was found to be an accurate indicator of the severity of coronary artery disease in STEMI patients more than HDL or LDL [114]. Overall, both LDL and HDL are important in reflecting cardiovascular health.

Differences were spotted between genders related to the association of high HDL and major adverse cardiovascular events. In hypertension patients, females were more prone to develop cardiovascular events than males. This study contradicts our expectations regarding the protective role of HDL in females, however, the population of this study is elderly patients (the average age between 67-68 years old) [115].

2.3.3. The role of HDL in reducing inflammation by endotoxin neutralization

Inflammation is induced by pathogen-derived molecular patterns (PAMPs) like endotoxin or by tissue damage and the release of danger-associated molecular patterns (DAMPs). Sepsis is a severe inflammatory response that usually occurs due to bacterial endotoxin and overproduction of cytokines released from inflammatory cells particularly Tumor necrosis factor-alpha (TNF-α) [116]. Sepsis has a global burden and is associated with high mortality and morbidity [117]. Sepsis incidence has increased over the past decade where a study conducted from 1979 to 2016 estimated 31.5 million sepsis cases with 5.3 million annual deaths [118].

Toll-like receptor 4 (TLR4) is recognizing about 5% of circulating LPS. TLR4 is a transmembrane glycoprotein highly expressed on the surface of macrophages and other immune cells [119]. TLR4 recognizes endotoxin (LPS) via its co-receptor MD-2. The binding of LPS in the calyx of MD-2 leads to TLR4 dimerization and initiation of signaling cascade leading to NF-κB complex (nuclear factor kappa-light-chain-enhancer of activated B cells) activation as well as other signaling pathways, consequently inflammation and cytokines production [120-122]. NF-κB was found to have a negative effect on ApoA-1 and HDL-C through the suppression of peroxisome proliferator-activated receptor alpha (PPARα), a key regulator of lipid metabolism in the liver [123]. Experimental evidence revealed the downregulation of TLR4 upon the increase of the HDL-C after transferring ApoA-1 genes in mice. The decrease of TLR4 expression occurred after 2 weeks from transferring the ApoA-1 gene, and the fold change was about 8.4. The decrease of TLR4 continues even after the administration of LPS in those mice, and the survival rate was noticed to be improved [124]. Another study on mice demonstrated that the infusion of reconstituted HDL reduced

inflammation, improved survival, decreased bacterial count, and prevented the organs damage that occurs usually as a consequence of sepsis [125].

More studies have also shown that structural and functional modifications occur in HDL particles during sepsis. For instance, serum amyloid A (SAA1) is one of the acute phase proteins associated with HDL. During sepsis, SAA-1 is overexpressed replacing the ApoA-1 in HDL. SAA-1 binds directly to LPS and shuttles it with HDL to promote clearance. Clinical evidence showed that displacement of HDL contents leads to HDL particle remodeling which impacts the anti-inflammation and antioxidant functions [126, 127]. SAA-1 enriched HDL is enlarged the HDL size and more denser, as a consequence, the mobility of HDL becomes slower [128]. Moreover, the concentration of the total HDL in serum is decreased due to the pathophysiological changes in sepsis. Several mechanisms behind that decrease in HDL such as human secretory phospholipase A2 (sPLA2). This sPLA2 is an acute phase protein upregulated in presence of LPS, which stimulates SAA1, leading to modifications in HDL particle size and compositions [129]. The interaction between LPS and HDL is mediated by a couple of proteins e.g. CETP, PLTP, and LPS-binding protein (LBP). It is important to mention that HDL and associated proteins have a high affinity to bind and eliminate LPS more than other lipoproteins [130]. HDL evolved with time to be an important part of the innate immune system protecting against endotoxins and overstimulation of immune systems [131]. However, the exact mechanism by which HDL neutralizes endotoxin and confers anti-inflammatory activity is not fully elucidated.

3. Apolipoproteins

Apolipoproteins are a group of proteins considered key elements in lipoproteins composition. They are responsible mainly for lipid binding and transportation and are classified into apolipoprotein A (apoA1, apoA2, apoA4, and apolipoprotein A-V (apoA5), apolipoprotein B (apo B48 and apo B100), apolipoprotein C (apo C-I, apo C-II, apo C-III, and apo C-IV), apolipoprotein D, E, F, H, L, and M [132]. The four classes of apolipoproteins; ApoA-I, ApoAII, ApoCs, and ApoE, are almost found in all lipoproteins [133].

3.1. HDL-associated apolipoproteins

Several apolipoproteins were found associated with HDL. Those apolipoproteins have different functions and vary in their affinity for binding HDL particles (Table 1). However, we are going to focus on three apolipoproteins ApoA-1, ApoM, and ApoD.

Table 1.1. Apolipoproteins associated with HDL and their functions and the reference paper.

Apolipoprotein	Function	Reference
ApoA-1	Reverse cholesterol transportation	[134]
ApoA-II	cholesterol binding	[135]
ApoC-I	Inhibits of lipoprotein binding to LDL receptor	[136]
ApoC-II	Triglyceride hydrolysis of VLDL and CM for energy regulation	[137]
ApoC3-III	Antiatherogenic properties, and Triglyceride homeostasis.	[138, 139]
ApoD	Molecule transportation e.g. Retinoic acid, sphingomyelins.	[140]
ApoE	Lipid transportation	[141]
ApoM	A ligand of S1P, anti-inflammatory, antioxidant effects	[142, 143]
ApoL 1	Lipid transportation	[144]
ApoJ	Extracellular chaperone	[145, 146]

3.2. Apolipoproteins A-1 (ApoA-1)

Apolipoproteins A-1 is the major component of HDL, located on chromosome 11 and composed of 3 helical chains with 45 kDa molecular weight produced in the liver and intestine [147]. ApoA-1 production and regulation is depending on the next factors; peroxisome proliferator-activated receptor-γ (PPARγ), the hepatocyte nuclear factor 4 (HNF4), Liver Receptor Homologue 1 (LRH1), and the ApoA-I Regulatory Protein 1 (ARP1/NR2F2) [148]. The construction of HDL starts with the assembling of two to four molecules of ApoA-I with ABCA1. ApoA-1 is helping in stabilizing the ABCA1 protein in the hepatocytes and enterocytes, allowing for the synthesis of lipid-poor apoA-1 HDL particles by mediating the

efflux of cellular phospholipids and free cholesterol [149]. Most of the ApoA-1 in serum was found associated with HDL, and about 8% of the total ApoA-1 was found lipid-free [134]. In addition, ApoA-1 mediated the cholesterol that effluxes from macrophages in ABCA1-dependent and -independent mechanisms [150].

ApoA-1 is modulating innate immunity and adaptive immunity. For instance, ApoA-1 represses both NF-kB and phosphatidylinositol-3-kinase (PI3K). Add to that stimulating the Activator of Transcription Factor 3 (ATF3) (Toll-like receptor down-regulator), which prevents of activation the pro-inflammatory chemokines release e.g. CCL2, CCL5, and CX(3)CL1 [151, 152]. Iqbal et. al. in vivo study confirmed a similar result. They studied the effect of acute administration of ApoA-1 and study reported a significant reduction of human monocyte chemotaxis; CCL2 and CCL5 [153]. The aforementioned action of ApoA-1 protects against atherosclerosis, and because of that, some studies consider ApoA-1 a biomarker for the prediction of cardiovascular diseases [154]. However, A recent meta-analysis of 15 randomized controlled trials proved that HDL/apoA-1 mimetics fail to reduce the atheroma size in the artery in acute coronary syndrome patients [155]. On some occasions, the ApoA-1 was linked to a negative impact on health. Zeng et. al. reported a bad prognosis and survival rate in kidney renal clear cell carcinoma patients, who have high expression of ApoA-1 mRNA at the time of surgery [156].

3.3. Apolipoprotein M (ApoM)

ApoM is a 23 to 25 kDa protein. The Human apoM gene is located in the major histocompatibility complex (MHC) class III locus on chromosome 6 [157]. ApoM belongs to the lipocalin family (a group of proteins that transport small hydrophobic molecules) and is produced mainly from the liver, and kidney. Several important proteins belong to this group

such as retinol-binding protein (RBP), apolipoprotein D, and MD-2, the co-receptor for toll-like receptor 4 (TLR4) [158].

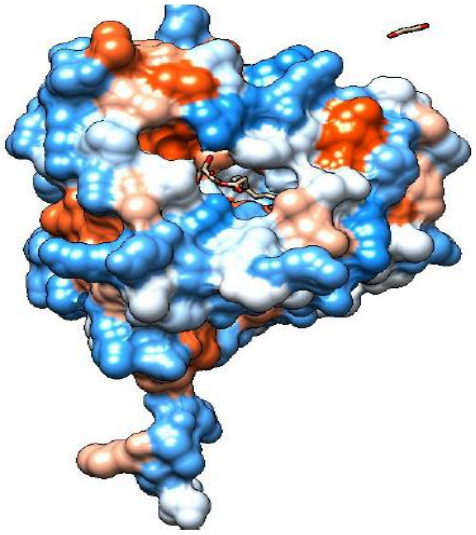

Figure 1. 3. 3D structure of ApoM generated using chimera. It is formed of a Lipocalin fold that consists of eight β-strands forming a β-barrel and an α-helix.

ApoM is required for HDL biogenesis, thereby 95 percent of plasma ApoM is found associated with HDL. ApoM is the carrier of sphingosine-1-phosphate (S1P), a bioactive lipid mediator that modulates vascular inflammation. Studies documented that ApoM-bound S1P is important for HDL antiatherogenic and anti-inflammatory effects [142]. Several transcription factors regulate ApoM expressions such as liver receptor homolog (LRH)-1, hepatic nuclear factor (HNF)-1α, and Forkhead box A2 (Foxa2). Any mutation in those factors could lead to ApoM dysregulation, thus HDL malfunctioning [159, 160]. However, it is not known if ApoM is a vitamin D-responsive gene i.e. contains a VDRE binding site. Structurally, ApoM has a typical lipocalin fold that consists of eight β-strands forming a β-barrel (pocket-like or calyx) and an α-helix [161].

ApoM is found associated mainly with HDL and to some lesser extent with LDL [162]. The terminal signal peptide of ApoM doesn't cleave from prior release to plasma as the case in the other proteins. This character helps ApoM in anchoring to HDL particle and preventing the dissociation between the two of them [163]. ApoM is a member of the lipocalin family [164]. This family has a special physical structure formed of 8 β-strands shaped a β-barrel-like or a calyx structure [158]. This unique structure is matching to some extent the MD2 unit in TLR4. MD2 is composed of 2 β-strands forming a cup-like structure [164]. The strong similarity of the structure of ApoM and MD2 suggests an analogous function of both of them, therefore may have an affinity for binding endotoxins.

3.3.1. ApoM function and S1P axis

Functionally, ApoM is known as a carrier of sphingosine-1-phosphate (S1P), a bioactive lipid mediator that modulates vascular inflammation. S1P is signaling sphingolipid produced intracellular and exerts through one of the G-protein coupled S1P-receptor family. This family includes S1P1, S1P2, S1P3, S1P4, and S1P5 receptors (S1P1-5). The vascular system is enriched with S1P1, S1P2, and S1P3, while S1P4 and S1P5 are found only in the hematopoietic and nervous system [165]. S1P is bound mainly with ApoM and to a lesser extent with albumin. The resulting complex ApoM/S1P is more stable in plasma [166, 167].

Some studies documented that the complex of ApoM/S1P is important for HDL antiatherogenic and anti-inflammatory effects [142]. Ruiz et.al. Report confirmed that the ApoM/ S1P complex was very crucial to suppress the vascular adhesion molecule-1 (VCAM-1) and E-selectin surface abundance during the inflammation. Whereas ApoM alone and HDL alone were not successful in conferring this adhesion suppression. Add to that the complex of albumin/ S1P didn't possess the same efficacy in suppressing those molecules as ApoM/ S1P complex. Indicating the importance of ApoM/S1P axis in maintaining a functional endothelial

barrier during inflammation [142]. The association of HDL/ApoM/S1P also might help HDL's vasoprotective and antiatherogenic effects [168, 169].

ApoM/S1P also was observed to be upregulated during the inflammation [170]. However, Winkler et. al. study reported a decline in S1P level in septic shock patients, they suggested that the drastic decrease in HDL level during septic shock is the reason behind such an effect [171].

S1P also interacts with endothelial scavenger-receptor class B type 1 (SR-BI). This SR-BI is also known as the cholesterol-sensing receptor. The importance of S1P is due to its role in facilitating the uptake of cholesterol from tissues by HDL particles [172]. Briefly, the associated S1P with HDL interacts with S1P1 to generate some proximity with SR-BI, later intracellular calcium is released and the cholesterol efflux and downstream signaling events will take place [173].

Certain disorders have been associated with the disturbance in ApoM levels and function for example plasma ApoM concentration was noticed to be decreased in type 2 diabetes patients [174] and heart failure [175]. Furthermore, the complications of diseases were seen to be positively associated with the change of ApoM level and therefore HDL function diabetes e.g. diabetic nephropathy [176]. Similarly, the change in ApoM activity due to genetic polymorphisms, for example, is noticed to increase the susceptibility to impacted HDL, and cholesterol metabolism, and have a higher chance of developing coronary artery diseases [177].

In several studies, ApoM was referred to as a protective agent. Bai et. al. studied ApoM in patients' tissues with primary liver cancer and discovered that the expression of ApoM gene was less in cancer tissue than in adjacent tissues. Furthermore, to understand the mechanistic relevance of the previous result they conducted in vitro and in vivo experiments using a mouse

model. The results showed that ApoM gene has a suppressive effect on the liver cancer cell. ApoM was found to inhibit cell proliferation and promotes apoptosis in cancerous cells through activation of cleaved-caspase-3, cleaved-caspase-9, and Bax/Bcl-2. In contrast, the deletion of the ApoM gene was found to exacerbate cancer progression via increasing migration and invasion [178].

Patients with type 2 diabetes were found to has a low concentration of ApoM in serum. ApoM/S1P complex exhibited a protective effect against insulin resistance through stimulating insulin production. ApoM activates AKT and AMPK pathways and increases SIRT1 expression, which an indicator of enhancement of the mitochondrial functions. The ApoM/S1P axis effects were mediated through activation of S1P1 and/or S1P3 [179].

However, most of the studies linked ApoM and S1P to a positive outcome on human health. Christoffersen et. al. research linked the overstimulation of the S1P1 receptor to the decrease in triglycerides uptake and brown adipose formation. In this study, they revealed that ApoM-deficient mice exhibited an increase in stimulating brown adipose tissue formation, increasing the triglyceride uptake, thereby protecting against diet-induced obesity [180]. Hajny et.al study also supports this claim, they examined the triglyceride turnover rate in the human ApoM transgenic mouse model. This model is designed for a higher concentration of ApoM and S1P levels than normal. Upon comparing ApoM transgenic with wild-type mice, researchers noticed a reduction in plasma in overall triglyceride turnover rate. Additionally, the rate of fatty acid uptake in subcutaneous adipocytes was lower than usual. This could be explained by the reduction of both plasma level of lipase and plasma level of fibroblast growth factor 21 (FGF21), as a response to the dramatic increase of ApoM and S1P level. Both lipase and FGF21 are very crucial for triglyceride clearance from plasma. The disturbance in their

level impacted causes an elevation in plasma triglycerides level after administrating a high-fat diet to ApoM transgenic mice [181].

Autophagy is an intracellular process where macromolecule such as endotoxins is degraded or recycled. The formed autophagosome is fused with the lytic lysosome to form autophagolysosomes that will degrade the engulfed molecules. Autophagy plays a critical role in maintaining balanced lipid metabolism in the liver [182]. Recently, ApoM deficiency was found to cause dysregulation in autophagy which resulted in altered lipid metabolism in the liver [183]. Autophagy is induced by LPS through a TLR4-TRIF-dependent pathway. While other TLR ligands are shown to induce autophagy via MyD88 and TRIF. Thus, TLRs are environmental sensors for the autophagy associated with innate immunity [184].

3.4. Apolipoprotein D (ApoD)

ApoD is another member of the lipocalin family associated with HDL and with the enzyme lecithin-cholesterol acyltransferase (LCAT) that converts cholesterol to cholesteryl ester. ApoD is secreted to plasma and enriched in brain testes, breast, and B cells. ApoD has a molecular weight of 33 kDa and is structurally similar to ApoM [185]. In contrast to other HDL-associated proteins like ApoA-1 which adopt a helix structure, ApoM and ApoD are lipocalins characterized by the typical β-sheet fold with calyx thereby, act as a carrier protein. ApoD binds hydrophobic ligands such as progesterone, retinoic acid, sphingomyelin pregnenolone, and arachidonic acid.[140]. ApoD plays a significant role in the neurological system, especially in the regeneration and healing of nerves. In vivo studies, ApoD was noticed to elevate tremendously during the healing process of sciatic nerve injury [186]. An increase in ApoD expression has been shown in some neurological diseases such as schizophrenia, bipolar disorder, and Multiple sclerosis [187]. Alzheimer's disease has been linked to the elevation of ApoD level in CSF and cortex [188, 189].

Moreover, in vivo studies, exhibit suppression of the innate immunity system by ApoD during the acute encephalitis induced by coronavirus OC43 in mice with restricted phospholipase A2 activity. Additionally, it has been demonstrated that overexpressing human ApoD in a mouse model of viral infection decreases T-cell infiltration into the CNS, lowers the production of pro-inflammatory cytokines including IL-1ß and TNF, and downregulates the activity of phospholipase A2 (PLA2)[190].

4. Apolipoprotein associated with others lipoproteins

4.1. Apolipoprotein B (ApoB)

ApoB is one of the apolipoprotein family linked to atherogenesis. ApoB is associated almost with all lipoproteins LDL, chylomicrons, VLDL, and IDL, (HDL is an exception) [191]. It is divided into two subclasses B-48, originating from the intestine, and apolipoprotein B-100 from liver. The complex of LDL and associated ApoB work as a ligand for LDL receptors. Any disturbance in ApoB occurs e.g. autosomal dominant familial defective ApoB, will affect the ApoB binding capacity to LDL receptor, thereby the LDL-cholesterol increases in the bloodstream [191]. Some studies considered ApoB as superior indicator of cardiovascular system status than the classical biomarkers. It can reflect the number of circulating atherogenic particles (LDL, chylomicrons, VLDL, and IDL). In some diseases e.g. coronary heart disease [192], and type 2 diabetes [193], ApoB was used as an early indicator for predicting the disease. Even in surgery, e.g. hepatocellular carcinoma patients who have curative resection of the liver ApoB was very useful predicting the outcomes. The patients with high values of ApoB had bad prognoses [194]. From another perspective, Sirniö et.al studied, the survival rate, systemic inflammatory biomarkers, and ApoB and ApoB/ApoA-1etc. of 144 colorectal cancer patients. They conclude that high serum ApoB levels associated with high

serum C-C Motif Chemokine Ligand 2(CCL2 levels) and higher levels of ApoA1 and ApoB, as well as a lower ApoB/ApoA1 ratio, are linked to better overall and cancer-specific survival. In addition ApoB/ApoA1 ratio was noticed to be correlated positively with serum levels of the next cytokines (IL-1ra, IL-6, IL-7, IL-8, IFNγ, CCL2 and PDGF-BB [195].

4.2. Apolipoprotein E (ApoE)

ApoE is 299 amino acid glycoprotein, produced mainly from the liver, macrophages, and astrocytes in the central nervous system [196]. The function of ApoE is similar to other lipoproteins, it is a carrier of cholesterol particles and helps lipid transportation. In addition to that ApoE is a carrier and regulator of amyloid-β (Aβ) in the brain. There are 3 subtypes of ApoE; ApoE2, ApoE3, and ApoE4. The difference between them is in one single amino acid [197, 198]. ApoE 4 was linked to the progression of neurological degenerative diseases e.g. Alzheimer's disease [198].

5. Vitamin D impact on HDL-associated proteins ApoA-1, ApoM, ApoD, and LL-37

The link between vitamin D and HDL-associated proteins, and in what way vitamin D affects the expression of apolipoproteins ApoA-1, ApoM, and ApoD in addition to LL-37 is still not fully clear. Some studies addressed part of this relationship. In vitro studies on HepG2 cells (hepatocellular carcinoma) revealed that hormonally active 1, 25-(OH)2 D3 was able to suppress ApoA-1 gene expression at the transcriptional level. This effect was mediated by VDR in addition to the vitamin D response elements in the ApoA-1gene. In this study, ApoA-1 secretion and mRNA levels were both inhibited in a dose-dependent manner after

administrating 1, 25-(OH)2 D3. A decrease in ApoA-1 promoter activity has been stated as well. [199].

Upon comparing the wild-type mice with VDR knockout ones, experiments illustrated an elevation in HDL-c in general and ApoA-1 expression in the liver. In addition to an increase in total cholesterol levels. Yet, some difference in serum concentration of HDL-c between female and male VDR knockout mice has been reported, where more increase has been noticed in males. Consequently, Apo A-1 mRNA was increased by 49.2% in males as well [200]. Indicating a stronger effect of vitamin D in suppressing the HDL and ApoA-1 in both genders and particularly in males. Due to species-specific differences, this could be a debated result since rodents and humans do not share similar regulatory mechanisms between HDL-c, ApoA-1, and VDR [201]. From another perspective, Vitamin D supplementation in human studies has a positive effect on HDL-c levels [202]. Radkhah et. al. systematic review suggested that vitamin D supplementation $\leq$12 weeks is increasing Apo-A1 levels, yet the data was not conclusive, and the link was not well elucidated [203].

A study demonstrates a direct relationship between ApoM and vitamin D receptors where it reported that ApoM results in overexpression of VDR [204]. However; the effect of vitamin D on ApoM expression is not known. The study of Yu et. al. on ApoM in Hepatocellular carcinoma cell lines using the CRISPR/Cas9 techniques revealed an interesting result. Knocking out ApoM genes in SMMC7721 cell lines was noticed to inhibit tumor cell death, and induce tumorigenicity characteristics i.e. increase the proliferation rate. This was followed by a huge reduction in VDR expression. Further investigations demonstrated that VDR overexpression was able to reverse the previous effect and suppresses the tumorigenicity. Therefore, ApoM can suppress the tumorigenicity characteristics in hepatocellular carcinoma cell lines via enhancing the expression of VDR [205]. In some diseases such as Systemic lupus

erythematosus (SLE), ApoM is inversely correlated with disease activity [206]. Similarly, ApoM was inversely related to the risk of type 2 diabetes [207]. Of note, ApoM level during vitamin deficiency is not yet addressed in the literature.

ApoD expression is affected in the presence of 1,25(OH)2 D3. ApoD was found to increase up to 5 folds in the presence of D3 which is more than the increase resulting from other steroid hormones (ApoD ligands). This increase is associated with an inhibitory effect on some cancers such as breast and prostate cancer cells [208, 209]. Those data are an indication of a direct relationship between ApoA-1, ApoM, ApoD, and vitamin D, although this association is not fully clear.

Vitamin D is known to be a potent inducer of cathelicidins (LL-37/ hCAP18) which are small cationic peptides that possess antimicrobial properties towards both gram-negative and gram-positive bacteria [210]. Cathelicidin (LL-37/ hCAP18) plays an important role in innate immunity by inhibiting the interaction between LPS and LPS-binding protein, preventing TLR4 activation, and resulting in decreasing TNF-α release [211-213]. Furthermore, LL-37 was found to suppress inflammation and cell death via inhibiting the IL-1β expression and caspase-1 activation [214]. Proteomics studies documented that LL-37 is found to be associated with lipoproteins such as HDL, VLDL, and LDL [215]. Further, the LL-37 gene has a vitamin D response element (VDRE) in its promoter thus it is highly induced by vitamin D [216]. However, the exact role of this association between HDL, LL-37, and Vitamin D in endotoxin clearance is still not fully understood.

The rationale of the study

Vitamin D deficiency and dyslipidemia are very common in Qatar. Vitamin D is known to exert anti-inflammatory properties. Patients with dyslipidemia have a low level of HDL and

are most likely to have accompanied Vitamin D deficiency. Low HDL is a risk factor for many chronic inflammatory diseases including atherosclerosis, cardiovascular disease, and metabolic syndrome. HDL possesses anti-inflammatory properties as well as antioxidant activity; however, the exact mechanism of the anti-inflammatory activity of HDL is not clear. Also, It is not clear why vitamin D deficiency is associated with dyslipidemia i.e low HDL and high LDL and triglycerides. It is not known whether vitamin D affects HDL biogenesis by affecting the expression of HDL-associated proteins e.g. ApoM. Inflammation is underlying many chronic diseases like metabolic syndrome, diabetes, atherosclerosis, and autoimmune diseases. Sepsis leads to high mortality rates and is a global health crisis. According to WHO sepsis affects more than 30 million people annually worldwide, with 6 million deaths. Sepsis is characterized by uncontrolled inflammation leading to an exacerbated immune response with cytokine storm triggered by lipopolysaccharides (LPS) endotoxin released from the Gram-negative bacteria [217]. Studies point to an important role of HDL and HDL-associated proteins ApoM, ApoD, ApoA-1, and LL-37 in sepsis However it is not clear if they have a role in endotoxin detoxification process, meaning minimize the cytokines released by clearing the endotoxins out of the system. The nature of the relationship between HDL and associated proteins (ApoM, ApoD, ApoA-1, and LL-37) and Vitamin D and their role in endotoxins clearance is not fully understood.

Hypothesis and Study Objectives

In this study, we are investigating the mechanisms by which HDL exerts anti-inflammatory properties. We hypothesize that HDL-associated proteins mainly ApoM, ApoD, and host defense cationic peptides LL-37 exert endotoxin neutralization activity. It is confirmed that LL-37 binds to LPS electrostatically and neutralizes its activity thus LL-37 is an immune modulator with anti-inflammatory activity.

TLR4 is the main endotoxin (LPS) sensor and upon activation, it leads to the secretion of pro-inflammatory mediators such as cytokines, chemokines, and lipid mediators like arachidonic acid and prostaglandins. Increased levels of pro-inflammatory mediators lead to subclinical inflammation, which in turn contributes to the pathogenesis of several chronic diseases.

The source of LPS in otherwise healthy humans is the gut microbiota where all microbial PAMPs are cleared by systemic circulation and in the liver. However, in cases of infection, endotoxin is released in large amounts in circulation leading to potent TLR4 activation and massive cytokine storm leading to sepsis. Also, sepsis increases gut barrier permeability which allows more LPS leaking to the circulation. Endotoxin neutralization is a very critical process required to maintain homeostatic immune responses. The majority of endotoxin molecules are cleared by HDL, however, the exact mechanism is not understood.

We hypothesize that HDL-associated proteins play an important role in the process of clearing endotoxin and preventing it from activating the TLR4 receptor. ApoM and ApoD are lipocalins that share high 3D homology to MD-2 the TLR4 co-receptor. TLR4 does not signal without MD-2 they form a dimer of 2 TLR4 and 2 MD-2 to form a complex that initiates an inflammatory signal once LPS is bound to MD-2. Therefore, we propose that ApoM, ApoD, and ApoA-1 bind LPS and anchor it to HDL, which prevents TLR4-MD-2 activation (competition for LPS). Further, cationic peptides such as LL-37, which is vitamin D inducible, bind to LPS and neutralize it [218]. It is not known if vitamin D induces ApoM expression. The goal is to investigate whether ApoM and ApoD contribute to LPS neutralization similar to LL-37 and whether vitamin D deficiency affects HDL-associated proteins (ApoM, ApoD, ApoA-1, and LL-37) expression. To achieve this goal the following specific aims will be investigated

Aim I- Transilatinal studies

Investigate the levels of HDL-associated proteins (ApoM, ApoD, ApoA-1, and LL-37) in VitD deficient and dyslipidemic adults. (metabolomics and proteomics approach)

Vitamin D deficiency is associated with low HDL; ApoM is critical for HDL biogenesis and ApoM deficiency result in very low HDL. However, it is not known whether VitD deficiency affects ApoM, ApoD, or ApoA-1 expression. ApoD and LL-37 are confirmed to be VitD-responsive genes. Also, the impact of vitamin D and dyslipidemia on metabolomic and proteomic profiles including the expression of ApoM, ApoD, ApoA-1, and LL-37 is not revealed yet.

We hypothesize that vitamin D deficiency leads to reduced expression of HDL-associated proteins (ApoM, ApoD, ApoA-1, and LL-37) consequently altering HDL composition, biogenesis, and function. Also, we anticipate a major change in metabolomic and proteomic profiles. Therefore, VitD deficiency could reduce the functionality including the anti-inflammatory properties of those apolipoproteins. In this aim we will answer the following question:

Q: What is the subclinical inflammatory status during vitamin D deficiency? What is the correlation between vitamin D and monocyte percentage?

Q: Is there an association between vitamin D status and HDL-associate proteins ApoM, ApoD, ApoA1, and LL-37? How does low vitamin D status affect HDL biogenesis and metabolomic and proteomic profiling?

To understand why vitamin D deficiency is associated with subclinical inflammatory status and low HDL (quantity and function), we will use data obtained from the Qatar Biobank database including proteomic and metabolic data.

Aim II- In Vitro studies

Determine the role of HDL-associated proteins ApoM an example in endotoxin neutralization.

Endotoxin is the most potent inflammatory molecule that binds to the TLR4-MD-2 receptor complex and induces inflammatory mediators release as well as ROS release. Endotoxin leak from the gut is cleared in circulation and liver, which prevents inflammation. HDL plays a crucial role in endotoxin clearance as it is estimated that 95% of endotoxin molecules are cleared via HDL. However, the mechanism by which HDL neutralizes endotoxin is not known. Further, it is not known why ApoM has a high affinity to bind HDL more than the other lipoproteins. We hypothesize that ApoM contributes to endotoxin clearance through its association with HDL. Here we will investigate the mechanism of endotoxin neutralization by HDL and examine the role of HDL-associated proteins ApoM in neutralizing endotoxin and reducing inflammation. To understand how HDL neutralizes endotoxin, the following questions will be experimentally addressed:

Q: Does ApoM bind to LPS directly through physical interaction or electrostatic charge attraction?

Q: What are endotoxin structural requirements for effective neutralization?

Q: Does HDL presence is important for ApoM neutralization activities? Or ApoM is alone enough to equalize HDL?

Aim III - In Vitro studies

Identify the effect of vitamin D and HDL-associated proteins, ApoM, ApoD, ApoA-1, and LL-37 expression in Monocytes.

Monocytes play a central role in innate immunity and inflammation as these immune cells mediate the secretion of large quantities of pro-inflammatory cytokines. Altered lipid homeostasis due to reduced HDL biogenesis impacts reverses cholesterol transfer (RCT) which leads to an increase in the monocytes' transformation to macrophages, subsequently inducing foam cell formation. These pro-inflammatory foam cells are a component of atherosclerotic plaque and exacerbate atherosclerosis. Vitamin D exerts anti-inflammatory effects by reducing cytokines IL-1β and IL-6 released from macrophages [219]. Vitamin D possesses immune-modulatory effects by inducing or suppressing many genes containing VDRE, thereby affecting pathophysiology and cellular responses during perturbation.

To understand why vitamin D deficiency is associated with dyslipidemia and how it affects HDL biogenesis, we will experimentally answer the following questions:

Q: Does vitamin D induces ApoM and other HDL-associated proteins expression in Monocytes (THP1)? Are ApoM, and ApoA-1 vitamin D responsive genes similar to LL-37 and ApoD? Is vitamin D affect the cytokine released from monocytes besides affecting HDL-associated proteins?

CHAPTER 2: VITAMIN D AND INFLAMMATION

Vitamin D deficiency is associated with subclinical inflammatory status. Healthy subjects with a low level of vitamin D have an elevated level of CRP and higher serum concentration of the pro-inflammatory cytokines TNF-α and IL8 than the vitamin D-sufficient subjects [220]. Monocytes are a vital key factor in innate immunity and they formed up to 5% of circulating nucleated cells [221]. Monocytes regulate cellular homeostasis and activate several pro-inflammatory pathways leading to augmenting the inflammatory status [222]. Both forms of vitamin D the hormonally active 1,25(OH)2D3 and the non-active one; 25(OH)D3, have a direct impact on the human monocytes. In dose-dependent- matter, they suppress the p38 phosphorylation in monocytes, and the production of IL-6, and TNF-α [223]. On the other hand, vitamin D attenuates monocytes' ability to chemo-attract other monocytes or macrophages to atherosclerotic plaque sites by downregulating the chemoattractant protein 1 (MCP-1) [224]. In addition, vitamin D has a great impact on lipoproteins profile particularly HDL. Low baseline level of 25(OH)D3 found to be associated with disturbance in HDL-C level or function [225, 226]. Therefore, Monocyte-to-HDL Ratio (MHR) could be a good indicator reflecting the subclinical inflammatory status resulted from low level of vitamin D. Here, in this paper we shed light on the novel biomarker of subclinical inflammation Monocytes percentage and HDL (MHR) inverse association with vitamin D deficiency in young healthy adult population in Qatar. This study answering the next questions; what is the inflammatory status during the vitamin D deficiency, what is the correlation between vitamin D and Monocytes percentage? The paper was published in Nutrients journal and released in 2020 [227]. Similar conclusion has been confirmed by following studies such Matteis et. al. where

they revealed an inverse association of MHR and vitamin D and considered it not just is a good indicator in reflection of the subclinical inflammatory status during vitamin D deficiency, but also could predict the presence of vitamin D deficiency in the healthy population [228].

This paper is a translational study conducted to fulfill part of Aim I and to spot the light on the inflammatory status represented by Monocytes percentage and HDL during the vitamin D deficiency in the population in Qatar. This study answers the next questions; what is the inflammatory status during vitamin D deficiency, and what is the correlation between vitamin D and monocyte percentage? The paper has been published in the Nutrients journal and released in 2020 [227].

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Serum 25-hydroxyvitamin D is Inversely Associated with Monocyte Percentage to HDL Cholesterol Ratio among Young Healthy Adults in Qatar.

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Abstract: Low serum 25-hydroxyvitamin D [25(OH)D] is linked to an altered lipid profile. Monocytes play an important role in inflammation and lipid metabolism. Recently, the monocyte percentage to HDL-cholesterol ratio (MHR) has emerged as a novel marker of

inflammation. We investigated the association between serum 25(OH)D concentrations and MHR and serum lipids in young healthy adults. Data from the Qatar Biobank were utilized to investigate the relation between serum 25(OH)D and inflammation and serum lipid concentrations in healthy Qatari adults using multivariate regression analysis. Prevalence of serum 25(OH)D concentrations <12 ng/mL (deficiency), 12–20 ng/mL (insufficiency), and ≥20ng/mL (sufficiency) were 55.8%, 29.9%, and 14.3%, respectively. Serum 25(OH)D was significantly inversely associated with monocyte percentage, MHR, total cholesterol, LDL-cholesterol, and triacylglycerol in multivariable-adjusted analysis. MHR could be a potential biomarker to predict cardiometabolic diseases among young healthy Qataris.

Keywords: Vitamin D; 25-hydroxyvitamin D; HDL; monocyte percentage; MHR; inflammation

1. Introduction

The classical function of vitamin D is to maintain the homeostasis of calcium and phosphorous. Vitamin D can be obtained from diet and skin exposure to the sun's UVB light. Regardless of the source, in the liver, vitamin D is converted to 25-hydroxyvitamin D [25(OH)D], a major circulatory vitamer. Further, in the kidney, 25(OH)D is converted to 1,25 dihydroxyvitamin D [1,25$(OH)_2$D] by 25-hydroxyvitamin D-1α-hydroxylase [2]. Non-calcemic effect of vitamin D is through the action of 25$(OH)_2$D via vitamin D receptors. Low circulating concentration of 25(OH)D is highly prevalent in Qatar. Previous studies conducted in 2012 reported an estimated 90% of the Qatari population suffer from various degrees of

vitamin D insufficiency and deficiency [229]. More recently, Al-Dabhani reported that 64% of the population in Qatar suffers from vitamin D deficiency [27].

The impact of low vitamin D on health outcomes is well documented [230]. Specifically, vitamin D deficiency is related to several chronic inflammatory diseases [231] such as metabolic syndrome, obesity, and cardiovascular diseases (CVD) [232, 233]. Therefore, by improving serum vitamin D may lead to reduced inflammation and CVD [234]. Zughaier et al. [219] reported that the hormonally active $1,25(OH)_2D$ leads to a significant decrease in IL-6 and IL-1β gene expression in monocytes exposed to an inflammatory stimulus such as lipopolysaccharide.

Monocytes are central immune cells that display a wide range of homeostatic and immune response functions. Because monocytes are major secretors of pro-inflammatory mediators such as cytokines, an increase in monocyte percentage may be an indication of sub-clinical inflammation [235, 236]. Additionally, HDL is a major component of total cholesterol that has been known to have anti-inflammatory and protective functions, hence dubbed as the good cholesterol [237]. Thus, when monocyte percentage is elevated and HDL is reduced, the monocyte to HDL cholesterol ratio (MHR) is elevated which suggests a homeostatic perturbation and sub-clinical inflammation. Additionally, vitamin D deficiency has been linked to dyslipidemia such as low HDL-cholesterol [238-240]. However, it is not very clear about the mechanism through which vitamin D exerts its effects on lipids [57, 58].

Recently, MHR has been recognized as a novel biomarker of subclinical inflammation [241-243]. The total number of monocytes is inversely related to HDL and MHR is reported to be elevated in many disorders such as hypertension [244], atherosclerosis [245], and diabetic nephropathy [246]. Elevated MHR is associated with disease severity in CVD such as coronary

artery stenosis [247, 248]. MHR is also associated with a chronic inflammatory condition such as chronic obstructive pulmonary disease [249]. MHR was reported in many studies as an independent prognostic biomarker in many diseases associated with high inflammatory status particularly disorders linked to cardiac disorders or atherosclerotic events. For example, in infective endocarditis patients, MHR alone predicted in-hospital death with relatively higher sensitivity (74.4%) and specificity (57.6) [250]. In acute ischemic stroke, patients with higher MHR showed higher susceptibility to develop clinical complications such as intracranial hemorrhage [251]. However, the association between serum 25(OH)D and MHR is not known yet. Therefore, in this study, we investigated the relationship between serum 25(OH)D concentration and subclinical inflammation biomarker, MHR in healthy young adults in Qatar.

2. Materials and Methods

2.1. Study Design and Study Participants

This study is a cross-sectional, retrospective study based on the data collected by the Qatar Biobank (QBB). The study sample broadly represents the population of Qatar. Briefly, the QBB collected data on Qataris and non-Qataris who have been living in the country ≥15 years. The participants were ≥18 years old. Data on general health and lifestyle, diet, cognitive function, and physical and clinical measurements were collected. Further, several health and clinical biomarkers were measured in blood, urine, and saliva. The detailed methodologies were described elsewhere [252, 253]. Ethical IRB approval (QBB-RES-ACC-0237-0142) and confidentiality agreements were obtained before conducting this study. The total number of participants in this study was 874, however, 14 participants were excluded as gender was missing. Hence the initial analysis included 860 participants (men, n = 399; women, n = 461).

The inclusion criteria were young healthy Qatari adults between the ages of 18 and 40 years who did not have any co-morbidities. The exclusion criteria included those using vitamin D supplements and those with CVD, diabetes, hypertension, kidney disease, liver disease, pregnancy, cancer, and critical illness. Thus, the sample contained apparently healthy subjects. Vitamin D status was defined as deficiency, insufficiency, and sufficiency if the serum 25(OH)D concentrations were <12 ng/mL, 12-<20 ng/mL, ≥20 ng/mL, respectively. This classification was based on the Institute of Medicine's recommendations [254].

2.2. Physical and biochemical measurements

Participants' height, weight, and BMI were measured with light clothing by trained nurses. Bodyweight was measured using the TANITA BC-418 MA instrument. BMI was computed using weight in kg divided by height in m^2. Venous blood samples were collected from participants after overnight fasting. Blood specimens were sent to Hamad Medical Corporation Laboratories (College of American Pathologist Accredited Laboratory) for further analysis. Monocytes, white blood cells (WBC), lymphocytes, and neutrophils were measured as part of the differential while blood cell count. All the blood biomarkers such as serum 25(OH)D, plasma glucose, serum HDL cholesterol, serum total cholesterol, serum LDL cholesterol, and serum triacylglycerol were measured all at once. Serum 25(OH)D concentration (included both vitamin D_2 and vitamin D_3 fractions) was measured using electrochemiluminescence immunoassay (LIAISON® 25-hydroxyvitamin D Total Assay, DiaSorin Inc., Stillwater, MN, USA). Plasma glucose was measured with the enzymatic/amperometric method (Nova Statstrip and Roche Accu-Check Inform II devices). Serum total cholesterol was measured with enzymatic CHOD-PAP method. HDL cholesterol Plus Third Generation Method was used to measure the serum HDL cholesterol. LDL cholesterol Plus Second Generation Method was

used to measure the serum LDL cholesterol. Serum triacylglycerol was measured with enzymatic GPO-PAP method. Detailed methodologies were described elsewhere [252, 255].

2.3. Statistical Analysis

Baseline characteristics according to the serum 25(OH)D concentrations were presented as frequencies and percentages for categorical variables and as means (SD) for continuous variables. Data were tested for normality using Shapiro-Wilk test. Histograms were constructed to detect the normality. Although MHR and 25(OH)D concentrations were not normally distributed based on the significance, the histogram revealed that the data were very close to normal. Therefore, regression analysis was performed on non-transformed data. Comparisons between participants with serum 25(OH)D concentrations (sufficient, insufficient, and deficient) were performed using chi-squared Tests (or Fisher exact tests for cells <5) for categorical variables. ANOVA was used for normally distributed numerical variables and Kruskal-Wallis tests were used for non-normally distributed interval variables. Accordingly, Chi-Squared was used for gender, while ANOVA was used for age, BMI, WBC, monocyte, lymphocyte, neutrophil, neutrophil percentage to HDL ratio (NHR), total cholesterol, LDL, and HDL. Kruskal-Wallis test was used for MHR, lymphocyte percentage to HDL ratio (LHR), WBC percentage to HDL ratio, and triglycerides. Additionally, baseline characteristics between men and women were also reported for selected characteristics. The differences between men and women for monocyte percentage, serum HDL cholesterol, and MHR were tested with an independent, 2-tailed *t*-test.

An association between serum25(OH)D and inflammatory markers and serum lipids were analyzed with multinomial logistic regression using gender, age, BMI, and smoking as confounding variables. In this analysis, the sample size varied from 702 to 706 depending on

the variable. We also performed a forward stepwise multinomial regression adding each confounding variable at a time to study the impact of each confounding variable on the relationship between serum 25(OH)D and MHR. In the multinomial logistic regression analysis, 25(OH)D ≥ 20 ng/mL category was used as a reference category. Multinomial logistic regression coefficients (β) and their 95% confidence intervals (95% CI) were reported. Further, an association between serum 25(OH)D concentrations and inflammatory markers and serum lipids were analyzed with multivariable linear regression using gender, age, BMI, and smoking as confounding variables. In this analysis, all serum 25(OHD, inflammatory markers, and serum lipids were used as continuous variables. Multivariable regression coefficients (β) and their 95% confidence intervals (95% CI) were reported. Additionally, we performed a restrictive Cubic Spline adjusted regression analysis to assess whether the relationship between serum 25(OH)D concentrations and MHR was linear. For simplicity, we did not report cubic spline analysis between serum 25(OH)D and other inflammatory markers and serum lipids. All statistical analyses were two-sided. A $p < 0.05$ was considered statistically significant. Analyses were performed using the Stata statistical software package 16 (Stata Corp, College Station, TX, USA).

3. Results

3.1. Serum Vitamin D Concentrations in Young Healthy Adults in Qatar

Table 1 shows the baseline characteristics based on serum 25(OH)D concentrations. In this study, the prevalence of serum 25(OH)D <12 ng/mL and ≥20 ng/mL were 55.8% (n = 488) and 14.2% (n = 125), respectively. The mean BMI was significantly higher in the vitamin D deficient group (28.3 kg/m^2) in comparison with a sufficient group (26.4 kg/m^2). Monocyte

percentage was significantly higher in participants with vitamin D deficiency compared to sufficiency. In the vitamin D sufficient group, the MHR mean was significantly lower (5.1) compared to the vitamin D deficiency group (5.8) or the vitamin D insufficiency group (5.7) ($p < 0.011$).

Table 2. 1. *Characteristics of the study population based on serum 25(OH)D concentrations (n = 860)* [1].

	Vitamin D Deficiency (serum 25(OH)D, <12 ng/mL)	Vitamin D Insufficiency (serum 25(OH)D, 12-<20 ng/mL)	Vitamin D sufficiency (serum 25(OH)D, ≥20 ng/mL)	*p*-Value [2]
n	488	261	125	
Gender [3]				
Women	274 (56%)	131 (51%)	56 (46%)	ns
Men	214 (44%)	130 (50%)	55 (44%)	
Age (years)	28.8 (6)	30.3 (5.9)	29.8 (5.8)	0.002
Body mass index, kg/m^2	28.3 (6.8)	27.6 (5.3)	26.4 (5.5)	0.008
White blood cells, cells/10^9 L	6.8 (2.0)	6.8 (2.0)	6.7 (1.8)	ns
Monocyte, %	7.5 (1.9)	7.3 (1.9)	6.9 (1.6)	0.014
Lymphocyte, %	35.6 (8.6)	35.1 (9.4)	36 (9.1)	ns
Neurtophil, %	53 (9.8)	54.1 (10.4)	53.5 (10.1)	ns
Monocyte % to HDL ratio	5.8 (2.3)	5.7 (2.5)	5.1 (1.8)	0.011
Lymphocyte % to HDL ratio	27.3 (10.1)	26.9 (11.1)	26 (8.4)	ns
Neutrophil % to HDL ratio	41 (14)	42 (16.2)	39 (12.2)	ns
Total cholesterol, mmol/L	4.8 (0.8)	4.8 (0.7)	4.6 (0.8)	ns

	Vitamin D Deficiency (serum 25(OH)D, <12 ng/mL)	Vitamin D Insufficiency (serum 25(OH)D, 12-<20 ng/mL)	Vitamin D sufficiency (serum 25(OH)D, ≥20 ng/mL)	*p*-Value [2]
HDL-cholesterol, mmol/L	1.4 (0.4)	1.4 (0.4)	1.4 (0.3)	ns
LDL- cholesterol, mmol/L	2.8 (0.8)	2.8 (0.7)	2.7 (0.7)	ns
Triacylglycerol, mmol/L	1.2 (0.7)	1.1 (0.7)	1.0 (0.5)	ns
Glucose, mmol/L	5.0 (0.7)	5.0 (0.9)	4.9 (0.7)	ns

[1] Data are presented in mean (±SD) for continuous measures and n (%) for categorical measures; ns: not significant. To convert ng/mL to nmol/L, multiply with 2.496.[2] Serum vitamin D categorization was based on Institute of Medicine guidelines.[3] Significance in Chi-Squared test for proportions or ANOVA for continuous measurements

Additionally, gender differences in selected characteristics were described in Figures 1 and 2. Monocyte percentage was significantly lower in women compared to men, while the HDL was significantly higher in women compared to men ($p < 0.001$). The MHR was significantly lower in women compared to men (Figure 2A). However, the MHR was significantly lower in women compared to men in vitamin D deficient group ($p < 0.004$) but not in vitamin D insufficiency or in sufficiency group (Figure 2B).

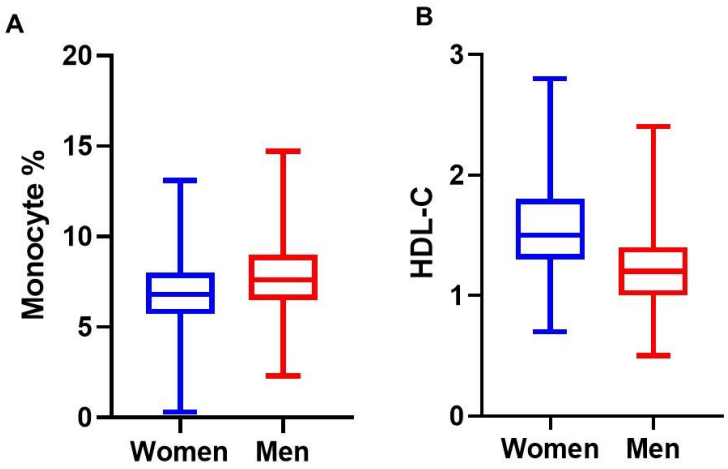


Figure 2. 1. Differences in selected baseline characteristics between men and women. (A): Monocyte percentages in women and men (6.95 vs. 7.87; $p < 0.001$ for independent, 2-tailed t-statistic). (B): HDL cholesterol concentrations in women and men (1.56 vs 1.24 mmol/L; $p < 0.001$ for independent, 2-tailed t-statistic).

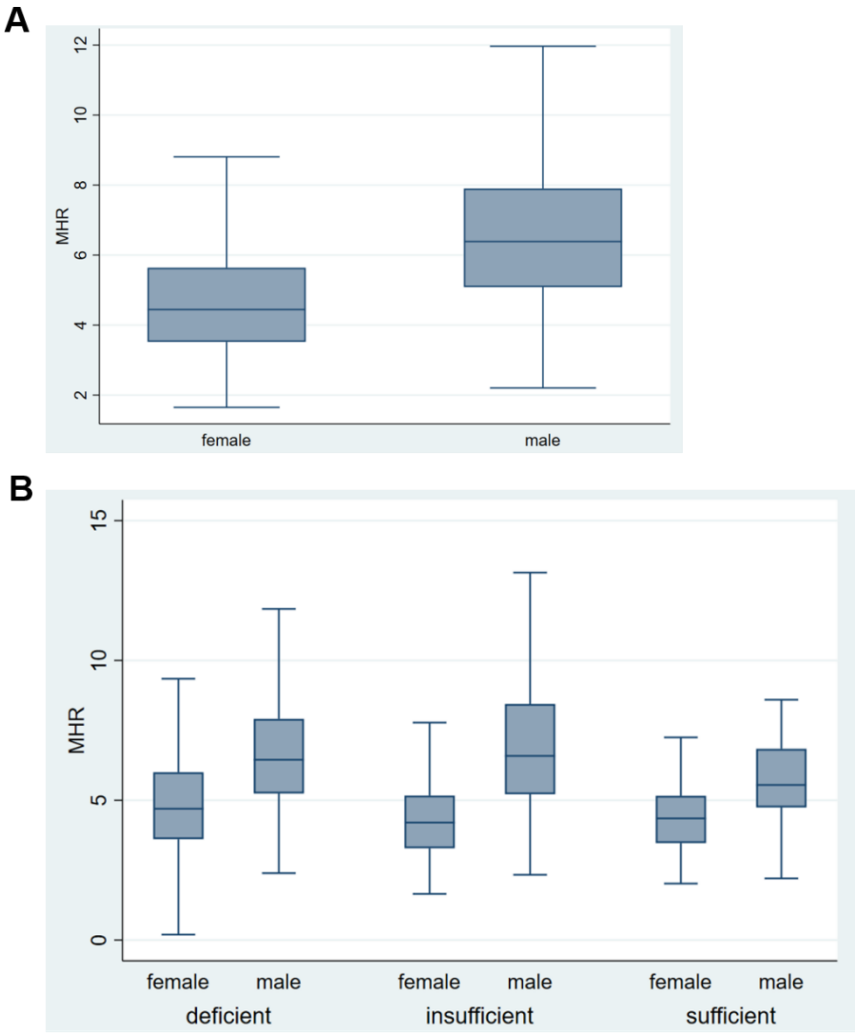


Figure 2. 2. Comparison of MHR based on serum 25(OH)D concentrations in healthy young adults in Qatar. Serum 25(OH)D concentrations were defined as deficiency (<12 ng/mL), insufficiency (12-<20 ng/mL), and sufficiency (≥20ng/mL). (**A**): Boxplots of MHR in women compared to men ($p < 0.001$ for t-statistic). (**B**): Boxplots of MHR for men and women within vitamin D deficient ($p < 0.004$ for independent, 2-tailed t-statistic), vitamin D insufficient ($p < 0.58$ for independent, 2-tailed t-statistic), vitamin D sufficient ($p < 0.21$ for independent, 2-tailed t-statistic) categories. To convert ng/mL to nmol/L, multiply with 2.496. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; MHR, monocyte percentage to HDL cholesterol ratio; VitD, vitamin D.

3.2. Association between serum 25(OH)D Concentrations and Inflammation Biomarkers and Serum Lipids

The association between serum 25(OH)D concentrations and inflammatory markers and serum lipids (categorized form) are presented in Table 2. In the multivariable regression analysis, a significant association between serum 25(OH)D and MHR was observed in the deficient group, ($\beta = 0.19$; $p < 0.005$) in comparison to the sufficient category. Also, in vitamin D insufficient participants, the regression coefficient was 0.15 ($p < 0.03$). The monocyte percentage coefficient was statistically significant in vitamin D deficient participants 0.19 ($p < 0.006$) in comparison to the vitamin D sufficient category. In contrast, no associations were observed between serum 25(OH)D concentration and lymphocyte percentage, neutrophil percentage, LHR, and NHR. Interestingly, in vitamin D deficient participants, serum lipids such as serum total cholesterol ($p < 0.014$), serum LDL-cholesterol ($p < 0.03$), and serum triacylglycerol ($p < 0.04$) but not serum HDL-cholesterol were significantly associated with serum 25(OH)D. Plasma glucose was not significantly associated with serum 25(OH)D concentrations.

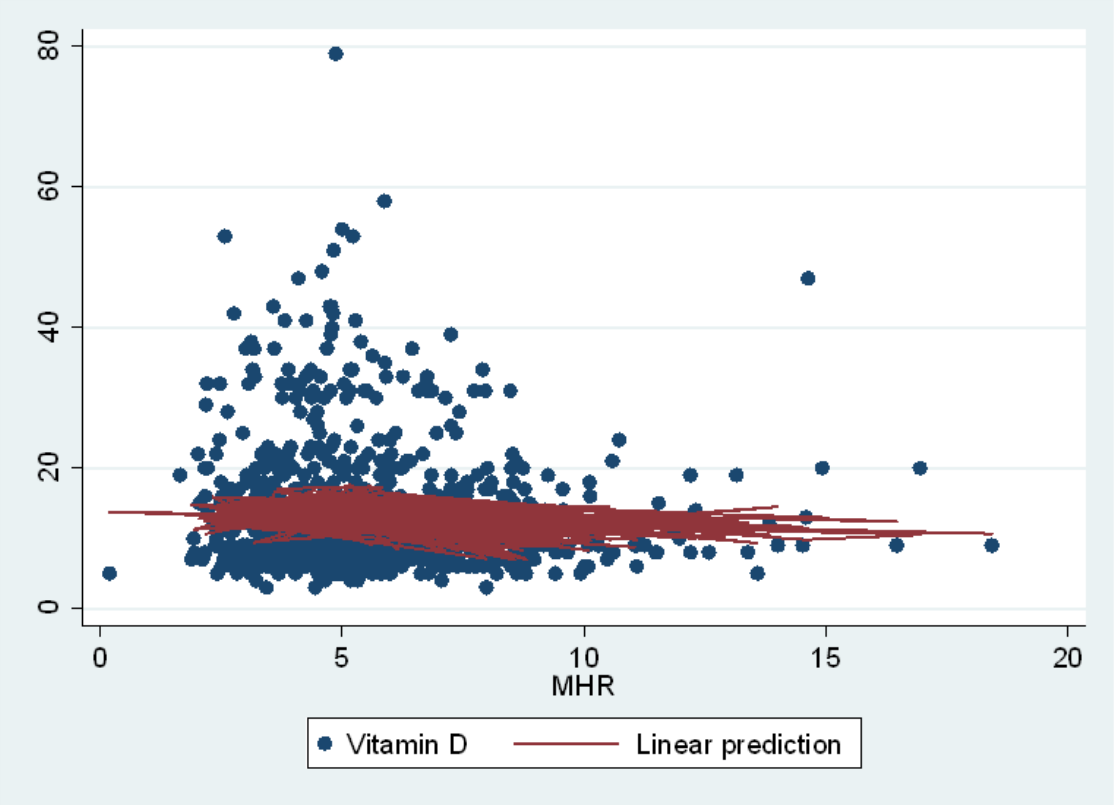
Table 2. 2. Association between serum 25(OH)D concentrations and inflammatory markers and serum lipids in young healthy adults in Qatar [1].

	Vitamin D Deficiency (<12 ng/mL) [2]		Vitamin D Insufficiency (12-<20 ng/mL) [2]		Continuous Association [3]	
	β (95% CI)	***p*-Value**	**β (95% CI)**	***p*-Value**	**β (95% CI)**	***p*-value**
White blood cell, cells/10^9 L (*n* = 702)	0.01 (−0.11, 0.14)	ns	0.02 (−0.11, 0.15)	ns	0.09 (−0.22, 0.41)	ns
Monocyte % (*n* = 702)	0.2 (0.06, 0.34)	0.006	0.09 (−0.05, 0.24)	ns	−0.54 (−0.19, −0.2)	0.002
Lymphocyte % (*n* = 702)	−0.01 (−0.03, 0.02)	ns	−0.02 (−0.04, 0.01)	ns	0.01 (−0.06, 0.08)	ns
Neurtophil % (*n* = 702)	−0.01 (−0.03, 0.02)	ns	0.01 (−0.01, 0.04)	ns	0.02 (−0.03, 0.08)	ns
Monocyte % to HDL ratio (*n* = 701)	0.19 (0.06, 0.32)	0.005	0.15 (0.02, 0.29)	0.03	−0.38 (−0.66, −0.1)	0.008
Lymphocyte % to HDL ratio (*n* = 701)	0.01 (−0.02, 0.04)	ns	0.01 (−0.02, 0.04)	ns	−0.02 (−0.08, 0.04)	ns
Neutrophil % to HDL ratio (*n* = 701)	0.02 (−0.004, 0.04)	ns	0.01 (−0.01, 0.02)	ns	0.001 (−0.04, 0.04)	ns
Total cholesterol, mmol/L (*n* = 706)	04 (0.08, 0.72)	0.014	0.25 (−0.09, 0.58)	ns	−1.03, −1.81, −0.26)	0.009
HDL-cholesterol, mmol/L (*n* = 706)	−0.2 (−0.94, 0.54)	ns	−0.22 (−0.1, 0.58)	ns	0.54 (−1.42, 2.4)	ns
LDL- cholesterol, mmol/L (*n* = 703)	0.4 (0.05, 0.74)	0.03	0.27 (−0.1, 0.63)	ns	−0.98 (−1.84, −0.12)	0.026
Triacylglycerol, mmol/L (*n* = 706)	0.49 (0.02, 0.93)	0.04	0.31 (−0.16, 0.78)	ns	−1.06 (−2.0, −0.11)	0.028
Glucose, mmol/L (*n* = 706)	0.2 (0.18, 0.58)	ns	0.1 (−0.26,0.55)	ns	−0.34 (−1.2, 0.49)	ns

[1] Persons with chronic diseases and who were taking prescription medication were not included in the study. To convert ng/mL to nmol/L, multiply with 2.496. Abbreviations: 25(OH)D, 25-hydroxyvitamin D; β, regression coefficient; ns, not significant. [2] Multinomial logistic regression analysis was adjusted for gender, age, BMI, and smoking. Serum vitamin D categorization (deficiency, insufficiency, and sufficiency) was based on Institute of Medicine guidelines. Vitamin D sufficiency (≥20 ng/mL) was used as a referent

category. [3] Multivariable regression analysis was adjusted for gender, age, BMI, and smoking. Serum 25(OH)D and inflammatory markers and serum lipids were used as continuous variables.

Additionally, the multivariable associations between serum25(OH)D and inflammatory biomarkers and serum lipids (continuous form) are presented in Table 2. We observed a significant inverse relation between serum 25(OH)D and monocytes percentage, ($\beta = -0.54$; $p < 0.002$), MHR, ($\beta = -0.38$; $p < 0.008$), serum total cholesterol, ($\beta = -1.03$; $p < 0.009$), serum LDL cholesterol, ($\beta = -0.98$; $p < 0.026$), and serum triacylglycerol ($\beta = -1.06$; $p < 0.028$). The relationship in continuous variable regression analysis, between serum 25(OH)D and monocytes percentage, MHR, and serum total cholesterol, LDL cholesterol, and triacylglycerol was much stronger compared to the multinomial logistic regression.



Serum 25(OH)D had a linear relationship with MHR in Restricted Cubic Spline multivariable adjusted regression analysis when analyzed as continuous variables. In both logarithmic transformed and non-transformed analyses, we observed a significant linear relationship between serum 25(OH)D and MHR ($p < 0.001$) (Figure 3). In stepwise forward regression analyses, age, gender, BMI, and smoking were significantly related in all regression models. However for simplicity, in Figure 3, we only reported the multivariable adjusted relationship between non-transformed serum 25(OHD and MHR in a continuous form (without categorization of serum 25(OH)D concentrations).

Figure 2. 3. Restricted Cubic Spline Regression between serum 25(OH)D concentration and MHR after adjustment for age, gender, BMI, and smoking in young health adults in Qatar (n = 701). Serum 25(OH)D and MHR were used as continuous variables. Linear relationship between serum 25(OH)D and MHR was significant ($p < 0.001$). Abbreviations: 25(OH)D, 25-hydroxyvitamin D;MHR, monocyte percentage to HDL cholesterol ratio.

4. Discussion

We have investigated the relationship between serum 25(OH)D concentrations and MHR and serum lipids in healthy young Qatar population. This is the first study to report an inverse relationship between 25(OH)D and MHR. This relationship was measured in using 2 separate statistical procedures, i.e., stepwise multinomial adjusted logistic regression using serum 25(OHD as categorical variable and multivariable adjusted linear regression using 25(OH)D and inflammatory markers and serum lipids as a continuous variable. In categorized analysis, vitamin D deficiency was significantly related to MHR. In the continuous regression, serum 25(OH)D was significantly, inversely related to MHR and serum lipids. It is interesting to note that based on regression coefficients, the relationship was much stronger in the continuous regression analysis.

MHR is an emerging novel maker of CVD [256] such as ischemic stroke [257] and cerebral hemorrhage [258]. Elevated MHR is an indicator of systemic inflammation as well as oxidative stress [241]. In disorders like polycystic ovary syndrome, MHR was found to be more sensitive than the usual markers such as increased BMI and C-reactive protein (CRP) in predicting disease development [259]. Moreover, MHR is reported to predict the severity and complication in diseases such as obstructive sleep apnea, and MHR was used to anticipate the cardiovascular sequels [260]. MHR also demonstrated efficiency in predicting short-term mortality in patients with ST-segment elevation myocardial infarction [261].

Elevation in monocyte percentage can occur in infection, inflammation, and other cellular perturbations such as autoimmunity disorders, thus reflecting clinical inflammation [262]. Monocyte percentage elevation suggests more perturbation and inflammatory cytokine release, whereas absolute monocyte number vary among participants and gender. Therefore, in this

study, monocyte percentage is used to better reflect sub-clinical inflammation status. Vitamin D is known to exert anti-inflammatory effects on monocytes leading to reduced pro-inflammatory cytokines release and reprogramming of cells [219, 263]. Vitamin D deficiency is observed in various chronic inflammatory diseases indicating the anti-inflammatory effect of vitamin D [264] We found a significant inverse association between serum vitamin D and the subclinical inflammation marker, MHR, among healthy young adults. This relation could be explained by several mechanisms. Vitamin D is a suppressor of endoplasmic reticulum stress leading to downregulation of adhesion molecules such as PSGL-1, β(1)-integrin, and β(2)-integrin, consequently, decreasing the monocytes activation [265]. Furthermore, vitamin D possesses an immunomodulatory effect and regulates monocyte inflammatory responses by attenuating cellular signaling and pro-inflammatory genes activation, subsequently preventing cytokines release. For example, vitamin D attenuates TLR2 and TLR4 mediated signaling leading to reduction in TNFα release, and activation of intracellular inflammatory pathways like p38 and NF-kB pathway [266].

Epidemiological studies showed a significant relation between low concentrations of serum 25(OH)D and the risk of infections and hospitalization [267, 268]. For instance, the risk of acute lower respiratory tract infection is reported to be higher in children who suffer from vitamin D deficiency in the first two years of life [269]. In support, vitamin D supplementation demonstrated a protective effect against acute respiratory tract infections [270]. Further, vitamin D deficiency is prevalent in several inflammatory diseases such as inflammatory bowel disease [271], and rheumatoid arthritis [272]. In systemic lupus erythematosus, vitamin D induces reconstruction of the balance between B and T cells through stimulating an increase in CD4+ T cells and a decrease of memory B cells and anti-DNA antibodies [273]. Moreover, in cancer patients, a meta-analysis study illustrates an enhanced overall survival in patients who

have higher serum 25(OH)D concentrations [274]. The given data suggest that the importance of vitamin D as an anti-inflammatory and an antioxidant nutrient metabolite. A recent retrospective study found a negative relation between serum 25(OH)D concentrations and CRP (a marker of inflammation and cytokine storm), in COVID-19 patients, which again indicates a protective role of vitamin D in reducing inflammation [275].

The association between vitamin D deficiency and dyslipidemia i.e., low HDL, high LDL, and high triacylglycerol is well documented [240]. Studies reported a significant association between serum 25(OH)D concentrations and HDL. Subjects with sufficient serum 25(OH)D concentrations have higher concentrations of HDL, while deficient subjects have significantly lower HDL concentration [238, 276]. Further, sufficient HDL is shown to be protective in CVD and other chronic diseases [277]. However, in this young healthy adult cohort, we did not observe any significant associations between serum 25(OH)D concentrations and the HDL profile as this sample is selected to be devoid of co-morbidities, which may explain the lack of association in this study. Additionally, it has been known that anti-dyslipidemia drugs affect HDL cholesterol concentrations. Consequently, MHR measurements would be affected accordingly. However, we were unable to investigate the potential confounding effect on the relationship between serum 25(OH)D and MHR as this study sample did not contain subjects who were taking anti-lipidemic medications. Further, this might be interesting to study this confounding effect in older cohort.

The importance of this study is that it highlights the association between serum 25(OH)D concentrations and the novel biomarker MHR. This association could be utilized to predict the risk of progressing into diseases like metabolic syndrome and dyslipidemia. Recent studies investigated whether lymphocytes and neutrophils as predictors of inflammation. LHR and NHR along with MHR were good predictive biomarkers of inflammatory diseases such as

metabolic syndrome [278, 279]. In our study, we found no association between serum 25(OH)D and LHR and NHR. This is probably due to sample selection criteria; young healthy adults who do not have any underlying disorders. However, thus far, no study reported an association between 25(OHD concentrations and LHR and NHR.

The cross-sectional design of the study is one of the study limitations. Therefore, the cause and effect should not be assumed. Moreover, this study is conducted on healthy young adults, which explains the modest changes in HDL and MHR. If the study population is older, we may have a higher MHR and perhaps a stronger association between serum 25(OH)D concentrations and MHR. Further, in this study, we did not correct for general confounders like socioeconomic status as the cohort are all Qataris without drastic differences in demography, and with relatively high socioeconomic status. Therefore, the lack of adjustment for this variable, might not affected the outcome. Future studies are warranted to confirm the association of serum vitamin D with MHR in an older population with or without chronic inflammatory conditions. In conclusion, serum 25(OH)D concentrations is inversely associated with the MHR, a novel subclinical inflammation biomarker.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Qatar

Biobank, Doha, Qatar (QBB-RES-ACC-0237-0142) and confidentiality agreements were obtained before conducting this study. (

Informed Consent Statement Informed consent was obtained from all subjects involved in the study..

Data Availability Statement: Restrictions apply to the availability of these data. Data was obtained from Qatar Biobank (https://www.qatarbiobank.org.qa) under confidentiality agreement with Qatar University.

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CHAPTER 3: METABOLOMICS PROFILING OF VITAMIN D AND DYSLIPIDEMIA

The metabolome can reflect physiological and pathological modifications in an internal environment, giving an instantaneous picture of individual's health [280]. Thereby, huge changes in metabolomics signature occur during chronic diseases e.g. inflammatory bowel disease (IBD), cardiovascular diseases, and diabetes [281, 282]. Moreover, several alterations in the metabolomics profile were spotted during vitamin D deficiency and its treatment. Amrein et. al clinical trial performed on vitamin D deficient patients in intensive care units shows a significant alteration in metabolomics profile during a very short period in patients supplemented with high dose of vitamin D3 (540,000 IU). The change included several components of the sphingomyelin's family, plasmalogen, lysoplasmalogen and lysophospholipid metabolites. Those metabolites are involved in improving the mitochondrial function and the innate immunity, which reported to be altered during vitamin D deficiency. Some studies suggested that metabolomics approach is a superior predictor of disease than the biochemical markers. For instance dyslipidemia onset was detected with higher precision in HIV patients compared to the classical biochemical parameters, HDL, LDL, and triglycerides [283]. However, metabolomics signature alterations due to vitamin D and dyslipidemia are not investigated. This study summarizes the changes in metabolomics profiling during vitamin D deficiency in relation to dyslipidemia and provides insights on the enriched lipidomics and metabolomics pathways.

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Metabolomics Profiling of Vitamin D Status in Relation to Dyslipidemia

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Abstract: Vitamin D deficiency is a global disorder associated with several chronic illnesses including dyslipidemia and metabolic syndrome. The impact of this association with both dyslipidemia and vitamin D deficiency on metabolomics profile is not yet fully understood. This study analyses the metabolomics and lipidomic signatures in relation to vitamin D status and dyslipidemia. Metabolomics data were collected from Qatar Biobank database and categorized into four groups based on vitamin D and dyslipidemia status. Metabolomics multivariate analysis was performed using the orthogonal partial least square discriminate analysis (OPLS-DA) whilst linear models were used to assess the per-metabolite association

with each of the four dyslipidemia/vitamin D combination groups. Our results indicate a high prevalence of vitamin D deficiency among the younger age group, while dyslipidemia was more prominent in the older group. A significant alteration of metabolomics profile was observed among the dyslipidemic and vitamin D deficient individuals in comparison with control groups. These modifications reflected changes in some key pathways including ceramides, diacylglycerols, hemosylceramides, lysophospholipids, phosphatidylcholines, phosphatidylethanol amines, and sphingomyelins. Vitamin D deficiency and dyslipidemia have a deep impact on sphingomyelins profile. The modifications were noted at the level of ceramides and are likely to propagate through downstream pathways.

Keywords: vitamin D; 25-hydroxyvitamin D; dyslipidemia; metabolomics; lipidomics

1. Introduction

Vitamin D deficiency (serum 25 dihydroxy vitamin D (25(OH)D) concentrations <12 ng/mL) is a worldwide health problem affecting approximately 1 billion individuals globally, with vitamin D insufficiency (<20 ng/mL) affecting 50% of the population. The elderly, obese individuals, nursing home residents, and hospitalized patients have the greatest rates of vitamin D deficiency [285, 286]. In Qatar, Al-Dabhani et al. found that 64% of the 1205 individuals in their research cohort were vitamin D deficient and suffered vitamin-D-related morbidity [27].

In recent years, a growing body of epidemiological and experimental data has shown that low blood vitamin D levels are associated with a variety of metabolic illnesses, including dyslipidemia, obesity, type 2 diabetes, insulin resistance, and cardiovascular disease, including hypertension [232]. In a study by Jiang et al., it was revealed that low vitamin D levels were

inversely associated with LDL and triglycerides levels, whereas higher vitamin D levels were linked to high HDL [240]. HDL is dubbed as the “good” cholesterol, tasked with extracting excess cholesterol from peripheral arteries and transferring it to the liver for elimination in a process known as reverse cholesterol transfer [287]. As a result, guidelines recommend lowering triglyceride and LDL levels while increasing HDL levels. The activities of dyslipidemia medications, which reduce triglyceride and LDL levels while boosting HDL levels, support these guidelines.

Metabolic profiling has become an essential approach for identifying steady-state metabolite concentrations and researching metabolic system control [288]. Metabolomics and lipidomics studies assessing vitamin D in chronic diseases such as multiple sclerosis [289, 290], inflammatory bowel disease (IBD) [281], cardiovascular diseases (CVD) [282], and diabetes revealed significant changes in specific metabolites [282]. Vitamin D supplementation influenced the metabolomics profile of overweight/obese African Americans in a dose-dependent manner. The study reported significant changes in ceramides and sphingomyelins, specifically increased levels of N-stearoyl-sphingosine (d18:1/18:0) (C18Cer) and stearoyl sphingomyelin (d18:1/18:0) (C18SM) [291]. Changes in ceramides such as *N*-palmitoyl-sphingosine and levels of sphingomyelins such as sphingosine-1-phosphate have been associated with vitamin D deficiency [291]. Sphingolipids including ceramides and sphingomyelin are a group of lipids that play a role in cell membrane integrity, cellular stress, and inflammatory signaling. Ceramides are the source of sphingomyelin. Sphingosine-1-phosphate (S1P) glycosphingolipids and sphingomyelin are two physiologically active sphingolipids that are formed from the latter [292, 293].

The metabolic pathways linked with vitamin D insufficiency have been emphasized in the literature [239, 294]. However, the metabolic signature of vitamin D sufficiency and deficiency

in the context of dyslipidemia has not been investigated. In order to decipher the interplay between vitamin D status and dyslipidemia, this study profiled metabolic changes in four different categories of participants: vitamin D sufficient and normolipidemic (Group 1); vitamin D sufficient and dyslipidemia (Group 2); vitamin D deficient and dyslipidemia (Group 3); vitamin D deficient and normolipidemic (Group 4).

2. Materials and Methods

2.1. Study Design

This cohort is a cross-sectional, retrospective study based on data from 277 participants selected randomly from a larger cohort of 1820 subjects. The participants in the original cohort selected based on vitamin D level and dyslipidemia status. Those data were collected by the Qatar Biobank (QBB) [252, 253]. This study was performed in line with the World Medical Association Declaration of Helsinki–Ethical Principles for medical research involving human subjects. The Institutional Research Board of Qatar University QU-IRB form (1366-E/20), QBB-IRB form (EX-2020-QBB-RES-ACC-0237-0124), approved all protocols. All participants consented to the use of their samples for research. Vitamin D status was dichotomized according to serum 25 dihydroxy vitamin D (25(OH)D) concentrations into a deficient <12 ng/mL and a sufficient ≥ 20 ng/mL. This classification was based on the Institute of Medicine's recommendations [254]. Dyslipidemia was defined if any of the following cutoffs have been met: high total cholesterol (>6.2 mmol/L), high LDL-C (>4.1 mmol/L), and high TG (>2.3 mmol/L) [295].

The inclusion criteria were Qatari and non-Qatari healthy adults. The exclusion criteria included those using vitamin D supplements, pregnancy, and those with chronic diseases such as diabetes, high blood pressure, asthma, hay fever, blood clot, heart attack, angina, stroke,

emphysema/chronic bronchitis, hyperthyroidism hyperparathyroidism, Cushing syndrome, and cancer. The 277 participants were further divided into four groups according to the serum 25 (OH)D concentrations and dyslipidemia presence: vitamin D sufficient and normolipidemic (Group 1, n = 64); vitamin D sufficient and dyslipidemia (Group 2, n = 26); vitamin D deficient and dyslipidemia (Group 3, n = 88); vitamin D deficient and normolipidemic (Group 4, n = 99).

2.2. Physical and Biochemical Measurements

Participants' height and weight were measured by trained nurses. Bodyweight was measured using the TANITA BC-418 MA instrument. BMI was calculated by dividing weight in kg by height in m2. Waste to hip ratio (WHR) was calculated by dividing the waist by cm on the hip. Venous blood samples were collected from participants after overnight fasting. For biochemical measurements, blood specimens were sent to Hamad Medical Corporation Laboratories (College of American Pathologist Accredited Laboratory) for analysis. White blood cells (WBC), monocytes, lymphocytes, neutrophils, and eosinophils were measured as part of the differential white blood cell count. Serum 25(OH)D concentration (included both vitamin D2 and vitamin D3 fractions) was measured using electrochemiluminescence immunoassay (LIAISON® 25-hydroxyvitamin D Total Assay, DiaSorin Inc., Stillwater, MN, USA). Serum total cholesterol was measured with the enzymatic CHOD-PAP method. HDL cholesterol Plus Third Generation Method was used to measure the serum HDL cholesterol. LDL cholesterol Plus Second Generation Method was used to measure the serum LDL cholesterol. Serum triacylglycerol was measured with the enzymatic GPO-PAP method. Plasma glucose was measured with the enzymatic/amperometric method (Nova stat strip and Roche Accucheck Inform II devices). C peptide, HBA1C, and insulin were measured using the immunoassay method. Technical methodology details are previously described [252, 253].

2.3. Metabolomics and Lipidomic Profiling

In this study, 1158 metabolites that were annotated or previously identified by Metabolon were used in this study. The established procedures were applied for untargeted metabolomics of serum samples from all individuals as previously described [296]. Briefly, metabolite measurements were performed using Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and an Orbitrap mass analyzer with a 35,000 mass resolution. In short, methanol was used to extract serum samples to eliminate the protein fractions. Subsequently, the extracts were divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Later, the peaked were identified using Metabolon's hardware. Peaks were matched to existing library entries of pure standards of over 3,300 pure standard chemicals to identify compounds. Compounds were then classified into distinct groups based on their origins, as previously discussed [297].

Statistical analyses were carried out using IBM SPSS version 25, R version 3.6 (Armonk, N.Y, USA), and SIMCA 16.0.1 software (Umetrics, Umeå, Sweden). Baseline characteristics of four groups were presented in mean and (SD) for continuous variables. For skewed variables, the median was calculated. ANOVA was used for biomarkers; age, BMI, waist-to-hip ratio, white blood cell, monocyte, lymphocyte, neutrophil, eosinophil, C Peptide, total cholesterol, LDL, HDL-c, Triglyceride, HBA1C, glucose, insulin, and monocyte percentage to HDL-c ratio (MHR). *p*-value $p < 0.05$ was considered statistically significant.

A linear model was fit per metabolite (y-variable) that incorporated the explanatory categorical variable study group (featuring four levels Group 1, Group 2, Group 3 and Group 4) as well as the following confounders: BMI, age, the first two PCs from PCA analysis and gender. R package Emmeans was used to extract the desired pairwise contrasts. The *p* values were penalized for multiple testing using the FDR procedure. Enrichment analysis of pathway/structural classes of metabolites was performed on the list of metabolites with nominal *p* value less than or equal to 0.05 from each contrast based on the Fishers' exact test, followed by FDR multiple testing correction.

3. Results

3.1. General Characteristics of Participants

The total number of participants was 277 including 38.9% female and 61.1% male participants. The cohort was divided into four groups based on vitamin D status and serum lipids profile. Group 1 was composed of 64 participants who were vitamin D sufficient (greater than 20 ng/mL) with normolipidemia, hence considered as the control group. Group 2 was composed of 26 participants who were vitamin D sufficient with dyslipidemia. Group 3 was composed of 88 participants who were vitamin D deficient (less than 12 ng/mL) with dyslipidemia. Group 4 was composed of 99 participants who exhibited vitamin D deficiency with normolipidemia. Dyslipidemia was more prominent in the older age group, although they were vitamin D sufficient, as opposed to the younger age group with a higher prevalence of vitamin D deficiency. Higher BMI was observed in dyslipidemia groups in comparison with normolipidemic groups. Moreover, the inflammation marker monocyte to HDL ratio (MHR) was significantly higher in dyslipidemic groups (Group 2 and Group 3) in comparison with normolipidemic participants. The median for the glycemic variables C Peptide has the highest

value 2.76 (ng/mL) in Group 3 of participants with combined vitamin D deficiency and dyslipidemia. Additionally, HDL has the lowest value in this group, although it was low in both dyslipidemic and vitamin D deficiency groups. Of note, triglycerides levels among participants with combined vitamin D deficiency and dyslipidemia were almost double that of other groups. There were differences in the distribution of gender in the four studied groups. For instance, males were more dominant in Group 3 (dyslipidemia and vitamin D deficiency) than females, whereas females were more dominant in Group 4 (normolipidemia and vitamin D deficiency) (Table 1).

Using Pearson correlation, a strong negative association has been detected between HDL and vitamin D level in Group 3 (Vitamin D deficient and dyslipidemia) (Figure 1).

Table 3. 1. Characteristics of the participants' groups based on serum 25(OH)D concentrations and dyslipidemia.

	Total	**25(OH)D sufficient, Normolipidemia (G1)**	**25(OH)D sufficient, Dyslipidemia (G2)**	**25(OH)D deficient, Dyslipidemia (G3)**	**25(OH)D deficient Normolipidemia (G4)**	
	n = 277	**n = 64**	**n = 26**	**n = 88**	**n = 99**	***p* Value**
25(OH)D ng/ml	14.8 (8.3)	22 [20, 26] *	24.5 [22, 29.2] *	10 (1.8)	9.7 (1.8)	<0.001
Age	34.7 (10.8)	38.7 (11.04)	43.8 (8.9)	37.5 (9.2)	26 [21, 31] *	<0.001
Gender						
Males %	61.4	53.1	65.4	79.5	49	
BMI	27.6 (5.7)	26.8 (5.0)	27.9 (4.3)	29.4 (4.9)	26.5 (6.7)	0.002
HWR [a]	0.8 (0.1)	0.8 (0.1)	0.9 (0.1)	0.9 (0.1)	0.8 (0.1)	<0.001
WBC x103/µl	6.5 (1.8)	6.2 (1.2)	5.8 [5.0, 6.4] *	6.98 (2.0)	6.4 (1.7)	0.006
Monocyte %	7.8 (2.1)	7.6 (1.87)	8.1 (1.6)	7.84 (1.8)	7.5 [6.3, 8.8] *	0.750
Neutrophil %	53.1 (8.9)	54.0 (8.5)	50.4 (9.7)	52.58 (8.9)	53.8 (8.6)	0.266
Lymphocyte %	35.5 (7.8)	34.9 (7.8)	37.3 (8.5)	35.87 (8.1)	35.1 (7.2)	0.533
Eosinophil %	3.0 (2.0)	2.6 [1.5, 3.6] *	3.7 [2.2, 5.0] *	2.75 [2.1, 3.7] *	2.3 [1.3, 3.5] *	0.044
MHR [b]	6.7 (3.0)	5.6 [4.1, 7.22] *	7.01 (2.7)	7.2 [5.2, 8.7] *	5.7 [4.4, 7.5] *	0.001
Total Cholesterol (mmol/l)	4.9 (1.0)	4.7 (0.7)	5.9 (0.7)	5.6 (1.0)	4.2 (0.5)	<0.001
HDL-C (mmol/l)	1.29 (0.4)	1.4 (0.4)	1.25 (0.3)	1.11 [0.9, 1.3] *	1.3 (0.3)	<0.001
LDL (mmol/l)	2.96 (0.9)	2.8 (0.6)	3.79 (0.7)	3.5 (1.0)	2.4 (0.5)	<0.001
Triglycerides (mmol/l)	1.48 (1.1)	1.1 (0.4)	1.55 [1.1, 2.5] *	2.2 [1.2, 2.7] *	1.0 (0.3)	<0.001
HBA.1C %	5.3 (0.5)	5.4 [5.1, 5.5] *	5.49 (0.3)	5.4 (0.7)	5.2 (0.3)	0.015
Glucose (mmol/L)	5.14 (0.9)	5 [4.5, 5.4] *	5 [4.9, 5.6] *	5.1 [4.7, 5.6] *	4.9 (0.5)	0.004
Insulin (µU/mL)	18.4 (26)	7.9 [5, 12.9] *	9.2 [7.0, 19.4] *	14.2 [8.2, 29.8] *	9.6 [6.0, 13.0] *	<0.001
C. Peptide (ng/mL)	3.0 (2.3)	2.1 [1.4, 2.7] *	2.1 [1.7, 4.0] *	2.76 [2.1, 4.9] *	1.97 [1.5, 2.9] *	<0.001

Data is represented as mean (SD) or median (IQR) for skewed data. *p* value was in reference to control group (1) (Vitamin D sufficient, and Normolipidemic). [a] Hip-to-waist ratio [b] MHR Monocyte percentage to HDL Ratio * Represent Median [Interquartile range]

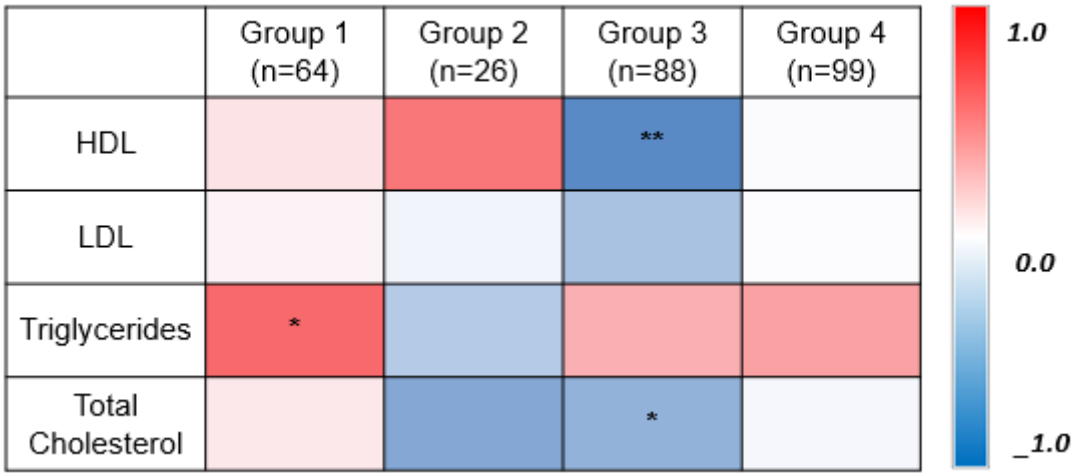

Figure 3. 1. The heat map illustrates Pearson correlation depicting the association between lipid profile components (HDL, LDL, Triglycerides, and Total cholesterol) and serum 25(OH)D among four groups. * $p \leq 0.05$, ** $p \leq 0.01$. Group 1 (Vitamin D sufficient and normolipidemic); Group 2 (Vitamin D sufficient and dyslipidemia); Group 3 (Vitamin D deficient and dyslipidemia); Group 4 (Vitamin D deficient and normolipidemic).

3.2. Metabolites and Lipids Analysis

The orthogonal partial least square discriminate analysis (OPLS-DA) of metabolites and lipids associated with vitamin D status stratified by dyslipidemia levels revealed two significant class-discriminatory components accounting for 95% of the variation in the data due to group membership. R2Y (cum) was 0.637, and Q2 (cum) was 0.229. Figure 2A shows a scatter plot of these two components, distinguishing all four groups in a two-dimensional space

representation. Interestingly, the first predictive component (x-axis) projects the dyslipidemia status whilst the second predictive component is an indicator of vitamin D levels. (Figure 2A). The relative abundance of metabolite enrichment is important, and thus, the variable influence on projection (VIP) list indicating top metabolites that differentiate the four groups is shown in appendix Table 1. The loading plot (Figure 2B) indicates the weights of the metabolites underlying the separation of subjects in the score plot in Figure 2A. The metabolic pathways that appeared confined to certain groups and not others are highlighted in color. These include ceramides, diacylglycerols, hemosylceramides, lysophospholipids, phosphatidylcholines, phosphatidylethanol amines and sphingomyelins (Figure 2B). Cholesterol (as a single metabolite) was assigned a high weight by the OPLS-DA, which indicates a high discriminatory capacity for group three; representing vitamin D deficient and dyslipidemic subjects. In addition, sphingomyelins/lysophospholipids are markers of vitamin D sufficiency and deficiency, respectively. Moreover, we noted an enrichment in ceramides, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and diacylglycerol in the dyslipidemic group as opposed to a marked presence of hexosylceramides in the normolipidemics controls.

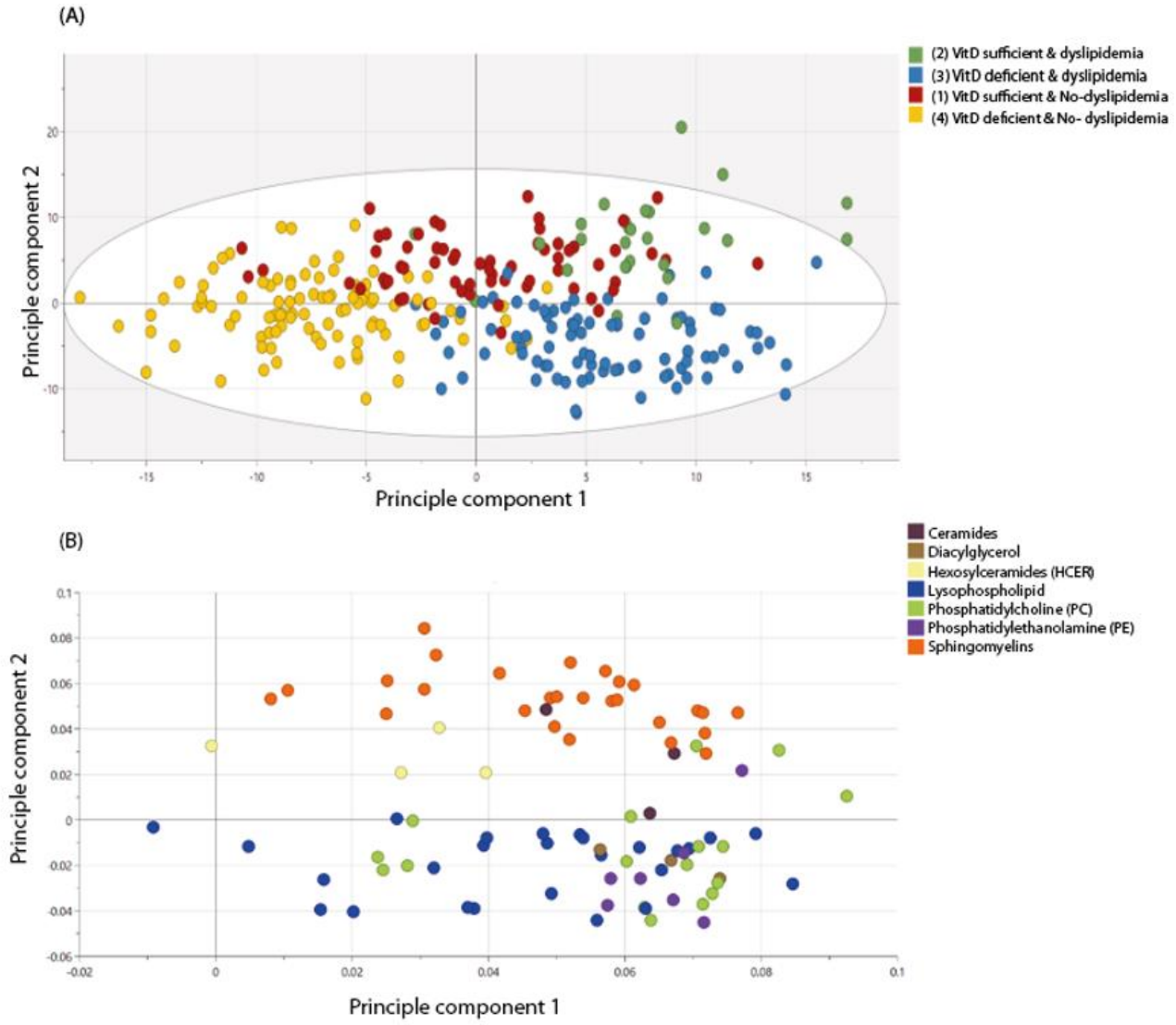

Figure 3. 2. Illustration of OPLS-DA analysis for the four groups. (**A**): represents a scatter plot of two principal components, distinguishing all four groups in a two-dimensional space. (**B**): represents a loading plot highlighting the weights of the metabolites in all four groups, indicating top metabolite responsible for group separation. R2Y (cum) was 0.637, and Q2 (cum) was 0.229. Vitamin D sufficient and normolipidemic (Group 1, n = 64); Vitamin D sufficient and dyslipidemia (Group 2, n = 26); Vitamin D deficient and dyslipidemia (Group 3, n = 88); Vitamin D deficient and normolipidemic (Group 4, n = 99).

Metabolomics and Lipidomic Signatures in the Participants' Groups

Linear regression analysis was used to find an association between group pairs, the cut-off value of significance for FDR, and the *p*-value was (≤0.05). Examples of Metabolomics signatures are shown using volcano plots in Supplementary Figure S1.

In vitamin D sufficient groups (Group 2 versus Group 1) (dyslipidemic versus normolipidemic participants), univariate analysis revealed twenty metabolites that were associated with dyslipidemia among vitamin D sufficient participants—namely cholesterol, 1-palmitoyl-2-docosahexaenoyl-GPE, 1-oleoyl-2-docosahexaenoyl-GPC, CMPF, and various sphingomyelins metabolites, which were significantly more abundant in the dyslipidemia group (Supplementary Table S1). Enrichment analysis revealed a marked upregulation of sphingomyelins in the dyslipidemia group at physiological levels of vitamin D (Table 2 and Figure 3).

In vitamin D deficient groups (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants), a wider range of complex lipids was found to plummet in the dyslipidemic group. Univariate analysis also highlighted other metabolites including oleoyl-linoleoyl-glycerol, retinol, cholesterol, alpha-tocopherol, and amino acids leucine, isoleucine, and valine were also found to differ in their levels in the dyslipidemic group with deficient vitamin D in comparison to their corresponding controls (Supplementary Table S2). Enrichment analysis revealed a marked upregulation of sphingolipids, fatty acids, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), lysophospholipid and ceramides (Table 2 and Figure 3).

In normolipidemics groups (Group 4 versus Group 1) of vitamin D deficient versus sufficient participants, eleven metabolites were significantly associated with vitamin D

deficient participants without dyslipidemia, i.e., Group 4 (Supplementary Table S3). Metabolites such as 1-oleoyl-2-docosahexaenoyl-GPC, ergothioneine, urea, valine, 4-methyl-2-oxopentanoate, arabo.te/xylo.te, 1-(1-enyl-oleoyl)-GPE (*p*-18:1), allantoin, methionine sulfone, 1-(1-enyl-stearoyl)-GPE (*p*-18:0), tryptophan), and most notably ergothioneine is higher in vitamin D sufficiency compared to deficiency (Supplementary Table S3). Enrichment analysis delineated the following significant categories: sphingomyelins, phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophospholipid, diacylglycerol, and ceramides (Table 2 and Figure 3).

In dyslipidemics groups (group 3 versus group 2) of vitamin D deficient versus sufficient participants, univariate analysis was significant for 15 metabolites, including hydroxy-CMPF and CMPF, which were significantly reduced in vitamin D deficient Group 3 (Supplementary Table S4). Other metabolites such as (docosahexaenoic (DHA; 22:6n3), S-methyl cysteine sulfoxide, 1-oleoyl-2-docosahexaenoyl-GPC, perfluorooctanoate (PFOA), tartro.te (hydroxymalo.te), 2-hydroxyglutarate, lysine, and sphingomyelin pathway followed the same pattern of regulation (Supplementary Table S4). The univariate analysis for other groups, e.g., Group 1 (vitamin D sufficient versus normolipidemic) and Group 3 (vitamin D deficient and dyslipidemia) didn’t reveal any significance difference in metabolites enrichment.

Table 3. 2. Metabolites enrichment among various groups based on vitamin D status with or without dyslipidemia.

Pathway	*p*. Value	FDR
Metabolites enriched in vitamin D sufficient groups; Normolipidemic versus vitamin D sufficient dyslipidemic participants (group 2 versus group 1)		
Sphingomyelins	1.97×10^{-13}	<0.001
Metabolites enriched in different levels in vitamin D deficient Normolipidemic versus vitamin D sufficient dyslipidemic participants (Group 4 versus Group 3)		
Phosphatidylcholine (PC)	4.42×10^{-13}	<0.001
Lysophospholipid	2.87×10^{-9}	<0.001
Sphingomyelins	5.46×10^{-9}	<0.001
Phosphatidylethanolamine (PE)	1.77×10^{-6}	<0.001
Hexosylceramides (HCER)	1.87×10^{-3}	0.032
Metabolites enriched in different levels in normolipidemics vitamin D sufficient versus deficient participants (Group 4 versus Group 1)		
Sphingomyelins	1.98×10^{-36}	<0.001
Phosphatidylcholine (PC)	1.09×10^{-16}	<0.001
Phosphatidylethanolamine (PE)	3.80×10^{-12}	<0.001
Lysophospholipid	9.51×10^{-10}	<0.001
Diacylglycerol	1.80×10^{-5}	<0.001
Ceramides	2.42×10^{-4}	0.004

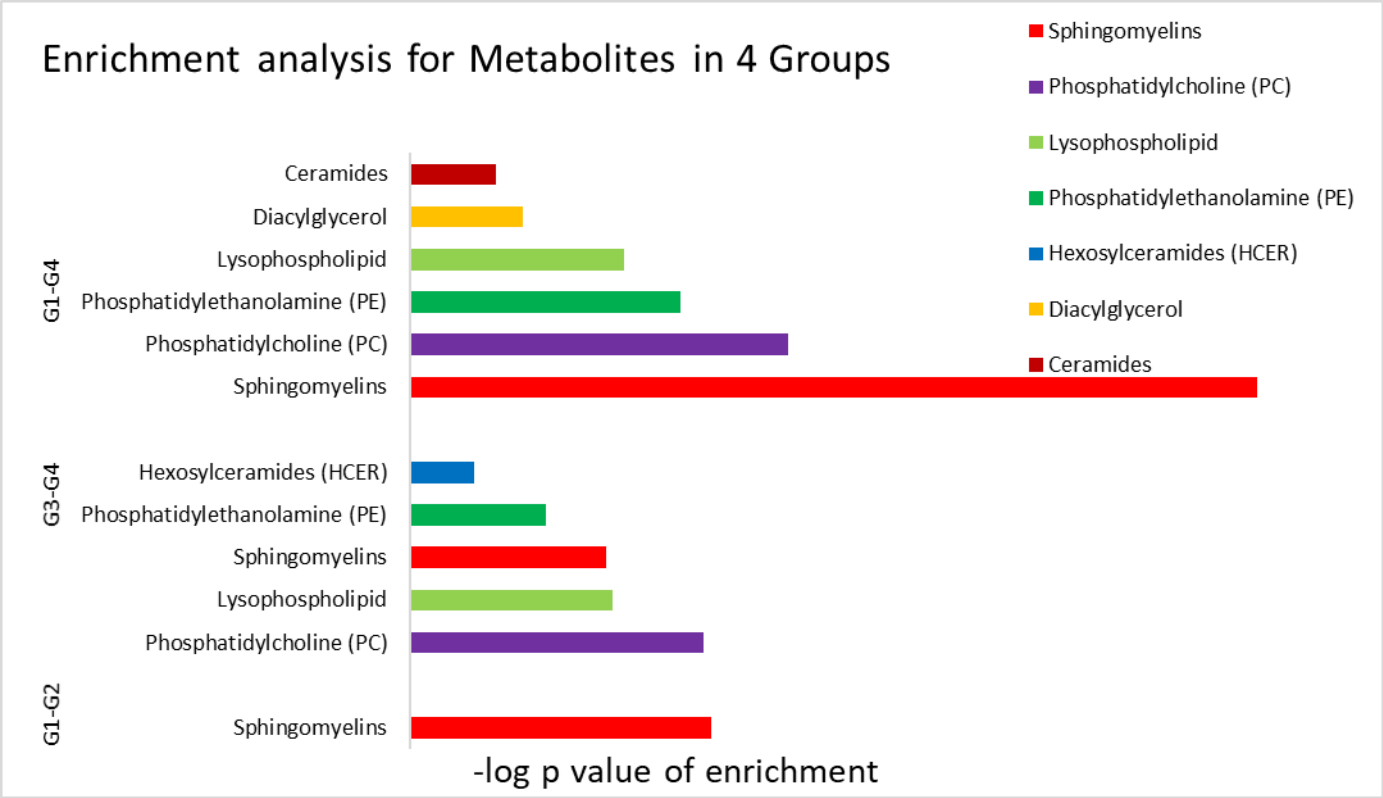

Figure 3. 3. Illustration of the enriched pathway in paired group contrasts. A significant alteration was prominent among sphingomyelins pathway components. Vitamin D sufficient and normolipidemic (Group 1, n = 64); vitamin D sufficient and dyslipidemia (Group 2, n = 26); vitamin D deficient and dyslipidemia (Group 3, n = 88); vitamin D deficient and normolipidemic (Group 4, n = 99).

3.3. Quantitative Measurement of Single Specific Metabolites Distribution among Participants

Sphingomyelin showed the highest value in the dyslipidemic vitamin D sufficient group, i.e., Group 2 (Figure 4A), whereas, N-stearoyl-sphingosine (d18:1/18:0) and N-palmitoyl-sphingosine (d18:0/16:0) were higher in the dyslipidemic and vitamin D deficient group individuals, i.e., Group 3 (Figure 4B,C). When comparing vitamin D sufficient and

normolipidemic, i.e., Group 1, with vitamin D deficient and dyslipidemic, i.e., Group 3, Palmitoyl sphingomyelin (d18:1/16:0) was significantly elevated in the latter group 3 (Figure 4D).

Interestingly, the amino acid ergothioneine was reduced in vitamin D deficient normolipidemic in comparison to vitamin sufficient participants (Figure 5A). Upon comparison between the different groups, 3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF) level was higher in vitamin D deficient and dyslipidemic, i.e., Group 3 (Figure 5B).

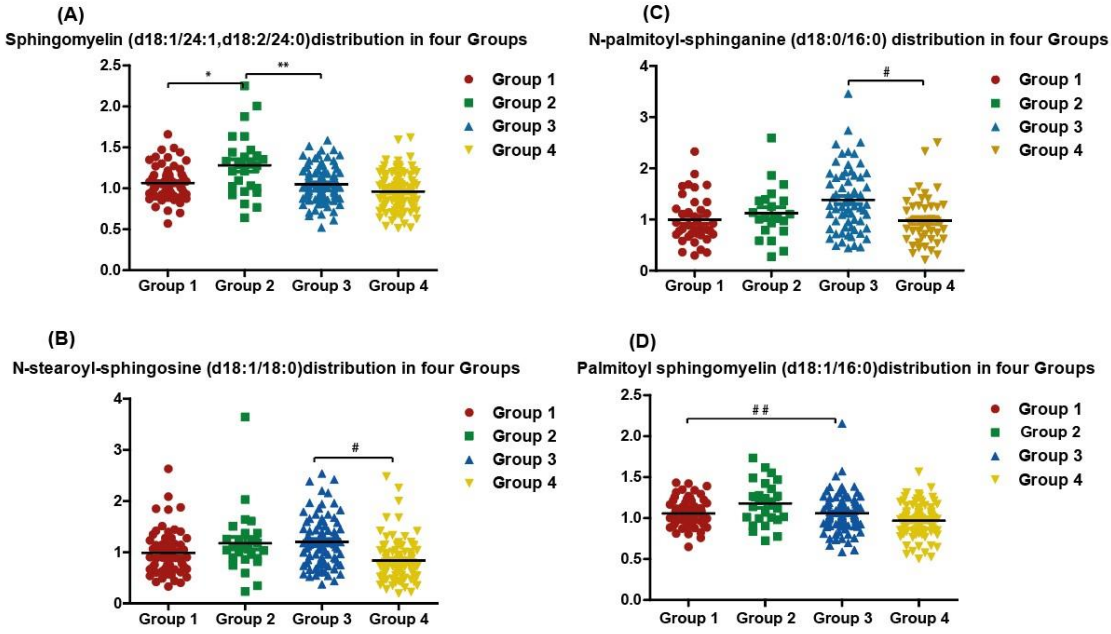

Figure 3. 4. Metabolites quantity distribution among four groups of participants. (**A**) sphingomyelin (d18:1/24:1,d18:2/24:0); (**B**) N-stearoyl-sphingosine (d18:1/18:0); (**C**) N-palmitoyl-sphingosine (d18:0/16:0); and (**D**) palmitoyl sphingomyelin (d18:1/16:0). Vitamin D sufficient and normolipidemic (Group 1, n = 64); vitamin D sufficient and dyslipidemia (Group 2, n = 26); vitamin D deficient and dyslipidemia (Group 3, n = 88); vitamin D deficient and normolipidemic (Group 4, n = 99). Y axis is log transformed abundance levels of metabolites. * Significant p value comparing Group 1 to Group 2. ** Significant p value

comparing Group 2 to Group 3. # Significant p value Group 3 to Group 4. # # Significant p value comparing Group 1 to Group 3.

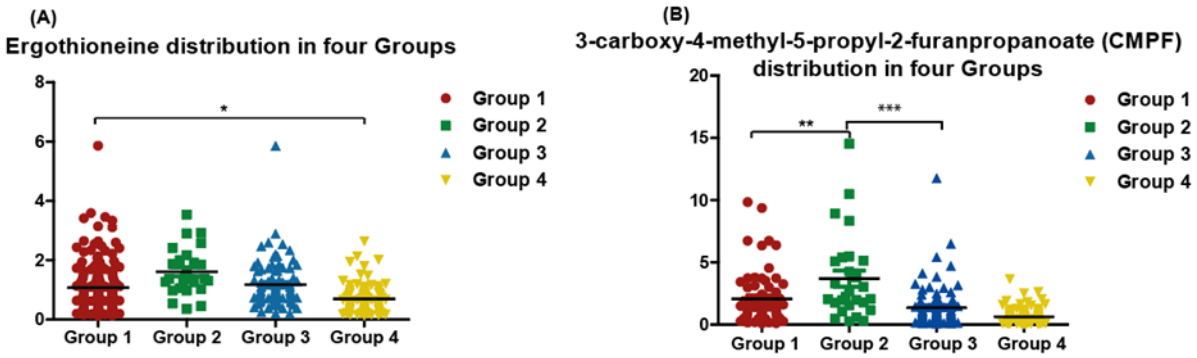

Figure 3. 5. Quantitative distribution of Ergothioneine (**A**) and 3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF) (**B**) among four groups. Vitamin D sufficient and normolipidemic (Group 1, n = 64); vitamin D sufficient and dyslipidemia (Group 2, n = 26); vitamin D deficient and dyslipidemia (Group 3, n = 88); vitamin D deficient and normolipidemic (Group 4, n = 99). Y axis is log transformed abundance levels of metabolites. * Significant p value comparing Ergothioneine levels in Group 1 (Vitamin D sufficient) to Group 4 (Vitamin D deficient). ** Significant p value comparing CMPF levels in Group 1 (normolipidemic) to Group 2 (dyslipidemic). *** Significant p value comparing CMPF levels in Group 2 (Vitamin D sufficient) to Group 3 Vitamin D deficient in dyslipidemic participants.

4. Discussion

Worldwide, vitamin D insufficiency and dyslipidemia are common [298, 299]. Vitamin D is well-known for its anti-inflammatory properties. This vitamin deficit may result in and be associated with a variety of illnesses [234]. The disruption in the lipids profile that occurs in

dyslipidemia is also associated with various chronic conditions, most notably metabolic syndrome and cardiovascular diseases [300]. Individuals featuring combined vitamin D insufficiency and dyslipidemia are predisposed to a variety of chronic diseases. However, the metabolomics signature for this group of individuals has not yet been thoroughly investigated. In this work, we examined the effect of vitamin D status on metabolomics signatures of participants with and without dyslipidemia both separately and combined.

The findings of this study revealed that dyslipidemia was more prevalent in the older age group, despite vitamin D sufficiency. Vitamin D deficiency, on the other hand, was more common among the young age group. Sphingomyelins were more notably altered in vitamin D deficiency without dyslipidemia as the sphingomyelin pathway was disrupted. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were found to be highly altered among vitamin D deficient participants with and without dyslipidemia. Ergothienone levels were substantially higher in vitamin D adequate people compared to vitamin D deficient persons. Vitamin D is synthesized from the main precursor 7-dehydrocholesterol present in the skin, thereby it may be affected by dyslipedmia status. Several studies and meta-analysis reported the associations between low vitamin D status and altered lipid profile, i.e., dyslipidemia [226, 301]. Further, vitamin D possesses immune modulatory effects that impact inflammation status and consequently lipid homeostasis including HDL-c biogenesis. Mousa et al. [227] observed the inverse association between vitamin D status and the subclinical inflammation marker monocyte percentage to HDL ratio (MHR) [227].

Sphingolipids, along with phosphoglycerides (also known as glycerophospholipids), are important components of cellular membranes, forming the two leaflets of the cellular membrane. The lipid bilayer structures protect the structure and stability of the cellular membrane. Phosphoglycerides include phosphatidylcholine (PC), phosphatidylethanolamine

(PE), and phosphatidylserine (PS). Phosphoglycerides are made up of glycerol units and fatty acids. The inner cellular membrane leaflet is mostly composed of phosphatidylethanolamine that accounts for 25% of all phospholipids and is crucial for distributing charges and membrane stability as it contains amine group [302, 303]. Sphingolipids are made up of a hydrophobic ceramide backbone and a lengthy hydrocarbon chain of amino alcohol. In mammalian cells, the primary sphingolipids are sphingomyelin (SM) and glycosphingolipids (GSLs) [304]. Sphingomyelins are key components of a nerve cell's myelin sheath. Sphingomyelin dysfunction has been linked to a number of autoimmune disorders, most notably multiple sclerosis [305]. Phosphoglycerides and sphingolipids are lipid metabolites that have a tight relationship with lipoproteins, notably HDL. HDL's primary lipid components are phosphatidylcholine (PC) and sphingomyelin (SM) [306]. However, increasing the quantity of sphingomyelin in HDL particles impairs HDL formation and maturation [307]. This study investigated the levels of sphingolipids and ceramides-related metabolites among the normolipidemic, dyslipidemic, vitamin sufficient and deficient individuals. N-stearoyl-sphingosine (d18:1/18:0), one of long chain ceramides, was noted higher in the dyslipidemic and vitamin D deficient group (group 3 population); rather contradicting with the findings by Chen et al. and Koch et al. Indeed, an increase in N-stearoyl-sphingosine (d18:1/18:0) upon the increase of vitamin D was noted after the administration of vitamin D3 supplementation in a dose and time-dependent manner [308]. The noted increase in ceramide despite the reduction in vitamin D levels could be due to the effects of dyslipidemia on the metabolomic profile [309].

The lysophospholipids category is made up of lipids that act as an intermediary in the creation of other lipids in cells. They are classified as either lysoglycerophospholipids or lysosphingolipids [310]. Our findings show an increase in lysophospholipids in the vitamin D

deficient and dyslipidemic groups. Lysophospholipids are linked to several diseases such as asthma and chronic obstructive pulmonary [311]. One of the lysosphingolipids is sphingosine 1-phosphate (S1P), which is a bioactive sphingolipid that functions as a signaling molecule [312]. It regulates innate and adaptive immunity and is implicated in atherosclerosis, in addition to modulating numerous physiological and biological variables such as cell migration and proliferation [313, 314]. The majority of S1P in the blood is associated with HDL [315]. Unlike in tissue, S1P is present in large amounts in the blood; however, it is rapidly degraded [316]. ApoM is an apolipoprotein that binds to S1P on HDL particles and has a significant influence on HDL biogenesis [97]. Vitamin D, on the other hand, has been shown to diminish the expression of S1P1, S1P2 receptors while increasing the expression of S1P3, S1P4 receptors [317, 318]. As a result, lysosphingolipids (mostly S1P) have an indirect influence on dyslipidemia through modulating HDL synthesis. Simultaneously, vitamin D reduces the S1P action by targeting the S1P primary receptors.

In vivo studies linked ceramides to diabetes and insulin resistance. For instance, feeding mice with a high-fat diet resulted in an increase in long chain ceramides, leading eventually to obesity and insulin resistance [319, 320]. N-palmitoyl-sphingosine (d18:1/16:0) (C16Cer) particularly was linked to cardiovascular disease and obesity-related insulin resistance [321, 322]. In vitro investigations revealed that vitamin D has a direct effect on both phosphoglycerides and palmitoyl sphingomyelin (d18:1/16:0), stearic, linoleic, and arachidonic acid while increasing palmitic acid [323]. N-palmitoyl-sphingosine and N-stearoyl-sphingosine specifically were linked to cardiovascular mortality [321]. Treatment with vitamin D metabolites, on the other hand, activated the sphingomyelin pathway, resulting in a large rise in ceramide concentration and a proportionate reduction in sphingomyelin [324]. In this investigation, we report that in vitamin D deficient groups, various sphingomyelins with

long acyl chain-like (C18, C17, and C24) acyl chain-like sphingomyelins were enriched in the dyslipidemia group compared to the normolipidemic group. This finding is consistent with that of Hanamatsu et al., who reported that serum sphingomyelins with saturated acyl chains (C18:0, C20:0, C22:0, and C24:0) are raised in obese people and may be linked to the development of metabolic syndrome [325]. In the vitamin D deficient and dyslipidemic group, we observed an increase in sphingomyelins with long acyl chain-like (C18, and C17), as well as an increase in ceramides and phosphoglyceride products such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). This might imply that patients with low serum vitamin D concentrations as well as dyslipidemia have an excess of both phosphoglycerides and sphingolipids.

Ergothioneine is an amino acid that is mostly generated by fungus and bacteria and is considered a potent antioxidant. It cannot be synthesized by the human body, but it can be obtained through dietary sources such as mushrooms and black beans [326]. Ergothioneine levels were shown to be low in the aged population, particularly those with cognitive impairment [327]. Ergothioneine levels have also been reported to be lower in several neurological illnesses, such as Parkinson's [328]. Ergothioneine was reported to reduce oxidative stress and inflammatory indicators such as allantoin (urate oxidation), 8-hydroxy-2'-deoxyguanosine (responsible for DNA damage), 8-iso-prostaglandin F2 (responsible for excessive lipid peroxidation), and C-reactive protein [329]. In this study, ergothioneine levels in the vitamin D adequate group were higher than those in the deficient group with normolipidemic status. This might be due to dietary factors; for example, foods high in vitamin D also contain high levels of ergothioneine.

5. Conclusions

Vitamin D deficiency and dyslipidemia have a profound impact on several individual metabolites including CMPF and Ergothioneine, in addition to multiple pathways, particularly sphingmyelins and ceramides. The modifications were also noted on the downstream pathways including phosphatidylcholine (PC), and phosphatidylethanolamine (PE). This impact was highest among subjects with combined vitamin D deficiency and dyslipidemia.

Limitation of the Study

The history of medication, particularly drugs which have an impact on calcium and phosphorus metabolism and self-prescribed lipid lowering agents, was missing. This could have influenced data, in addition to other unmeasured factors including environmental and behavioral factors, e.g., dietary habit. However, the linear model could report some confounders, helping to narrow the gap. An additional limitation is the lack of in vitro or in vivo studies to validate the result.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Volcano plots of metabolites enrichment among participants' groups. Vitamin D sufficient and normolipidemic (Group 1, n=64); Vitamin D sufficient and Dyslipidemia (Group 2, n=26); Vitamin D deficient and Dyslipidemia (Group 3, n=88); Vitamin D deficient and normolipidemic (Group 4, n=99); Table S1: Metabolites with significantly different levels in vitamin D sufficient groups (Group 2 versus Group 1) (Dyslipidemic versus normolipidemic participants); Table S2: Metabolites with significantly different levels in vitamin D deficient groups (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants); Table S3: Metabolites with significantly different levels in normolipidemics groups (Group 4 versus Group 1) vitamin D deficient versus sufficient

participants; Table S4: Metabolites with significantly different levels in dyslipidemics groups (group 3 versus group 2) vitamin D deficient versus sufficient participants; Appendix 1: The variable influence on projection (VIP) list indicating top metabolites that differentiate the four groups.

Author Contributions: Conceived the study: S.M.Z.; data collection: S.M.Z. and H.M.; data analysis: H.M., M.A.E., I.D. and S.M.Z. Supervision: S.M.Z. Writing manuscript draft: H.M., S.M.Z. Critically reviewing and finalizing manuscript: S.M.Z., H.M., M.A.E., I.D. and S.K.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Qatar Biobank, Doha, Qatar (QBB-RES-ACC-0237-0142) and confidentiality agreements were obtained before conducting this study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Restrictions apply to the availability of these data. Data was obtained from Qatar Biobank (https://www.qatarbiobank.org.qa. Accessed on 12, August 2022) under confidentiality agreement with Qatar University. Supplementary data are freely available.

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Table S1

Metabolites with significantly different levels in ***vitamin D sufficient groups*** (Group 2 versus Group 1) (Dyslipidemic versus *n*ormolipidemic participants)					
Metabolite Names	**Sub-Pathway**	**Estimate**	**Std.Error**	**P.value**	**FDR**
sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*	Sphingomyelins	0.27	0.06	1.34E-05	*<0.001*
1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)*	Phosphatidylethanolamine (PE)	0.67	0.16	2.71E-05	*<0.001*
Cholesterol	Sterol	0.22	0.05	3.17E-05	*<0.001*
sphingomyelin (d18:2/21:0, d16:2/23:0)*	Sphingomyelins	0.29	0.07	3.56E-05	*<0.001*
sphingomyelin (d18:1/24:1, d18:2/24:0)*	Sphingomyelins	0.22	0.05	6.71E-05	*<0.001*
sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)*	Sphingomyelins	0.30	0.07	8.17E-05	0.01
sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*	Sphingomyelins	0.20	0.06	2.91E-04	0.02
sphingomyelin (d18:2/16:0, d18:1/16:1)*	Sphingomyelins	0.19	0.05	3.82E-04	0.02
sphingomyelin (d18:1/24:1, d18:2/24:0)*	Sphingomyelins	0.23	0.07	5.90E-04	0.03
sphingomyelin (d18:2/23:1)*	Sphingomyelins	0.26	0.07	7.16E-04	0.03
sphingomyelin (d18:2/24:1, d18:1/24:2)*	Sphingomyelins	0.20	0.06	7.18E-04	0.03
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)*	Phosphatidylcholine (PC)	0.36	0.11	1.31E-03	0.04
sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)*	Sphingomyelins	0.21	0.06	1.31E-03	0.04
sphingomyelin (d18:1/20:0, d16:1/22:0)*	Sphingomyelins	0.21	0.06	1.55E-03	0.05
hydroxy-CMPF*	Fatty Acid, Dicarboxylate	1.17	0.37	1.55E-03	0.05
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	Fatty Acid, Dicarboxylate	1.27	0.40	1.57E-03	0.05
sphingomyelin (d18:1/20:1, d18:2/20:0)*	Sphingomyelins	0.18	0.06	1.59E-03	0.05
sphingomyelin (d18:2/14:0, d18:1/14:1)*	Sphingomyelins	0.24	0.07	1.60E-03	0.05
behenoyl sphingomyelin (d18:1/22:0)*	Sphingomyelins	0.20	0.06	1.80E-03	0.05
palmitoyl dihydrosphingomyelin (d18:0/16:0)*	Dihydrosphingomyelins	0.21	0.07	1.81E-03	0.05

Supplementary Table S2

Metabolites with significantly different levels in ***vitamin D deficient groups*** (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants)

Metabolite Names	Sub-Pathway	Estimate	Std.Error	P.value	FDR
Cholesterol	Sterol	-0.24	0.04	$2.70E^{-10}$	*<0.001*
N-palmitoyl-sphinganine (d18:0/16:0)	Ceramides	-0.27	0.04	$3.54E^{-09}$	*<0.001*
1-palmitoyl-2-dihomo-linolenoyl-GPC (16:0/20:3n3 or 6)*	Phosphatidylcholine (PC)	-0.46	0.08	$6.03E^{-09}$	*<0.001*
1-stearoyl-GPC (18:0)	Lysophospholipid	-0.27	0.05	$8.86E^{-09}$	*<0.001*
1-stearoyl-GPE (18:0)	Lysophospholipid	-0.40	0.07	$4.08E^{-08}$	*<0.001*
1-palmitoyl-GPC (16:0)	Lysophospholipid	-0.24	0.04	$4.21E^{-08}$	*<0.001*
1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)*	Phosphatidylcholine (PC)	-0.65	0.12	$6.73E^{-08}$	*<0.001*
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	Phosphatidylethanolamine (PE)	-0.74	0.14	$1.54E^{-07}$	*<0.001*
oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	Diacylglycerol	-0.57	0.11	$2.18E^{-07}$	*<0.001*
1-linoleoyl-2-arachidonoyl-GPC (18:2/20:4n6)*	Phosphatidylcholine (PC)	-0.37	0.07	$2.87E^{-07}$	*<0.001*
1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	Phosphatidylethanolamine (PE)	-0.60	0.11	$3.06E^{-07}$	*<0.001*
1-linolenoyl-GPC (18:3)*	Lysophospholipid	-0.54	0.10	$3.53E^{-07}$	*<0.001*
1-stearoyl-2-linoleoyl-GPE (18:0/18:2)*	Phosphatidylethanolamine (PE)	-0.48	0.09	$5.21E^{-07}$	*<0.001*
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	Phosphatidylcholine (PC)	-0.43	0.08	$7.40E^{-07}$	*<0.001*
sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)*	Sphingomyelins	-0.27	0.05	$7.49E^{-07}$	*<0.001*
1-myristoyl-2-palmitoyl-GPC (14:0/16:0)	Phosphatidylcholine (PC)	-0.68	0.13	$8.95E^{-07}$	*<0.001*
1-palmitoleoyl-GPC (16:1)*	Lysophospholipid	-0.37	0.07	$9.32E^{-07}$	*<0.001*
behenoyl sphingomyelin (d18:1/22:0)*	Sphingomyelins	-0.23	0.05	$9.32E^{-07}$	*<0.001*
retinol (Vitamin A)	Vitamin A Metabolism	-0.26	0.05	$9.77E^{-07}$	*<0.001*
oleoyl-linoleoyl-glycerol (18:1/18:2) [1]	Diacylglycerol	-0.54	0.11	$1.20E^{-06}$	*<0.001*
1-oleoyl-GPC (18:1)	Lysophospholipid	-0.24	0.05	$1.19E^{-06}$	*<0.001*
1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	Phosphatidylethanolamine (PE)	-0.68	0.14	$1.74E^{-06}$	*<0.001*
alpha-tocopherol	Tocopherol Metabolism	-0.21	0.04	$3.20E^{-06}$	*<0.001*
1-linoleoyl-GPE (18:2)*	Lysophospholipid	-0.41	0.09	$3.68E^{-06}$	*<0.001*

Metabolites with significantly different levels in ***vitamin D deficient groups*** (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants)

Metabolite Names	Sub-Pathway	Estimate	Std.Error	P.value	FDR
1-palmitoyl-GPE (16:0)	Lysophospholipid	-0.36	0.08	3.87E-06	*<0.001*
1-arachidonoyl-GPE (20:4n6)*	Lysophospholipid	-0.29	0.06	4.49E-06	*<0.001*
1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	Phosphatidylcholine (PC)	-0.52	0.11	5.11E-06	*<0.001*
sphingomyelin (d18:1/14:0, d16:1/16:0)*	Sphingomyelins	-0.24	0.05	5.27E-06	*<0.001*
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	Phosphatidylcholine (PC)	-0.26	0.06	8.22E-06	*<0.001*
1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	Phosphatidylcholine (PC)	-0.28	0.06	1.33E-05	*<0.001*
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	Phosphatidylethanolamine (PE)	-0.33	0.08	1.84E-05	*<0.001*
sphingomyelin (d18:1/20:0, d16:1/22:0)*	Sphingomyelins	-0.20	0.05	2.19E-05	*<0.001*
1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)	Phosphatidylcholine (PC)	-0.25	0.06	2.56E-05	*<0.001*
sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*	Sphingomyelins	-0.19	0.04	3.20E-05	*<0.001*
Tryptophan	Tryptophan Metabolism	-0.15	0.04	3.77E-05	*<0.001*
Glycerophosphoethanolamine	Phospholipid Metabolism	-0.17	0.04	4.42E-05	*<0.001*
1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)*	Phosphatidylethanolamine (PE)	-0.43	0.10	4.48E-05	*<0.001*
sphingomyelin (d18:2/14:0, d18:1/14:1)*	Sphingomyelins	-0.22	0.05	4.60E-05	*<0.001*
1-linoleoyl-GPC (18:2)	Lysophospholipid	-0.20	0.05	4.76E-05	*<0.001*
sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)*	Sphingomyelins	-0.19	0.05	6.19E-05	*<0.001*
sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*	Sphingomyelins	-0.16	0.04	6.38E-05	*<0.001*
sphingomyelin (d18:2/21:0, d16:2/23:0)*	Sphingomyelins	-0.21	0.05	6.54E-05	*<0.001*
1-arachidonoyl-GPC (20:4n6)*	Lysophospholipid	-0.21	0.05	8.38E-05	0.01
linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]*	Diacylglycerol	-0.52	0.13	1.12E-04	0.01
glycosyl ceramide (d18:1/20:0, d16:1/22:0)*	Hexosylceramides (HCER)	-0.32	0.08	1.33E-04	0.01
3-methyl-2-oxovalerate	Leucine, Isoleucine and Valine Metabolism	-0.21	0.05	1.53E-04	0.01
sphingomyelin (d18:1/19:0, d19:1/18:0)*	Sphingomyelins	-0.21	0.06	1.61E-04	0.01
4-methyl-2-oxopentanoate	Leucine, Isoleucine and Valine Metabolism	-0.21	0.06	1.72E-04	0.01

Metabolites with significantly different levels in ***vitamin D deficient groups*** (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants)					
glycosyl-N-stearoyl-sphingosine (d18:1/18:0)	Hexosylceramides (HCER)	-0.30	0.08	1.86E-04	0.01
1-palmitoleoylglycerol (16:1)*	Monoacylglycerol	-0.64	0.17	1.93E-04	0.01
sphingomyelin (d17:1/14:0, d16:1/15:0)*	Sphingomyelins	-0.27	0.07	2.03E-04	0.01
sphingomyelin (d18:2/16:0, d18:1/16:1)*	Sphingomyelins	-0.15	0.04	2.07E-04	0.01
1-linoleoylglycerol (18:2)	Monoacylglycerol	-0.70	0.19	2.40E-04	0.01
1-stearoyl-GPE (18:0)	Lysophospholipid	-0.48	0.13	2.48E-04	0.01
1,2-dipalmitoyl-GPC (16:0/16:0)	Phosphatidylcholine (PC)	-0.19	0.05	2.55E-04	0.01
1-palmitoyl-GPI (16:0)	Lysophospholipid	-0.44	0.12	2.83E-04	0.01
1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)	Phosphatidylinositol (PI)	-0.16	0.04	4.06E-04	0.02
sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)	Sphingomyelins	-0.18	0.05	4.27E-04	0.02
palmitoylcarnitine (C16)	Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	-0.26	0.07	4.56E-04	0.02
1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6)	Phosphatidylcholine (PC)	-0.25	0.07	4.80E-04	0.02
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)*	Phosphatidylcholine (PC)	-0.14	0.04	5.33E-04	0.02
glycerophosphorylcholine (GPC)	Phospholipid Metabolism	-0.09	0.03	5.46E-04	0.02
Indoleacetate	Tryptophan Metabolism	-0.19	0.06	6.00E-04	0.03
myristoyl dihydrosphingomyelin (d18:0/14:0)*	Dihydrosphingomyelins	-0.22	0.06	6.38E-04	0.03
Leucine	Leucine, Isoleucine and Valine Metabolism	-0.10	0.03	6.84E-04	0.03
N-stearoyl-sphingosine (d18:1/18:0)*	Ceramides	-0.27	0.08	7.25E-04	0.03
1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*	Phosphatidylinositol (PI)	-0.28	0.08	7.48E-04	0.03
1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)*	Plasmalogen	-0.27	0.08	8.48E-04	0.03
1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	Phosphatidylcholine (PC)	-0.23	0.07	8.98E-04	0.03
sphingomyelin (d18:2/18:1)*	Sphingomyelins	-0.17	0.05	9.54E-04	0.04
1-arachidonoyl-GPI (20:4)*	Lysophospholipid	-0.29	0.09	9.82E-04	0.04
isovalerylcarnitine (C5)	Leucine, Isoleucine and Valine Metabolism	-0.33	0.10	1.20E-03	0.04
1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)	Phosphatidylinositol (PI)	-0.23	0.07	1.22E-03	0.04

Metabolites with significantly different levels in vitamin D deficient groups (Group 4 versus Group 3) (Normolipidemic versus dyslipidemic participants)					
N-palmitoyl-sphingadienine (d18:2/16:0)*	Dihydroceramides	-0.41	0.13	1.33E-03	0.04
glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	Hexosylceramides (HCER)	-0.17	0.05	1.32E-03	0.04
dihomo-linolenoyl-choline	Fatty Acid Metabolism (Acyl Choline)	-0.43	0.13	1.46E-03	0.05
xanthure.te	Tryptophan Metabolism	-0.34	0.11	1.55E-03	0.05
N-acetylneurami.te	Aminosugar Metabolism	-0.20	0.06	1.55E-03	0.05
1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	Phosphatidylcholine (PC)	-0.13	0.04	1.60E-03	0.05
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)*	Phosphatidylcholine (PC)	-0.26	0.08	1.66E-03	0.05
1,2-dilinoleoyl-GPC (18:2/18:2)	Phosphatidylcholine (PC)	-0.32	0.10	1.79E-03	0.05
sphingomyelin (d17:2/16:0, d18:2/15:0)*	Sphingomyelins	-0.18	0.06	1.83E-03	0.05

Supplementary Table S3

Metabolites with significantly different levels in ***normolipidemics groups*** (Group 4 versus Group 1) vitamin D deficient versus sufficient participants					
Metabolite Names	**Sub-Pathway**	**Estimate**	**Std.Error**	**P.value**	**FDR**
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)*	Phosphatidylcholine (PC)	-0.31	0.09	4.04E-04	0.02
Ergothioneine	Food Component/Plant	-0.48	0.13	4.94E-04	0.02
Urea	Urea cycle; Arginine and Proline Metabolism	-0.19	0.05	6.73E-04	0.03
4-methyl-2-oxopentanoate	Leucine, Isoleucine and Valine Metabolism	-0.20	0.06	6.76E-04	0.03
Valine	Leucine, Isoleucine and Valine Metabolism	-0.13	0.04	1.12E-03	0.04
arabo.te/xylo.te	Pentose Metabolism	-0.23	0.07	1.13E-03	0.04
1-(1-enyl-oleoyl)-GPE (P-18:1)*	Lysoplasmalogen	-0.27	0.08	1.18E-03	0.04
Allantoin	Purine Metabolism, (Hypo)Xanthine/Inosine containing	-0.19	0.06	1.39E-03	0.05
methionine sulfone	Methionine, Cysteine, SAM and Taurine Metabolism	-0.22	0.07	1.43E-03	0.05
1-(1-enyl-stearoyl)-GPE (P-18:0)*	Lysoplasmalogen	-0.28	0.09	1.59E-03	0.05
Tryptophan	Tryptophan Metabolism	-0.12	0.04	1.68E-03	0.05

Supplementary Table S4

Metabolites with significantly different levels in ***dyslipidemics groups*** (group 3 versus group 2) vitamin D deficient versus sufficient participants					
Metabolite Names	**Sub-Pathway**	**Estimate**	**Std.Error**	**P.value**	**FDR**
hydroxy-CMPF*	Fatty Acid, Dicarboxylate	-2.08	-5.87	$1.28E^{-08}$	*<0.001*
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	Fatty Acid, Dicarboxylate	-2.09	-5.41	$1.44E^{-07}$	*<0.001*
docosahexaenoate (DHA; 22:6n3)	Long Chain Polyunsaturated Fatty Acid (n3 and n6)	-0.52	-4.18	$3.90E^{-05}$	*<0.001*
S-methylcysteine sulfoxide	Methionine, Cysteine, SAM and Taurine Metabolism	-0.62	-3.92	$1.11E^{-04}$	0.01
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)*	Phosphatidylcholine (PC)	-0.41	-3.76	$2.05E^{-04}$	0.01
sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)*	Sphingomyelins	-0.22	-3.70	$2.61E^{-04}$	0.01
perfluorooctanoate (PFOA)	Chemical	-0.51	-3.62	$3.56E^{-04}$	0.02
sphingomyelin (d18:1/24:1, d18:2/24:0)*	Sphingomyelins	-0.19	-3.52	$5.05E^{-04}$	0.02
hydroxypalmitoyl sphingomyelin (d18:1/16:0(OH))**	Sphingomyelins	-0.21	-3.48	$5.81E^{-04}$	0.03
tartro.te (hydroxymalo.te)	Food Component/Plant	-0.34	-3.43	$6.93E^{-04}$	0.03
sphingomyelin (d18:2/21:0, d16:2/23:0)*	Sphingomyelins	-0.23	-3.40	$7.73E^{-04}$	0.03
2-hydroxyglutarate	Fatty Acid, Dicarboxylate	0.23	3.34	$9.57E^{-04}$	0.04
Lysine	Lysine Metabolism	-0.13	-3.27	$1.23E^{-03}$	0.04
sphingomyelin (d18:2/24:1, d18:1/24:2)*	Sphingomyelins	-0.18	-3.23	$1.38E^{-03}$	0.05
1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)*	Phosphatidylethanolamine (PE)	-0.48	-3.16	$1.73E^{-03}$	0.05

CHAPTER 4: PROTEOMIC PROFILING OF VITAMIN D AND DYSLIPIDEMIA

Proteomics is an important tool to understand the cellular environment. The interaction between proteins is an accurate indicator of the physiological and pathophysiological modifications in the biological system [330]. HDL-associated proteins levels are affected by the changes in the internal environment. For example, ApoM is polymerized in presence of high glucose level conditions such as diabetes, which in turn impacts protein's function and attenuates its ability to bind to S1P, consequently causes a disruption and malfunctioning of HDL particle biogenesis [331]. Another example is ApoA-1 which is found in a very high concentrations in atherosclerotic human arterial tissue, but not associated to HDL particle. ApoA-1 in atherosclerotic tissues was oxidized and dysfunctional and it's ability to activate LCAT enzyme was dramatically reduced by 90%. In addition, ApoA-1 ability to accept cholesterol was also reduced by 80% leading to significant decrease in this function [332]. Here, we used proteomics analysis to understand the relationship between vitamin D deficiency as a potent anti-inflammatory effector and HDL- associated proteins (ApoA-1, ApoM, and ApoD) in dyslipidemia. The first part of the study is focused on the variation of apolipoproteins levels among participants with and without vitamin D deficiency and dyslipidemia. Since the relationship between vitamin D, HDL- associated proteins, inflammatory mediators, and other factors in immune system are highly complex, we also performed a holistic serum proteomic profile analysis of 277 participants with different levels of vitamin D and lipidomic status. We observed the enrichment of the cytokines pathways to be prominently altered in participants with combined vitamin D deficiency and dyslipidemia. The data indicate the anti-inflammatory roles of vitamin D and HDL, however, the causality and directionality of this cross-talk is not deciphered. In this chapter we aimed to elucidate the bigger picture of complex proteomics

pathways responsible for the alteration in proteomics signatures observed during vitamin D deficiency and dyslipidemia.

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Vitamin D status affects the proteomic profile of HDL-associated proteins and inflammatory mediators in dyslipidemia

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Abstract

Background: Vitamin D deficiency and dyslipidemia are highly prevalent globally and impacts human health. Vitamin D is essential for bone metabolism and also exerts immune modulatory effects. Vitamin D deficiency is associated with many chronic diseases with inflammatory nature. Dyslipidemia is characterized by a reduction in HDL and an increase in LDL and triglyceride levels. Several studies reported evidence for the association between vitamin D deficiency and low HDL. However, the exact mechanism by which vitamin D impacts HDL biogenesis and anti-inflammatory potential is not clear. Here we investigated the proteomics profiles of participants with and without vitamin D deficiency and dyslipidemia.

Materials and methods: 1301 proteins of 274 participants with or without vitamin D deficiency and dyslipidemia were analyzed and categorized into four groups according to vitamin D and dyslipidemia status. Data were obtained from the Qatar biobank database.

Results: Proteomics enrichment analysis of HDL-associated apolipoprotein showed that ApoM and ApoD were downregulated in participants with a combination of dyslipidemia and vitamin D deficiency. Whereas inflammation-induced acute phase proteins like SAA1 and SOD1 were overexpressed in those participants. Pathway enrichment analysis revealed reflected the high inflammatory status of participants with vitamin D deficiency and dyslipidemia. The enriched pathways were i.e. MAPK pathway, JAK-STAT signaling pathway, Ras signaling pathway and cytokine-cytokine receptor interaction, AGE-RAGE, ErbB signaling, and cancer pathways.

Conclusions:

Proteomic profiling revealed major alteration in HDL- associated proteins among participants with or without vitamin D deficiency and dyslipidemia. The inflammation pathways were enriched in vitamin D deficiency, whereas cancer pathways and inflammation pathways were heightened in participants who has a combination of vitamin D deficiency and dyslipidemia.

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1. Introduction

Vitamin D deficiency is reported widely across the globe. More than half of the population (64%) in Qatar have serum 25(OH)D concentrations below 12 ng/mL, which is the cutoff value of vitamin D deficiency [27]. Recently, studies linked vitamin D deficiency to several health problems including disorders associated with high inflammatory status e.g. metabolic syndrome, dyslipidemia, chronic cardiovascular diseases, obesity, and autoimmune diseases [1]. Vitamin D precursors are produced in the skin by UVB sunlight. These vitamin D precursors are converted into 25-hydroxyvitamin D (25(OH)D) metabolites by the vitamin D 25-hydroxylase enzyme. The production of the hormonally active form of vitamin D takes place in the kidney, where the 25(OH)D is converted to 1,25 dihydroxyvitamin D (1,25(OH)2D3) via 25-hydroxyvitamin D-1α-hydroxylase enzyme. Other tissues besides kidenys produce the active form 1,25(OH)2D such as skin, immune cells, intestinal epithelium, parathyroid gland, prostate, and breast [2]. The Active form of vitamin D 1,25(OH)2D binds to the vitamin D Receptor (VDR). This interaction stimulates a cascade of signaling pathways, resulting in the up or down-regulation of a number of gene targets. Therefore, the disturbance in vitamin D homeostatic level is associated with the disturbance in the expression of downstream targets. Vitamin D deficiency occurs when the serum concentration of 25(OH)D

is less than 12 ng/mL, due to lack of exposure to sunlight or poor diet habits [254]. Some cases of vitamin D deficiency has genetic predisposition such as familial hypertension due to polymorphisms of the VDR gene rs3847987. Patients with this disease suffer from hypertension, diabetes, insulin resistance, and vitamin D deficiency [29-31]. VDR polymorphisms are also observed to heighten the risk of developing metabolic syndrome particularly the VDR ApaI variant, which is linked to hypertriglyceridemia levels. Other variants e.g. BsmI and TaqI were associated with a lower level of HDL-C[333]. This might indicate the role of vitamin D and VDR in regulating lipidomic and lipoprotein in the blood.

Vitamin D is constructed from cholesterol as a backbone [334]. While the main function of lipoproteins is organizing the trafficking and distribution of cholesterol. High-density lipoprotein (HDL) belongs to the lipoproteins family and plays a crucial role in dyslipidemia and coronary heart disease (CVD) [335]. HDL's function is to eliminate the excess cholesterol from peripheral tissues and the bloodstream, to be transferred to the liver for recycling. This process is called reversed cholesterol transfer (RCT). The disruption in RCT affects many tissues, specifically the cardiovascular systems [336]. Furthermore, HDL is a carrier for several immune modulators and antimicrobial peptides such as cathelicidin or LL-37[336]. The gene encoding LL-37 has VDRE binding element and thus is one of the vitamin D target genes [337].

Both Vitamin D and HDL production and function are affected by several proinflammatory cytokines. For example, TNF-α attenuated HDL biogenesis by reducing the cellular cholesterol transporter ABCA1 expression [338]. A suppression of TNF- α by an anti-TNF-α agent aided in increasing HDL cholesterol level [339]. Similarly, low vitamin D level has been linked to overexpression of proinflammatory cytokines such as TNF-α and IL-8 [220, 340]. However, studies didn’t show a conclusive result regarding the ability of vitamin D

supplementation to reduce TNF-α levels [341, 342]. Although the combination of anti-TNF-α and vitamin D supplementation was found to improve the clinical manifestations of inflammatory disease e.g. Crohn's disease [343].

From another perspective, vitamin D found to affect the proteomic profile or expression levels of proteins. The administration of 1,25-dihydroxyvitamin-D3 in vivo studies was shown to impact the proteomic profile in mice brains with multiple sclerosis. The modifications were including proteins that induced myelin repair and were involved in calcium binding activity, e.g. calretinin, S10A5, and secretagogin. In addition to proteins linked to mitochondrial function, e.g. NADH-ubiquinone oxidoreductase chain 3, and acyl-coenzyme A synthetase [344]. Moreover, these proteomic profile modifications could predict the risk of cardiovascular disorders. For instance, the modifications of HDL proteins e.g. paraoxonase/arylesterase 1 (PON1), paraoxonase/arylesterase 3 (PON3), LCAT, and apolipoprotein A-IV enable the cardiovascular disorders risk prediction in patients with chronic kidney disease [345, 346].

Al-Daghri et. al used MALDI profiling to study the alteration of the proteomic profile during vitamin D deficiency in obese participants. They reported a change in apolipoprotein CIII, apolipoprotein B100, alpha-1-antichymotrypsin, and complement C3 in participants with high BMI compared to those with low BMI. Maldi mass spectrometry was used to allocate the change of Apo A1, Apo B100, and lipoprotein lipase. However, the results were not conclusive, although, there was an increase in expression levels in obese subjects that has been reported using the classical immunoblotting methods. The author suggested that proteomic profile alteration occurred in the total protein [347].

Vitamin D deficiency is associated with high inflammatory status and disturbance in lipid profile. Vitamin D serum concentration is inversely associated with the level of tumor

necrosis factor receptor 2 TNFR-2 and C-reactive protein (CRP). In contrast, the anti-inflammatory cytokines IL-10 and HDL are suppressed during vitamin D deficiency [348]. Jin et. al. clinical study showed that the increase in serum 25(OH)D in pregnant women highly impacted the lipid profile by decreasing total cholesterol, triglyceride, LDL-c, and HDL-c. In the same studies, the CRP level also was inhibited by high lipid metabolism [349].

Previous studies reported that both vitamin D deficiency and Dyslipidemia status can modify the proteomic presentation to some extent. However, the effect of combined vitamin D deficiency and dyslipidemia on inflammatory markers and apolipoproteins is not clear.

2. Material and Methods

2.1.Study Design

This cohort is retrospective cross-sectional. Data from 274 participants was obtained from the QBB database (https://www.qatarbiobank.org.qa/). Participants were selected randomly from a larger cohort of 1820 subjects. This study was performed in line with the World Medical Association Declaration of Helsinki–Ethical Principles for medical research involving human subjects. The Institutional Research Board of Qatar University QU-IRB form (1366-E/20), and QBB-IRB form (EX-2020-QBB-RES-ACC-0237-0124), approved all protocols. All participants consented to the use of their samples for research. The cohort was divided into 4 groups according to vitamin D status (sufficient and deficient) and the presence or absence of Dyslipidemia. Serum 25 dihydroxy vitamin D (25(OH)D) concentrations were considered deficient <12 ng/mL and a sufficient ≥ 20 ng/mL[28]. Dyslipidemia status was determined according to the following criteria: high total cholesterol (>6.2 mmol/L), high LDL-c (>4.1 mmol/L), and high TG (>2.3 mmol/L) [295]. Accordingly, the four groups were: vitamin D sufficient and normolipidemic (Group 1, n = 64); vitamin D sufficient and

dyslipidemia (Group 2, n = 26); vitamin D deficient and dyslipidemia (Group 3, n = 85); vitamin D deficient and normolipidemic (Group 4, n = 99).

The inclusion criteria were adults devoid of comorbidities except for dyslipidemia and vitamin D deficiency. The exclusion criteria included using vitamin D supplements, pregnancy, and those with chronic diseases such as diabetes, high blood pressure, asthma, hay fever, blood clot, heart attack, angina, stroke, emphysema/chronic bronchitis, hyperthyroidism hyperparathyroidism, Cushing syndrome, and cancer.

2.2.Biochemical Measurements

QBB team collected venous blood samples from participants after their consent and sent them to Hamad Medical Corporation Laboratories (College of American Pathologist Accredited Laboratory) to be analyzed. Serum 25(OH)D concentration was analyzed using electrochemiluminescence immunoassay (LIAISON® 25-hydroxyvitamin D Total Assay, DiaSorin Inc., Stillwater, MN, USA) and both vitamin D2 and vitamin D3 fractions were measured. Lipid profiles, Alanine aminotransferase ALT and AST, Total Bilirubin, Total Protein, Albumin, Alkaline Phosphatase, Creatinine, Urea, Uric Acid, and Sex Hormone binding Globulin were measured using automatic Biochemistry Analyzers at Qatar Biobank and Hamad General Hospital as previously described [252, 253]. Proteomic analysis has been done by QBB using SOMAscan aptamer-based affinity proteomics platform (Somalogic, Boudler, CO) more details and protocols are mentioned in Thareja et. al. study [350].

2.3.Statiscal analysis

Baseline characteristics were reported as means (SD) for normally distributed values and as median (interquartile) for the non-normally distributed variables. The linearity was assessed for ApoM, ApA-1, ApoD, and vitamin D levels. One-way ANOVA was used to detect the significant difference in mean for 122 proteins in the 4 groups. Whereas the T-test was used to address the differences in the significance of apolipoproteins (ApoA-1, ApoB, ApoD, ApoE, ApoM, and ApoL-1) means between the groups (Group1 versus Group 2), (Group1 versus Group 3), (Group1 versus Group 4), (Group2 versus Group 3), (Group2 versus Group 4), and (Group3 versus Group 4). The gender difference in protein level was reported for ApoM and ApoA-1, and lipocalin 2. All statistical tests were 2-tailed tests and the P value was considered significant if <0.05. SPSS and Graphpad Prism 5 were used for data analysis.

2.4.Protein expression enrichment analysis and co-expression analysis

The total number of analyzed proteins was 1301 proteins for 274 participants. Gender and BMI were considered as a confounder. To calculate the protein expression, we used the ProTExA workflow which facilitates differential expression analysis and co-expression network analysis as well as pathway analysis [351]. For each of the six comparison groups, we start the analysis by performing statistical analysis and filtering to highlight the genes of the differentially expressed proteins. For this purpose, we used the "default pre-processing scheme" option, which deletes rows that include NA and empty values. For entries that include protein names separated by semi-colon, the script keeps only the first name. For duplicate proteins, the script keeps one row per protein, that contains the mean value of all the duplicated rows per sample. This is followed by the normalization process where the pipeline uses the

LIMMA statistical analysis package[352] that requires the dataset to be normalized and transformed to log2 scale. For this purpose, we use the quantile algorithm as our normalization method of choice and log2 transformation. The next step involved performing enrichment analysis to identify top-scored pathways, where we used p value <= 0.05 as our threshold but without implementing any filtering for the fold change threshold. This will give us a list of high-quality differentially expressed proteins, which we then use to perform further downstream analysis. The first such analysis involves enrichment analysis. We decided to do three types of enrichment analysis: i) functional enrichment analysis using InterPro (InterPro Domain 2019) ii) pathway enrichment analysis using KEEG human pathway 2019 and iii) phenotype enrichment analysis using Human Phenotype Ontology. For all types of enrichment analysis, we use p value <= 0.05 for selecting a significant enriched result. Other than enrichment analysis, we also investigate the co-expression of the differentially expressed proteins. We used the Maximum Relevance Minimum Redundancy network method (MRNET) algorithm to infer the network [353].

2. Result

Cohort baseline characteristics

The total number of participants in this cohort was 277, three participants were excluded due to missing data. The cohort is categorized into 4 groups based on vitamin D status and serum lipids profile. Group 1 with 64 participants who were vitamin D sufficient (greater

than 20 ng/mL) with normolipidemia. Group 2 with 26 participants who were vitamin D sufficient with dyslipidemia. Group 3 with 85 participants who were vitamin D deficient (less than 12 ng/mL) with dyslipidemia. Group 4 with 99 participants who exhibited vitamin D deficiency with normolipidemic. Group 1 was considered the control and a reference point for the other groups. The baseline characteristics of participants in this current study were reported in our previous study on metabolomic profiling for the same cohort. Variables like gender, BMI, WBC, Monocyte, C-Peptide, and lipid profile components including total cholesterol, HDL, LDL, and TG [284]. In this paper, we investigated Liver and kidney function tests, since those systems have a great impact on lipidomic profile components and vitamin D metabolism.

The liver function test was compared among the four groups and ALT values were within the normal range for all (Table 1). However, a significant variation in the median was observed, particularly in the fourth group i.e. participants with vitamin D deficiency and normolipidemic, where the median was on the lower borderline (9.5 U/L). Moreover, the dyslipidemic groups (Group 2 and Group 3) has a slight increase in median compared to the control Group 1 (vitamin D sufficient with normolipidemia). No significant difference was observed among other liver biomarkers AST, total Bilirubin, total protein, albumin, and alkaline phosphatase. Whereas, kidney function biomarker creatinine was slightly higher in both Group 2 and Group 3 (the dyslipidemic groups) in comparison with other normolipidemic groups. The highest creatinine level was observed in Group 3 vitamin D deficient participants with dyslipidemia. Furthermore, uric acid was the highest among groups with dyslipidemia i.e. group 2 (338.2 µmol/l) participants (vitamin D sufficient with dyslipidemia) and group 3 (302.5 µmol/l) participants (vitamin D deficient with dyslipidemia). In contrast, the lowest value of uric acid was seen in group 4 participants (vitamin D deficiency with normolipidemia). Sex Hormone binding Globulin is produced in the liver and binds three steroid hormones:

estrogen, dihydrotestosterone (DHT), and testosterone was not statistically significant in the 4 groups (Table 1).

Table 4. 1. Baseline characteristics of participants in this study

	VitD sufficient, No dyslipidemia (G1)	VitD sufficient, Dyslipidemia (G2)	VitD deficient, Dyslipidemia (G3)	VitD deficient, No dyslipidemia (G4)	
Number of subjects	n=64	n=26	n=85	n=99	
	Mean (SD) (SD)	**Mean (SD)**	**Mean (SD) (SD)**	**Mean (SD) (SD)**	**P value**
Vitamin D (ng/ml)	24.41(7.4)	25.91(7.0)	10.04 (1.9)	9.77 (1.8)	<.001
Age	38.67(11.0)	47.93 (23.3)	39.09 (17.3)	28.39 (15.0)	<.001
Liver biomarker					
ALT(GPT) (U/L)	13 (2)	21*[21]	19.5 (9.1)	9.5 (0.7)	0.044
AST(GOT) (U/L)	15 (2.6)	20 (7.3)	35.5 (21.9)	17.5 (3.5)	0.389
Total Bilirubin (µmol/L)	7.2 (3.2)	9.5* [13.4]	8.8 (9)	9.7 (8)	0.302
Total Protein (g/L)	72.3 (3)	73.5* [9]	59 (14.1)	77.5 (6.3)	0.935
Albumin (g/L)	46.3(2.9)	43* [6]	48.5 (10.6)	49 (2.8)	0.856
Alkaline Phosphatase (U/L)	63 (10.8)	76* [53]	58.5 (3.5)	58.5 (3.5)	0.947
Kidney biomarker					
Creatinine (µmol/L)	68.5 (14.2)	73.0* [16.5]	74.9 (16.5)	64.0 (14.8)	<.001
Urea (mmol/l)	4.8 (1.3)	4.9* [1.8]	5.4 (7.8)	4.6 (6.2)	0.493
Bicarbonate (mmol/l)	26.1 (2.1)	26.0* [3.5]	25.7 (3)	26 (2.8)	0.646
Uric Acid (µmol/l)	264.7(52.3)	338.2*[117]	302.5(106.8)	214.5 (40.3)	<.001
Sex Hormone binding Globulin (nmol/L)	47*[26.5]	37*[24.1]	29.2*[21]	36.8* [34.2]	0.092

Data are represented as mean (SD) or *Median [Interquartile] for skewed data. p-value was considered significant if the value < 0.05. VitD: vitamin D metabolite 25(OH)D3.

Impact of vitamin D deficiency on HDL-associated proteins expression

To understand the cross-talk or impact of vitamin D deficiency on lipid profile homeostasis, we investigated the expression of the HDL-associated protein using proteomics profiling. Specifically, apolipoproteins concentrations vary according to vitamin D and lipid profile status (Table 2). The data indicate that ApoA-1 has the lowest expression values in Group 4 (vitamin D deficiency with normolipidemia), but the combination of dyslipidemia and vitamin D deficiency increased ApoA-1 (Figure 1A). ApoB is significantly increased in dyslipidemia as expected since ApoB is mainly associated with LDL. Although, vitamin D deficiency combined with dyslipidemia slightly reduced ApoB compared to dyslipidemia with vitamin D sufficiency. Therefore, vitamin D deficiency status has a modifying effect on ApoB **(**Figure 1B). ApoD is reduced during combined dyslipidemia and vitamin D deficiency only (Figure 1C). Whereas, ApoE is increased in dyslipidemia and the combination of dyslipidemia and vitamin D deficiency has a major effect on increasing ApoE (Figure 1D).

Dyslipidemia reduced ApoM and when combined dyslipidemia with vitamin D deficiency ApoM reduction was more pronounced (Figure 1E). This is the most supportive evidence that vitamin D status affects HDL levels as ApoM is essential for HDL biogenesis. Vitamin D deficiency increased ApoL1, which has a limited impact on dyslipidemia (Figure 1F).

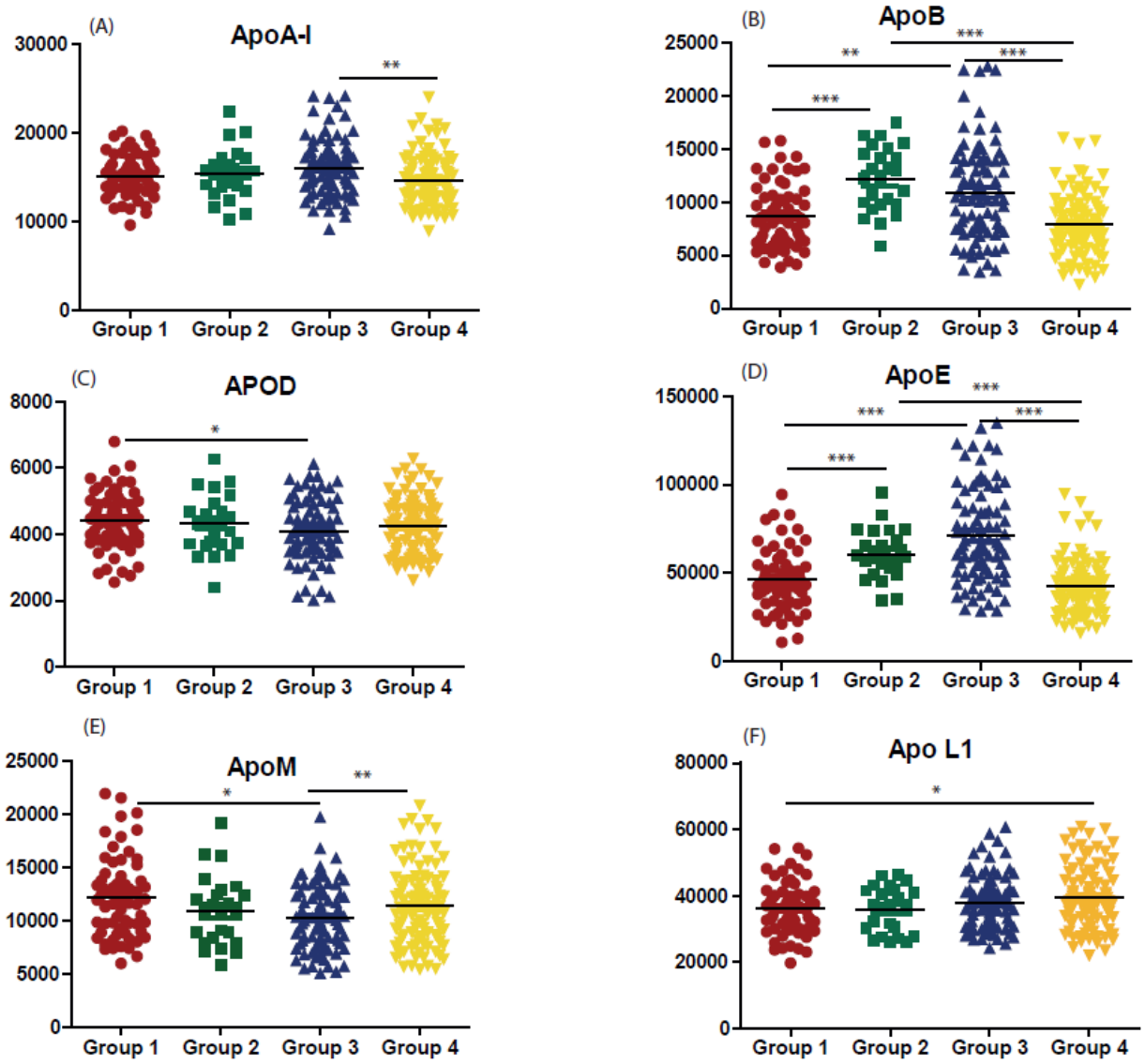

Figure 4. 1. Apolipoproteins concentrations are represented with their means among the four groups. Group 1 (n=64) represents participants who were vitamin D sufficient with normolipidemia (control group). Group 2 (n=26) participants who were vitamin D sufficient with dyslipidemia. Group 3 (n=85) represents participants who were vitamin D deficient with dyslipidemia. Group 4 (n=99) represents participants who exhibited vitamin D deficiency with normolipidemia. **A**: ApoA-1; **B**: ApoB; **C**: ApoD ; **D**: ApoE; **E**: ApoM and **F**: ApoL1.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Gender effect on apolipoproteins concentrations

Since HDL levels are higher in females than males, we further investigated apolipoprotein concentrations to identify if there is a gender-based difference. Here we report that ApoA-1 has a significant gender-based difference among participants with vitamin D deficiency without dyslipidemia. Males expressed significantly higher levels and ApoA-1 compared to females (Figure 2A). In contrast, ApoM was higher in females compared to males in the control group 1 which represents the normal physiological conditions, and in vitamin D deficient group 4 without dyslipidemia (Figure 2B). On the other hand, combined vitamin D deficiency and dyslipidemia (group 3) seem to impact ApoM levels in men more than women although statistically not significant, but lower levels of ApoM are visible (Figure 2B). ApoD has not had a significant variation in groups' levels although females have higher ApoD levels than males in all groups (Figure 2C). ApoM and ApoD belong to the lipocalin family, therefore we investigated lipocalin 2 (LCN2) expression among the four groups in this study. Lipocalin 2 is a carrier protein and anti-inflammatory molecule that increases during acute phase inflammation and was used as a control for gender differences. No significant difference was observed in lipocalin 2 expression between males and females. However, there was an obvious reduction in lipocalin 2 levels in Group 3 participants with combined vitamin D deficiency and dyslipidemia (Figure 2D).

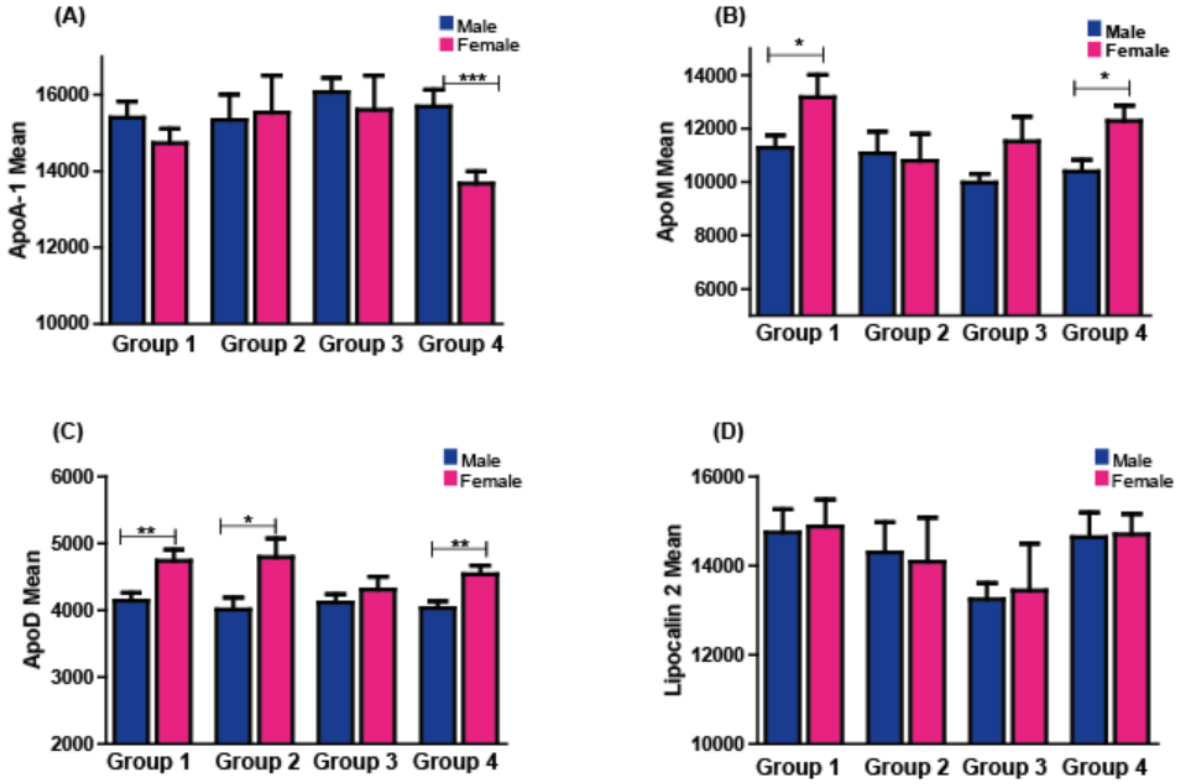

Figure 4. 2. The difference of means between males and females in four groups of Apolipoproteins (ApoA-1, ApoM, ApoD) and Lipocalin 2. Group 1 (n=64) represents participants who were vitamin D sufficient with normolipidemia (control group). Group 2 (n=26) participants who were vitamin D sufficient with dyslipidemia. Group 3 (n=85) represents participants who were vitamin D deficient with dyslipidemia. Group 4 (n=99) represents participants who exhibited vitamin D deficiency with normolipidemia. **A**: ApoA-1; **B**: ApoM; **C**: ApoD ; **D**: Lipocalin 2 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

HDL-associated proteins expression profile during vitamin D deficiency and dyslipidemia

Table 2 shows the expression of various HDL-associated proteins among participants included in the four groups (group 1 (n=64) vitamin D sufficient participants with normolipidemic (control group); group 2 (n=26) vitamin D sufficient participants with dyslipidemia; group 3 (n=85) vitamin D deficient participants with dyslipidemia and group 4 (n=99) vitamin D deficient participants with normolipidemia). The expression of proteins such as ApoM, ApoA-1, and Serum amyloid A (SAA), which directly affects HDL biogenesis was significantly varied among the four groups of participants. ApoM protein level was significantly reduced among dyslipidemia groups 2 and 3 compared to healthy controls group 1. Whereas ApoA1 was slightly elevated in those groups as expected. Apo E and its isoforms ApoE2, Apo E3, and Apo E4 were tremendously elevated in group 3 (vitamin D deficient with dyslipidemia). SAA protein level reflects the disturbance in HDL level. In this study, we observed that SAA1 protein, in particular, has its lowest value in the dyslipidemic groups (groups 2 and 3) but it was enriched in group 4 suffering from vitamin D deficiency without dyslipidemia. The concentrations of cathepsin family components including (Cathepsin B, G, and S), which are also found associated with HDL particles, had insignificant fluctuation among the four groups. In contrast, Cathepsin V level was decreased in Group2 vitamin D sufficient with dyslipidemia, while its highest value was recorded in Group 4 (vitamin D deficiency with normolipidemic) (Table 2).

Table 4. 2. HDL-associated proteins semi-quantitative among the four groups

	VitD sufficient, No dyslipidemia (G1)	**VitD sufficient, Dyslipidemia (G2)**	**VitD deficient, Dyslipidemia (G3)**	**VitD deficient, No dyslipidemia (G4)**	
Number of subjects	**n=64**	**n=26**	**n=85**	**n=99**	
Protein	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**P value**
ApoM	12166 (3800)	10960 (3184)	10286 (3009)	11474 (3786)	**0.01**
ApoA-1	15091 (2283)	15418 (2766)	15974 (3239)	14609 (2843)	**0.01**
ApoD	4423 (867)	4317 (853)	4154 (1002)	4299 (880)	0.36
ApoB	8725 (2940)	13052 (5364)	10927 (4556)	8644 (7819)	**0.00**
ApoE	46210 (17154)	60738 (13826)	72484 (28737)	42396 (15366)	**0.00**
Apo E2	288792 (36396)	296457 (26392)	312578 (36880)	289620 (37046)	**0.00**
Apo E3	24253 (53475)	278302 (31064)	307414 (69002)	230874 (50492)	**0.00**
Apo E4	251335 (47162)	278980 (31551)	306950 (54539)	245443 (44302)	**0.00**
ApoL-I	36136 (7813)	36758 (8073)	37997 (8078)	39817 (9604)	**0.051**
SAA1	3490 (3952)	2644 (2181)	2722 (2926)	3574 (6756)	0.572
Cathepsin B	1474 (336)	1445 (375)	1389 (261)	1369 (318)	0.17
Cathepsin G	944 (412)	895 (241)	957 (394)	1088 (738)	0.19
Cathepsin S	836 (183)	867 (186)	818 (164)	809 (153)	0.41
Cathepsin V	2011 (817)	1816 (510)	1871 (500)	2351 (827)	**0.00**

HDL-associated proteins concentrations in the four groups represented by Mean (SD), the p-value was considered significant for value < 0.05. VitD: vitamin D metabolite 25(OH)D3.

Semi-quantitative expression of cytokines and inflammatory mediators during vitamin D deficiency and dyslipidemia

Subclinical inflammation is a common feature during vitamin D deficiency and dyslipidemia. To understand how vitamin D deficiency affects HDL anti-inflammatory potential, we examined the expression of cytokines and inflammatory mediators among the participants in this study. Table 3 compares the expression level of various proinflammatory

cytokines and other inflammatory mediators among the four groups as explained above. Here, we selected a few commonly reported proinflammatory cytokines however, no significant changes were observed for the majority of these cytokines levels among the four groups except for CXCL8 and IL-18Ra (Table 3). The chemokine CXCL8 (IL8) concertation was significantly upregulated in the dyslipidemic group (group 2). A similar increase was also seen in the antimicrobial peptide LEAP-1 or hepcidin, the major iron-regulating hormone. Both dyslipidemic groups (group 2 and group 3) had an elevated level of LEAP-1. Whereas the normolipidemic groups (group1 and 4) has diminished level of hepcidin / LEAP-1, particularly group 4 (vitamin D deficiency with normolipidemia). The antimicrobial protein Lipocalin 2 was observed to be raised in the control group, while the combination of vitamin D deficiency and dyslipidemia (group 3) lead to a decrease in Lipocalin 2. Lipopolysaccharide Binding Protein (LBP), which plays a role in inflammation and endotoxin detoxification was also reduced in participants with vitamin D deficiency and dyslipidemia (Table 3).

Table 4. 3. Cytokines and inflammatory mediators, semi-quantitative levels

	VitD sufficient, No dyslipidemia (G1)	VitD sufficient, Dyslipidemia (G2)	VitD deficient, Dyslipidemia (G3)	VitD deficient, No dyslipidemia (G4)	
Protein	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**P value**
CRP	66258 (38968)	76270 (44954)	63440 (37231)	61669 (43577)	0.423
LEAP-1	11227 (10184)	12076 (8005)	11502 (7596)	7652 (7283)	**0.00**
Lipocalin 2	14814 (3144)	14216 (2833)	13293(3253)	14633 (3526)	**0.02**
LBP	98273 (15325)	98491 (12265)	93028 (14788)	98845 (15693)	**0.05**
Ferritin	9656 (7930)	11689 (8169)	12279 (9111)	7766 (7694)	**0.00**
TLR4	422 (191)	359 (91)	421 (242)	409 (218)	0.58
TLR4:MD-2 complex	4588 (936)	4404 (804)	4499 (762)	4584 (857)	0.72
TNF-α	516 (176)	481 (60)	485 (112)	488 (110)	0.42
IFN-ɤ	750 (363)	635 (77)	691 (589)	681 (147)	0.56
IL-1 β	2316 (1192)	2098 (351)	2180 (603)	2360 (2214)	0.78
IL-1 α	757 (664)	684 (362)	657 (223)	1049 (2490)	0.33
IL-2	1512 (327)	1522 (296)	1415 (250)	1501 (707)	0.53
IL-6	374 (100)	415 (177)	417 (119)	398 (99)	0.13
IL8 (CXCL8)	732 (180)	1294 (2589)	804 (441)	708 (114)	**0.01**
IL-10	212 (63)	198 (24)	258 (248)	222 (105)	0.17
IL-12	334 (206)	279 (99)	308 (159)	351 (317)	0.436
IL-13	702 (186)	715 (259)	688 (166)	699 (266)	0.95
IL-22	1155 (1318)	961 (574)	889 (332)	935 (468)	0.16
IL-18 Ra	9757 (2661)	9203 (2321)	10779 (2841)	9715 (3087)	**0.02**
IL-18 BPa	8118 (2173)	8190 (1749)	7934 (1956)	7845 (2202)	0.80

Cytokines and inflammatory markers concentration in the four groups, represented by Mean (SD), the p-value was considered significant for value < 0.05. VitD: vitamin D metabolite 25(OH)D3.

Holistic proteomic profile analysis of the participants with or without vitamin D deficiency and dyslipidemia

The global effect of vitamin D deficiency and dyslipidemia on serum proteomics profile was investigated. Herein, the variations in proteins expression and the involved pathways among different groups with or without dyslipidemia were examined and the top 20 most enriched proteins in each group were listed in Table 4. To illustrate the effect of dyslipidemia on proteomic profile, we first compared Group 1 participants who were vitamin D sufficient with normolipidemia (control group) and Group 2 participants who were vitamin D sufficient with dyslipidemia (Figure 3A). The data revealed that two of the apolipoproteins ApoE and ApoB were highly significant or enriched. ApoE is the main apolipoprotein associated with chylomicrons, and ApoB is the main apolipoprotein associated with LDL. As expected, ApoE and ApoB were upregulated in participants with dyslipidemia in addition to LDL receptor protein LRP1B.

Several proteins involved in the immune system were significantly enriched as well such as the pro-inflammatory cytokines IL7, and the protein-coding lysosomes in the neutrophil AZU1. The TNF ligand CD70 was downregulated in the same category. The most significant pathway was the PI3K-Akt signaling pathway, followed by pathways in cancer, MAPK signaling pathway, Ras signaling pathway, JAK-STAT signaling pathway, cytokine-cytokine

receptor interaction, prostate cancer, fluid shear stress and atherosclerosis, transcriptional misregulation in cancer, and hematopoietic cell lineage (Table 4 and Figure 3A).

To illustrate the effect of combined vitamin D deficiency and dyslipidemia on the proteomic profile, we compared Group 1 (control group) and Group 3 representing participants who were vitamin D deficient with dyslipidemia. In this comparison, ApoE and LDL receptor protein LRP1B were elevated. Furthermore, some proteins involved in inflammation were significantly upregulated e.g. the phosphatidylethanolamine-binding protein PEBP1. In addition, SOD1 which is a main antioxidant against free superoxide radicals was also significantly high. HTRA2 protein that plays a role in inducing apoptosis was also enriched. On the contrary, the member of the TNF-receptor superfamily TNFRSF17 and immunoglobulin member LSAMP were decreased in combined vitamin D deficiency and dyslipidemia. Furthermore, the appetite stimulator and energy homeostasis protein GHRL was markedly downregulated**.** The holistic proteomics analysis showed the inflammation and cancer pathways were the most significantly enriched in combined vitamin D deficiency and dyslipidemia (pathways in cancer, PI3K-Akt signaling, MAPK signaling, cytokine-cytokine receptor interaction, Ras signaling, JAK-STAT signaling, Hepatitis B, ErbB signaling, AGE-RAGE signaling pathway in diabetic complications, and HIF-1 signaling pathway (Table 4 and Figure 3 B)**.**

The third comparison was to elucidate proteomic profile modifications by vitamin D deficiency where Group 1 (control) was compared to Group 4 of participants who exhibited vitamin D deficiency with normolipidemia. In this comparison, proteins involved in calcium or bone metabolism such as calcium-binding protein ANXA2, CAMK2D calcium/calmodulin-dependent kinase, and calcium and hydroxyapatite binding protein IBSP, bone mineralization regulator OMD, were increased in vitamin deficient participants. Insulin-like growth factor

binding protein (IGFBP3) also was increased (Figure 3C). In contrast, the FAS protein, the cell death, and TNF-receptor superfamily member, and Interleukin 5 specific subunit of a heterodimeric cytokine receptor IL5RA were downregulated (Figure 3C). Here, the top significant enriched pathways were related to inflammation i.e. Cytokine-cytokine receptor interaction, IL-17 signaling pathway, Natural killer cell mediated cytotoxicity, Chemokine signaling pathway, and Influenza A, which indicates high sub-inflammatory status. In addition to the PI3K-Akt signaling pathway, pathways in cancer, JAK-STAT signaling pathway, ErbB signaling pathway, and Glioma (Table 4 and Figure 3 C). The results confirm vitamin D physiological role in bone homeostasis and the immune modulatory or anti-inflammatory effects as well.

Lastly, to examine the effect of dyslipidemia in vitamin D deficient participants' proteomics profiles, we compared Group 3 with combined vitamin D deficiency and dyslipidemia to Group 4 with vitamin D deficiency only. Interestingly, the level of ApoE was decreased in vitamin D deficiency alone without dyslipidemia. Similar to the previous group comparison, calcium and bone mineralization proteins IBSP, and OMD, were significantly upregulated. Alpha-2-Macroglobulin A2M, Adiponectin protein ADIPOQ, and growth arrest GAS1were also upregulated. In contrast, Growth Hormone Receptor GHR, and matrix Metallopeptidase MMP3 were decresad. In this comparison, several pathways were involved in inflammation such as cytokine-cytokine receptor interaction, complement, and coagulation, and Apoptosis cascades were highly significant. Furthermore, pathways involved in cancer were also highly significant, including prostate cancer, melanoma, and pancreatic cancer. In addition to other pathways i.e.Focal adhesion, FC epsilon RI signaling pathway, VEGF signaling pathway, Axon guidance (Table 4 and Figure 3 D).

Table 4. 4. The most significant proteins expression levels between different groups.

	G1-G2				G1-G3		
Protein	**P value**	**FC**	**logFC**	**Protein**	**P value**	**FC**	**logFC**
APOE	5.60E-03	1.11	0.16	APOE	4.64E-09	1.21	0.27
CRYZL1	1.63E-05	1.32	0.4	CRYZL1	2.71E-09	1.38	0.47
CA2	4.42E-03	0.67	-0.57	GDI2	4.80E-06	1.29	0.37
LAMA1	4.75E-03	1.17	0.23	LAMA1	5.44E-08	1.25	0.32
LRP1B	1.23E-05	1.24	0.31	LRP1B	1.02E-08	1.23	0.3
APOB	2.15E-06	1.49	0.57	GHRL	2.71E-06	0.77	-0.39
ADAMTS13	5.45E-03	0.86	-0.22	HTRA2	1.77E-06	1.18	0.23
AZU1	1.70E-03	1.15	0.2	LSAMP	1.18E-07	0.83	-0.27
CD70	3.62E-03	0.86	-0.22	METAP2	4.03E-06	1.37	0.45
CDH12	4.19E-03	1.36	0.44	NEGR1	1.33E-06	0.87	-0.2
EIF5	1.28E-04	1.31	0.39	PEBP1	4.07E-06	1.29	0.36
FGF10	2.43E-03	1.16	0.21	PPA1	4.56E-06	1.29	0.37
FGFR2	2.66E-03	0.76	-0.4	PRKCI	4.28E-06	1.38	0.46
HDAC8	4.06E-03	1.12	0.16	PSMA6	4.37E-06	1.27	0.34
HDGFL2	5.56E-03	1.08	0.12	SHC1	3.31E-06	1.42	0.51
IL7	4.62E-03	1.27	0.35	SKP1	4.57E-06	1.23	0.3
MAP2K4	6.93E-04	0.82	-0.28	SMAD3	5.22E-07	1.43	0.51
S100A4	5.03E-03	1.2	0.27	SOD1	2.75E-07	1.21	0.28
SERPINC1	3.74E-03	0.92	-0.12	TNFRSF17	1.93E-06	0.83	-0.27
TGM3	8.53E-04	1.62	0.7	VTA1	2.37E-06	1.58	0.66

	G1-G4				G3-G4		
Protein	**P value**	**FC**	**logFC**	**Protein**	**P value**	**FC**	**logFC**
CGA	8.85E-04	0.66	-0.6	APOE	1.29E+01	0.81	-0.3
ANXA2	1.76E-03	1.15	0.2	CRYZL1	1.72E+01	0.66	-0.61
CA2	1.27E-03	0.77	-0.38	UNC5D	8.24E-07	1.25	0.32
CAMK2D	2.27E-03	1.4	0.49	LAMA1	7.49E+00	0.8	-0.33
LRP1B	1.46E-03	0.9	-0.15	LRP1B	2.15E+01	0.73	-0.45
IBSP	3.69E-05	1.29	0.36	IBSP	7.91E+00	1.36	0.44
OMD	3.74E-06	1.33	0.41	OMD	8.76E+00	1.37	0.45
SOST	8.92E-12	0.66	-0.59	SOST	9.66E+00	0.73	-0.45
FAS	1.38E-03	0.85	-0.24	A2M	2.81E-07	1.28	0.36
IGFBP3	1.67E-03	1.13	0.17	ACY1	5.01E-07	0.64	-0.65
IL5RA	5.77E-04	0.82	-0.29	ADIPOQ	8.47E+00	1.38	0.46
MYBPC1	1.03E-03	0.74	-0.43	ESM1	8.87E+00	1.31	0.39
NAGK	7.03E-05	1.18	0.24	GAS1	3.67E-07	1.12	0.16
NRG4	3.62E-04	0.83	-0.26	GHR	6.09E-07	0.79	-0.34
PGK1	2.55E-03	1.23	0.3	MMP3	9.24E+00	0.73	-0.45
PRSS1	6.86E-04	0.86	-0.22	NRP1	3.06E-07	1.16	0.21
PTH	1.26E-03	1.22	0.28	PIANP	1.23E-07	1.24	0.31
SERPINA1	8.67E-04	1.16	0.21	PLAT	1.75E-07	0.76	-0.4
SERPING1	9.71E-04	0.88	-0.18	SELL	8.35E-08	1.16	0.21
VEGFD	2.67E-03	0.91	-0.14	TFPI	2.46E-07	0.84	-0.25

The highest 20 expressed proteins are selected based on their statistical significance, $p < 0.05$ considerd significant. Values highlited in green represst the log fold change of downnregulated proteins.

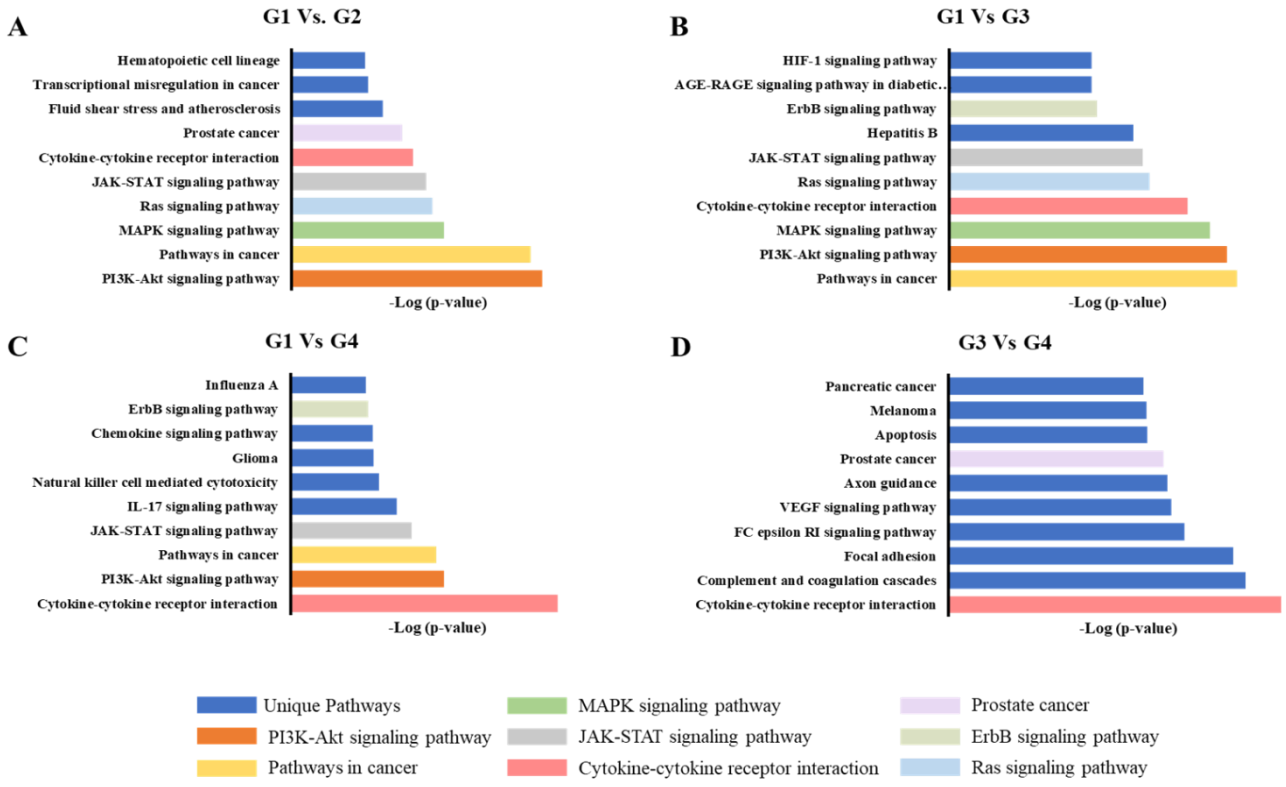

Figure 4. 3. Proteomics pathways enrichment during vitamin D deficiency and dyslipidemia. Bar graphs illustrates a comparison between the different groups in the level of statistical significance represented by -log of p value of enriched pathways. Group 1 (n=64) represents participants who were vitamin D sufficient with normolipidemia (contrl group). Group 2 (n=26) participants who were vitamin D sufficient with dyslipidemia. Group 3 (n=85) represents participants who were vitamin D deficient with dyslipidemia. Group 4 (n=99) represents participants who exhibited vitamin D deficiency with normolipidemia. **A:** The top

10 pathways with the highest level of statistical significance for Group 1 versus Group 2 (G1 Versus G2). **B**: Group 1 versus Group 3 (G1 versus G3). **C**: Group 1 versus Group 4 (G1 versus G2)**. D**: Group 3 versus Group 4 (G3 versus G4)**.** $p < 0.05$ considered significant.

Heatmap clustering of proteins expression differences observed in participants with or without vitamin D deficiency and dyslipidemia

The combination of vitamin D deficiency with dyslipidemia showed the most effects on proteomics profiles. Here, we focused on the differences in protein expression between Group 1 (the control group) and Group 3, which represents participants who were vitamin D deficient with dyslipidemia. The heatmap illustrates that many participants have a high level of the proteins involved in the inflammation process and the immune system was enhanced i.e. IL-1sR, IgA, and the immunoglobulin CD226. The data indicate the elevation of the inflammatory biomarkers reflecting on the association between vitamin D status and dyslipidemia (Figure 4).

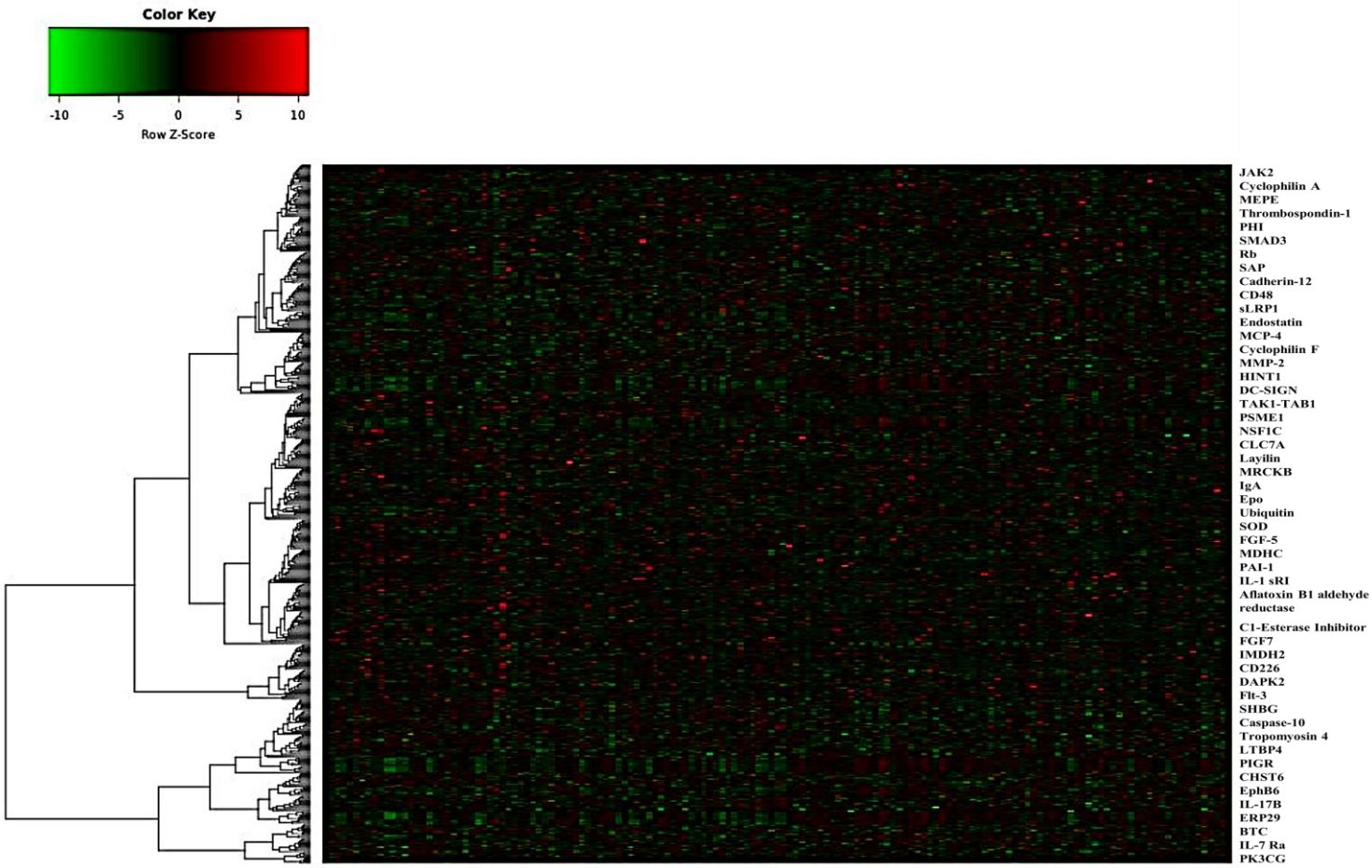

Figure 4. 4. Hierarchically clustered heatmap showing the expression of 50 most significant proteins between Group 1 (n=64) vitamin D sufficient with normolipidemia (control group), and Group 3 (n=85) of participants with combined vitamin D deficiency and dyslipidemia. Red and green represent expression abundance (green: low; red: high) as indicated in the legend.

4. Discussion

This study is designed to understand the molecular link between vitamin D deficiency and dyslipidemia specifically to address how vitamin D deficiency is associated with low HDL levels and reduced anti-inflammatory activity of HDL. Here we demonstrated that apolipoproteins concentrations varied according to vitamin D and lipid profile status. ApoA-

1, the major protein in HDL particles, expression is significantly reduced during vitamin D deficiency regardless of the presence of dyslipidemia. Clinical studies demonstrated that vitamin D supplementation increased ApoA-1 serum levels [203]. ApoA-1 is the backbone of HDL formation. ApoA-1 has been linked to the anti-inflammatory functions of HDL for instance ApoA-1 transgenic mice showed a decrease in TLR4 expression. This decrease was able to suppress the activation of TLR4 following the administration of LPS which consequently improved the survival rate of infected mice [124]. Our proteomics data here suggest that reduction in ApoA1 expression during vitamin D deficiency may directly contribute to the reduced anti-inflammatory potential of HDL. In contrast, ApoB and ApoE were elevated in dyslipidemia. This effect is due to their association with LDL and chylomicrons respectively [354]. The latter two lipoproteins usually increased during dyslipidemia and are associated with a high risk of cardiovascular disease[355].

The combination of both dyslipidemia and vitamin D deficiency has a drastic effect on reducing ApoM expression. This is the most supportive evidence that vitamin D status affects HDL levels as ApoM is essential for HDL biogenesis. However, there was a lack of studies demonstrating the relationship between ApoM and vitamin D. ApoM was found to induce the VDR, but when ApoM is reduced the VDR expression is reduced as well [204, 205]. Here, we provide evidence that ApoM is influenced by the level of vitamin D. ApoM is a member of the lipocalin family usually found bound to HDL and carries the S1P ligand [164]. The complex ApoM/S1P is crucial for HDL's antiatherogenic and anti-inflammatory effects [142]. Ruiz et.al. The report confirmed that the ApoM/ S1P complex was very crucial to suppress the vascular adhesion molecule-1 (VCAM-1) and E-selectin surface abundance during the inflammation. Whereas ApoM alone and HDL alone were not successful in conferring this adhesion suppression [142]. The double effect resulting from low concentrations of vitamin D

and dyslipidemia causes a dramatic reduction in ApoM expression; thereby exacerbating the disturbance in HDL level and function since ApoM is essential for HDL biogenesis [93]. HDL levels are known to be gender dependent. Our study shed light on how gender is impacting the expression of some apolipoproteins. For example, during vitamin D deficiency Males expressed higher levels of ApoA-1 compared to females. In contrast, ApoM was higher in females in normal physiological conditions and in vitamin D deficiency.

Further, ApoD was reduced during combined dyslipidemia and vitamin D deficiency. ApoD is lipocalin that binds hydrophobic ligands such as progesterone, retinoic acid, sphingomyelin pregnenolone, and arachidonic acid [140]. ApoD is found in body secretions and serum in large amounts more than any other apolipoproteins. ApoD is reported to decrease immune response during acute inflammation and decreases T-cell infiltration into the CNS, lower the production of pro-inflammatory cytokines including IL-1β and TNFα, and downregulate the activity of phospholipase A2 (PLA2)[190]. ApoD usually is increased in the presence of vitamin D. This increase is associated with an inhibitory effect on some cancers such as breast and prostate cancer cells [208, 209]. The reduction of ApoD level due to lack of stimulation from vitamin D in addition to dyslipidemia could indicate a deep disturbance in ApoD expression and immunological function.

SAA1 is an indirect indicator of HDL status as it plays a role in HDL remodeling and function. During the high inflammatory status such as sepsis, SAA1 replaced the ApoA-1 in HDL particle, and lead to a reduction in the anti-inflammatory function [96, 356]. Our findings demonstrated that SAA1 levels are highest during vitamin D deficiency. Together, the data indicate that SAA1 contributes to subclinical inflammation and dysfunction in HDL particle.

Iron homeostasis is known to be disturbed during inflammation and altered iron homeostasis is observed in various chronic inflammatory diseases. Hepcidin or LEAP-1 is the master iron-regulating protein produced from liver and iron-hepcidin-ferroportin axis is considered part of innate immunity. Hepcidin-ferroportin axis inhibits microbial growth during infection by depleting iron levels as it sequesters iron in macrophages [219, 357-359]. In our study, hepcidin (LEAP1) was found elevated in dyslipidemia but significantly reduced in normolipidemic participants with vitamin D deficiency.

The effect of dyslipidemia on the overall proteomic profile was observed to increase the expression of several proteins involved in the immune system such as IL7, and the protein-coding lysosomes in the neutrophil AZU1, which has antimicrobial activity. On the other hand, TNFα ligand family member CD70 was decreased. CD70 is highly expressed in activated lymphocytes, and macrophages [360]. CD 70 is a regulator of endothelial nitric oxide and reactive oxygen species. Knocking CD70 genes in endothelial cells resulted from impairment in eNOS expression and function and an increase in intracellular hydrogen peroxide subsequently exasperate the atherosclerotic events in humans [361]. Further, the most enriched pathways in dyslipidemia were including inflammatory responses and apoptosis MAPK pathway, JAK-STAT signaling pathway and Ras signaling pathway and cytokine-cytokine receptor interaction. Together, the data indicate the enrichment of inflammatory pathways during combined vitamin D deficiency and dyslipidemia.

The combination of vitamin D deficiency with dyslipidemia significantly enhanced the expression of inflammatory proteins e.g. the phosphatidylethanolamine-binding protein PEBP1 and the antioxidant enzymes superoxide dismutase (SOD1) which detoxify free superoxide radicals [362]. Moreover, SOD 1 was found to suppress the pro-inflammatory immune response [363]. Therefore the elevation of SOD1 here could be a compensatory mechanism

due to the increase in inflammatory cytokines. In the same context, HTRA2 which is known to induce apoptosis was elevated. In contrast, the member of the TNF-receptor superfamily TNFRSF17 and immunoglobulin member LSAMP were decreased. In addition to the previously mentioned pathways AGE-RAGE which disturbed in diabetes and ErbB signaling pathway. The data again suggest that a combination of vitamin D deficiency and dyslipidemia severely impacts the anti-inflammatory potential of HDL.

In this study, we report that vitamin D deficiency enhanced the expression of proteins related to calcium homeostasis and bone metabolism which again confirms the importance of vitamin D sufficiency for bone health. Calcium-binding protein ANXA2, CAMK2D calcium/calmodulin-dependent kinase, and calcium and hydroxyapatite binding protein IBSP, bone mineralization regulator OMD were upregulated. Of note, FAS, cell death, and TNF receptor was downregulated. FAS downregulation is associated with high tumorigenicity and was observed to be decreased in many cancers e.g. gastric cancer [364], prostate cancer [365], and bladder cancer [366].

In general vitamin D status has a great effect on HDL homeostasis and inflammation in the body. Yarparvar et. al. demonstrated that vitamin D levels were correlated positively with the anti-inflammatory cytokines IL-10 and negatively with the pro-inflammatory markers Tumor necrosis factor receptor 2 (TNFR-2) and high-sensitivity C-reactive protein (hsCRP) which lend support to our study findings. Moreover, the study found that healthy adolescents with low serum vitamin D have low HDL levels as well [348]. Sharif-Askari et. al. recent study also support that, where they studied the lipid profile in insulin-resistant individuals. They revealed that low concentrations of serum 25(OH) D were accompanied with low levels of HDL-C levels and increased circulatory levels of cytokines IL-6 and IL-8 [226]. Herein, we

confirmed that the combination of vitamin D deficiency and dyslipidemia has a great impact on the proteomic profile and is associated with inflammatory status among participants.

5. Conclusion

Proteomic profiling revealed major alterations in HDL- association proteins. It also revealed that inflammation pathways were enriched in vitamin D deficiency, whereas cancer pathways in addition to inflammation pathways were heightened in participants who has a combination of vitamin D deficiency with dyslipidemia.

CHAPTER 5: APOM BINDS ENDOTOXIN CONTRIBUTING TO NEUTRALIZATION AND CLEARANCE BY HDL

HDL-associated protein ApoM is important for HDL biogenesis and influences lipoproteins status in the body [142]. HDL is known to possess anti-inflammatory properties other than the main function in regulating the cholesterol efflux and transportation [152]. HDL anti-inflammatory activity is awed to its role in endotoxin (aka LPS) neutralization and clearance. Clinical studies documented reduced HDL levels in sepsis [367, 368]. LPS is a major component of the outer membrane of gram-negative bacteria, such as *E. coli, Salmonella* [369]. LPS stimulates the immune response through the activation of TLR4 to release pro-inflammatory mediators such as TNFα, IL6, IL-1β, and IL8. An overstimulation of the system results in devastating clinical manifestations including systemic inflammation, sepsis, septic shock, and death [120-122, 370].

In addition to its role in HDL biogenesis, ApoM is the carrier protein for the active sphingolipid S1P, which is also found to be reduced during sepsis [171, 371], supporting the hypothesis that ApoM may play a role in endotoxins neutralizations. In this paper we confirmed HDL neutralizing activity against LPS and show for the first time that ApoM binds LPS and therefore contributes to endotoxin neutralization.

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ApoM binds endotoxin contributing to neutralization and clearance by HDL

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Short title: ApoM binds LPS

Authors declare no conflict of interests

Abstract

Background: HDL possesses anti-inflammatory properties, however, the exact mechanism is not fully understood. Endotoxin is a potent inducers of TLR4 signaling, leading to inflammatory mediators' release. It has been estimated that TLR4 recognizes about 5% of circulating lipopolysaccharide whereas 95% is cleared by plasma lipoproteins, mainly HDL. ApoM is required for HDL biogenesis and 95 % of plasma ApoM is found associated with HDL, both are are significantly reduced during sepsis. **Aim:** The aim of this study is to investigate whether ApoM binds endotoxin and contributes to anti-inflammatory activity of HDL. **Methods:** Isothermal Titration Calorimetry (ITC) was used to determine the binding of ultrapure *E. coli* LPS to the recombinant ApoM protein. Purified human HDL and recombinant ApoM was used to investigate LPS neutralization using human and murine macrophages and computational simulation was performed. **Result**: ApoM shows high affinity for *E. coli* LPS, forming 1:1 complexes with Kd values below 1 μM, as revealed by ITC. The binding process is strongly exothermic and enthalpy-driven ($\Delta_r H$ = -36.5 kJ/mol), implying the formation of an extensive network of interactions between ApoM and LPS in the bound state. Computational simulation also predicted high-affinity binding between ApoM and *E. coli* LPS and the best scoring models showed *E. coli* LPS docking near the calyx of ApoM without blocking the pocket. The biological significance of this interaction was further demonstrated in macrophages where purified HDL neutralized an *E. coli* LPS effect and significantly reduced TNFα release from human THP-1 cells. **Conclusion**: ApoM binds LPS to facilitate endotoxin neutralization and clearance by HDL.

1. Introduction

Sepsis is a life threatening emergency resulting in high mortality and morbidity around the world. Around 20% of deaths are sepsis-related [372], which is caused by an exacerbated immunological reaction towards bacterial endotoxins or infection, leading to organ failure and death [373]. Endotoxin also known as Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, such as *E. coli, Salmonella, and Neisseria meningitidis* [369]. LPS activates the immune response via toll-like receptor 4 (TLR4) by binding to its co-receptor MD-2. TLR4 is a transmembrane glycoprotein highly expressed on the surface of immune cells, particularly macrophages [119]. TLR4 recognizes about 5% of circulating endotoxin / LPS [370]. The binding of LPS in the calyx of MD-2 leads to TLR4 dimerization and initiation of signaling cascade leading to pathways activation such as NF-κB complex (nuclear factor kappa-light-chain-enhancer of activated B cells), and releases some inflammation and cytokines mediators e.g. TNFα [120-122].

The clearance of endotoxin from circulation is mediated by HDL [374], which is known as good cholesterol owing to its role in reverse cholesterol transport and cardiovascular protection properties [375]. HDL belongs to the lipoproteins family and is composed of higher protein constituents relative to other members (e.g., LDL, VLDL, triglycerides) [376]. HDL's main function is to transfer cholesterol from peripheral tissues to the liver where it is recycled or excreted as bile salts. This process is known as reverse cholesterol transport (RCT). The HDL-associated apolipoproteins interact with numerous cellular receptors which enables the cholesterol efflux from cells to the HDL particle [98]. Moreover, apolipoproteins associated with HDL particles possess other functions like anti-inflammatory or antioxidative properties [87]. As a result, HDL particles undergo continuous remodeling in physical structure and constituents [99]. Importantly, inflammation disrupts the RCT pathway by reducing cholesterol

trafficking from macrophage foam cells to the liver, leading to dysfunctional HDL particles [100].

A reduction in HDL was observed during sepsis, suggesting a role of HDL in containing and suppressing the exacerbated immunological response [367, 377]. Studies have ascribed this decrease in HDL to the disturbance or dysfunction of the lipoproteins constituents leading to reduced HDL biogenesis [378, 379]. ApoA-1, the major protein associated with HDL is shown to bind to endotoxin potentially aiding in clearance from circulation [380].

ApoM is one of the HDL-associated apolipoproteins crucial for HDL biogenesis and may contribute to the anti-inflammatory function of HDL. Around 95 percent of plasma ApoM is found associated with HDL [381]. ApoM is a 25 kDa protein, produced mainly in liver and kidney, and belongs to the lipocalin family (a group of proteins that transport small hydrophobic molecules) [382]. Structurally, ApoM has a typical lipocalin fold that consists of eight β-strands forming a barrel [161]. ApoM is a lipid carrier and documented to shuttle the bioactive sphingosine 1 phosphate (SIP), where ApoM-S1P axis plays a critical role in modulating various diseases [381]. Similar to HDL, ApoM levels were also shown to be reduced during sepsis and severe inflammation [371, 383]. In this study, we examined the role of HDL and ApoM in endotoxin neutralization and provide evidence that ApoM binds to LPS to facilitate its clearance, consequently contributing to the anti-inflammatory effects of HDL.

2. Materials and Methods

2.1Reagents:

Human TNFα DuSet ELISA kit (R&D, Catalog Number: DY210), recombinant human ApoM protein (R&D, Catalog Number: 4550-AM-050), and recombinant human LL-37 (TOCRIS, Catalog Number: 5213). Highly purified endotoxins *Salmonella typhimurium, E. coli B55, Salmonella minnesota* and *Vibrio cholerae* were described previously [384] and new lots of endotoxins were purchased from Invivogen; SM Ultrapure (Invivogen, Catalog Number: tlr1-sm lps) and LPS-B5 Ultrapure (Invivogen, Catalog Number: tlrl-pb5lps). Commercial HDL derived from human plasma was purchased from (Sigma-Aldrich, Catalog Number: L8039. St. Louis, USA). Pooled human plasma HDL and LDL fractions were a kind gift from Dr Ngoc-Anh Le (Atlanta Veterans Affairs Medical Center, Decatur, GA; and Emory University School of Medicine, Atlanta, GA, USA).

2.2.Endotoxin neutralization studies by HDL and LDL:

Human THP-1 cells were cultured using RPMI 1640 supplemented with 5% penicillin-streptomycin and 10% Fetal serum bovine. Freshly grown THP-1 cells were adjusted to 1 $x10^6$ cell/ml and 250 µl aliquots were transferred into 96-well plate and then induced with 1ng/ml of LPS derived from *Salmonella typhimurium, E. coli B55, Salmonella minnesota* and *Vibrio cholerae* with or without 10 µl of purified HDL, LDL (stock solution at 1mg/ml) or PBS as control. The treated cells and controls were then incubated overnight at 37°C with 5% CO_2. The released TNFα was measured in the supernatants using ELISA method following manufacturer instructions as previously described [384].

2.3.Isothermal Titration Calorimetry:

A Nano ITC (TA Instruments) low-volume isothermal titration calorimeter was employed to study the interaction between human ApoM and LPS from E. coli. All solutions used for ITC experiments were dissolved directly in the same batch of PBS buffer, to avoid buffer mismatch dilution heats. All samples were thoroughly degassed before use to avoid bubble formation. In a typical experiment, the calorimetric cell was filled with 10 μM of ApoM and the syringe was loaded with a 100 μM LPS solution. The titration sequence consisted of an initial 0.9 μL injection, followed by 15 identical 2.54 μL injections at 300s intervals. Experiments were performed at 25°C and a stirring speed of 150 rpm was used to ensure a rapid equilibration of the mixture. Heat contribution from injectant dilution was accounted for in a separate experiment, by injecting LPS in a buffer solution following an identical titration protocol. These dilution effects were subsequently subtracted from the titration data to obtain the net binding isotherm, as a function of the overall LPS concentration in the cell. All ITC data were processed using the NanoAnalyze software (TA Instruments, New Castle, DE, USA).

Complex formation is an equilibrium interaction that can be described by a chemical equation of the form:

$$[ApoM] + [LPS] \overset{K_b}{\Leftrightarrow} [Complex]$$

where: [*ApoM*] and [*LPS*] are the concentrations of the non-complexed ApoM and LPS respectively, [Complex] represents the concentration of the ApoM-LPS complex, while K_b = 1/ K_d is the binding constant of the interaction.

The stoichiometry (moles of LPS bound per mol of ApoM) [N], the binding constant [K_b], and the binding enthalpy [$\Delta_r H$] of the reaction are obtained, along with their corresponding uncertainties, directly from fitting the ITC experimental data to a one set-of-sites binding model. The Gibbs free energy change ($\Delta_r G$) and the entropy change ($\Delta_r S$) of the complexation are then calculated from the equations: $\Delta_r G = RT \ln K_b = \Delta_r H - T \Delta_r S$, where R is the gas constant and T is the absolute temperature. The uncertainties of these parameters were estimated using error propagation calculations.

2.4.Molecular Docking Simulations of ApoM - E. coli LPS binding:

All computational procedures were carried out with the Molecular Operating Environment (MOE 2019.01, Chemical Computing Group, Montreal, Canada) software, using the Amber12: EHT force field with the reaction field electrostatics treatment. ApoM was treated as rigid for the docking simulations while conformational space was sampled for the LPS ligand (Template: PDB entry 2WEW). Briefly, 20.000 ligand conformations were generated by sampling their rotatable bonds and placed using the Triangle Matcher Method. Duplicate complex structures are then filtered out and the best 1.000 poses were scored according to the London dG empirical scoring function for an estimation of their binding energy [385]. The 100 top-scoring complexes were submitted to a more in-depth refinement step based on molecular mechanics and the structures produced were re-evaluated using the GBVI/WSA ΔG empirical scoring function to include solvation effects [386]. Ten or less structures are generated at this stage. Finally, the MOE 2019 LigX script was applied to the best pose to minimize the energies of both the ligand and the receptor, in order to get a more accurate estimation of the ligand affinity.

2.5.Endotoxin neutralization by recombinant ApoM:

E. coli LPS with different concentrations of 100 and 50 ng/ml were pre-incubated with recombinant ApoM protein (10 µg/ml) in a 96-well plate for 30 min. Freshly grown murine RAW264 macrophages were then added to a 96-well plate at $0.25x10^6$ cell/well and further incubated overnight. Nitric oxide release was measured as nitrite accumulation using the Greiss reaction as previously described [384]. Recombinant LL-37 is a host cationic peptide also known as cathelicidin was used as a positive control due to its ability to neutralize LPS immune-stimulatory activity [387].

2.6.Statistical Analysis:

Graphpad Prism 5 was used in the analysis and generating figures, T-test was calculated to compare HDL and LDL in relation to PBS (Cells treated with PBS were considered as a control). *P* value <0.05 is considered significant.

3. Results

HDL neutralized endotoxin and decreased TNF-α release from human THP-1 cells

The ability of purified HDL fraction to neutralize endotoxin was examined *in vitro.* TNF-α secretion from human THP-1 cells induced with various endotoxins in the presence and absence of HDL was measured. HDL (10 µg/ml) pre-incubation with various doses of endotoxins (2.5 to 0.3 pmole/ml) purified from *S. typhimurium, E. coli B55, S. minnesota* and

V. cholera prior to addition to THP-1 cells resulted in a significant reduction in TNF-α release (Figure 1).

Endotoxin concentrations used here are very low and thereby physiologically relevant. HDL neutralized endotoxins more effectively compared to LDL fraction and reduced TNF- α release especially at higher doses of endotoxins *S. typhimurium* (Figure 1A), *E. coli B55* (Figure 1B), *S. minnesota* (Figure 1C), and *V. cholerae* (Figure 1D). However, effective endotoxin neutralization by both HDL and LDL fractions was observed at lower doses of endotoxins.

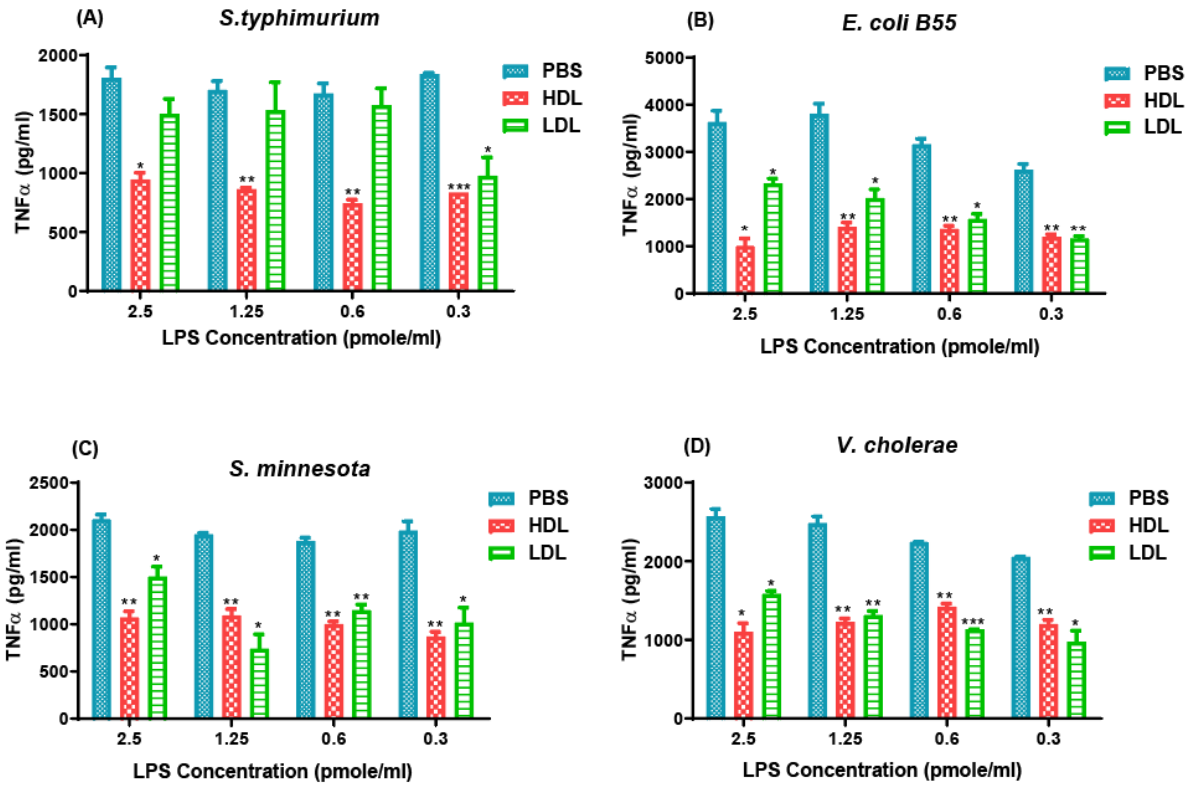

Figure 5. 1. Endotoxin neutralization by HDL decreased TNF-α release from human THP-1 cells. Endotoxin doses ranging between 2.5 to 0.3 pmol/ml were pre-incubated with purified HDL or LDL fractions (10 µg/ml) or PBS for 30 min prior to addition to THP-1 cells

($0.25x10^6$/ml) and further overnight incubation at 37°C. TNF-α release from induced THP-1 cells was measured using ELISA method. Endotoxins used were S. typhimurium (**A**), E. coli B55 (**B**), S. minnesota (**C**) and V. cholera (**D**). *P value <0.05, **P value <0.01 and ***P value <0.001.

Computational Docking Simulations of ApoM - *E. coli* LPS binding

ApoM, a lipid carrier protein, is critical for HDL biogenesis. Studies documented that both HDL and ApoM are reduced during sepsis. We examined our hypothesis that ApoM may bind to LPS potentially facilitating endotoxin clearance by HDL. To this end, computational docking simulations were performed to predict the potential binding between ApoM and *E. coli* LPS.

The best scoring model showed that *E. coli* LPS docks on the surface near the calyx of ApoM but does not block the S1P binding site inside the calyx (Figure 2). The simulation results suggest a very strong binding, with $\Delta_r G$ = - 41.46 kJ/mol (K_d = 52.8 nM) and root mean square deviation RMSD = 2.8 Å for the protein backbone C-α atoms. The interface binding sites between ApoM and *E. coli* is shown in Figure 2C (more details can be found in the supplementary information accompanying the article).

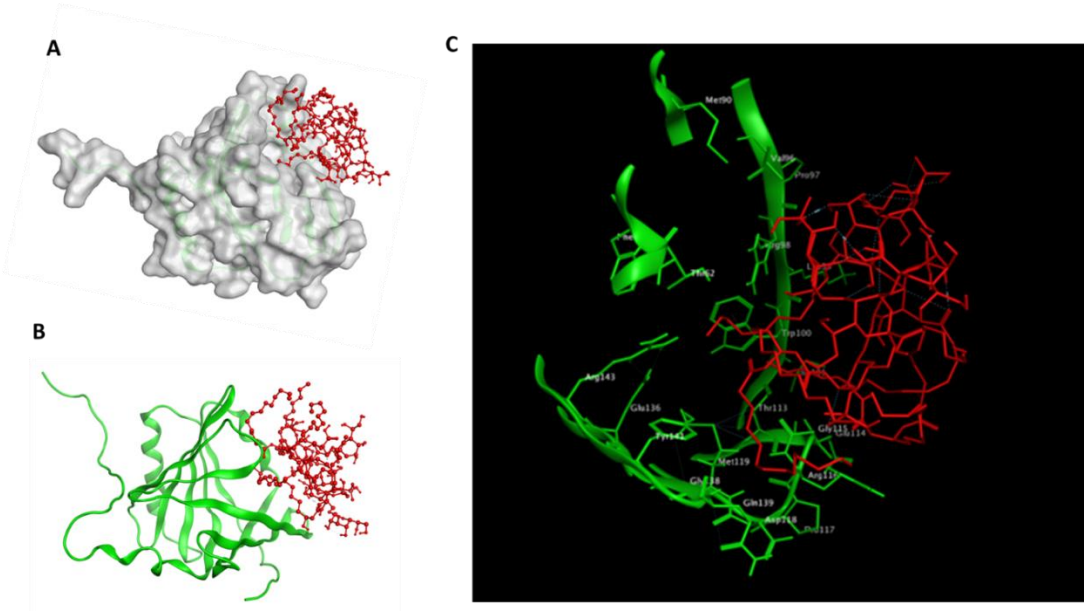

Figure 5. 2. Potential interaction between ApoM and E.coli LPS revealed by docking simulations. A: Top-ranked model of ApoM-E coli LPS complex; ApoM is shown as a grey van der Waals surface, while E. coli LPS is presented as a red ball-and-stick model. B: Top-ranked model with ApoM in ribbon representation (green) and E. coli LPS shown as a red ball-and-stick model. C: A closer view of the ApoM-E. coli LPS binding interface.

Isothermal titration calorimetry study of the ApoM - *E. coli* LPS interaction

ITC was used to confirm the molecular simulations results and study the interaction between LPS and ApoM in more detail. The titration was performed at T = 25°C, in PBS buffer and the ITC data are shown in Figure 3. ApoM shows high affinity for *E. coli* LPS, forming 1:1 complex with a dissociation constant below 1 μM (Table 1), similar to the predicted *in silico* values (137 nM and 52 nM respectively). The binding process is strongly exothermic

($\Delta_r H$ = -36.5 kJ/mol) and enthalpy-driven, implying the formation of an extensive network of interactions between ApoM and LPS when bound.

Table 5. 1. Thermodynamic profile of the ApoM - E. coli LPS interaction, as determined by ITC at 25°C.

Dissociation Constant [K_d] (nM)	**Stoichiometry [N]**	**Binding Enthalpy [ΔrH] (kJ/mol)**	**Entropic Term[-T·ΔrS] (kJ/mol)**	**Gibbs Free Energy Change [ΔrG] (kJ/mol)**
137.0 ± 3.4	1.06 ± 0.02	-36.5 ± 0.8	-2.7 ± 1.0	-39.2 ± 0.6

Table 5.1 footnotes: Dissociation constant [K_d], binding enthalpy change [$\Delta_r H$], entropic term change [$-T\cdot\Delta_r S$] and free energy change [$\Delta_r G$] for the interaction between LPS and ApoM at T = 25°C, in PBS buffer. Values and corresponding errors were derived from non-linear least square fit of the ITC data to a one-set-of-sites binding model and error propagation calculations.

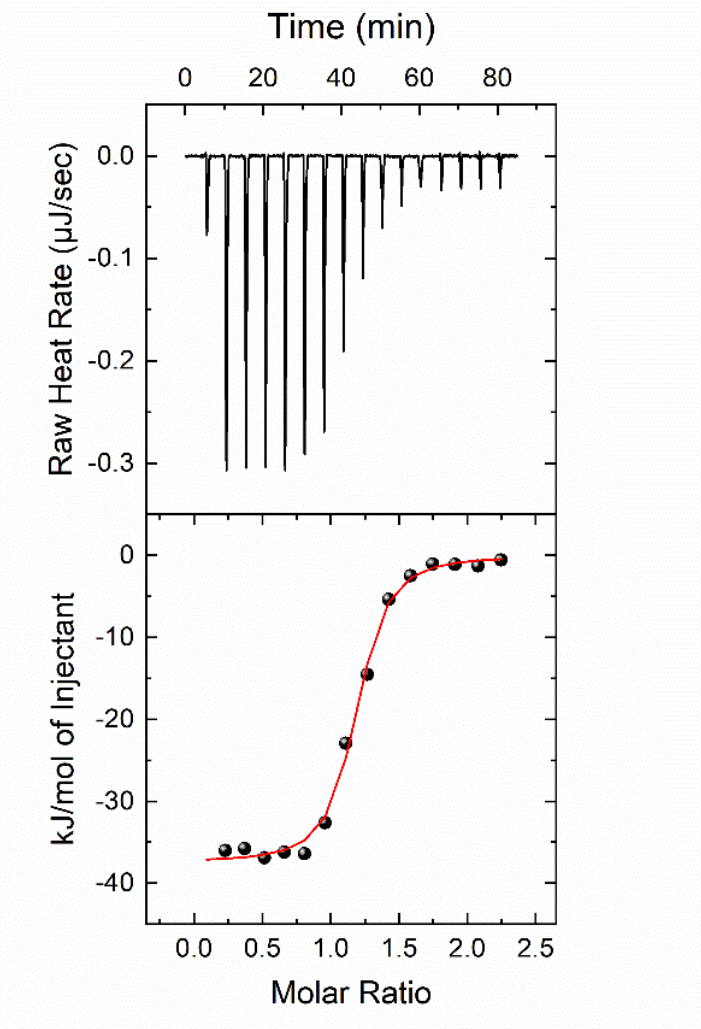

Figure 5. 3. ITC measurements of ApoM and E. coli LPS interactions. (Upper Panel) Change of power supply to the calorimetric cell during the titration of 100 µM of LPS solution into 10 µM of ApoM in PBS buffer at 25°C, after the subtraction of the appropriate reference experiments. Negative values indicate exothermic peaks. (Lower Panel) Integration of the area under each injection, normalized per mol of injectant and plotted as a function of the [LPS]/[ApoM] ratio at each point of the titration. Solid red line represents the non-linear least-square fit of the ITC data to a single-set-of-sites binding model.

ApoM neutralized *E. coli* LPS immune stimulatory activity *in vitro*

To examine ApoM's ability to neutralize endotoxin immune stimulatory activity, RAW264 macrophages were employed. Recombinant ApoM (10 µg/ml) where pre-incubated with *E. coli* LPS for 30 min and then used to induce RAW264 macrophages. The data suggest that ApoM neutralized *E. coli* LPS biological activity and inhibited nitric oxide release from RAW264 macrophages (Figure 4). Moreover, ApoM neutralizing effect was similar to LL-37 (Figure 5) which was used as a control due to its endotoxin-neutralizing activity [388]. Taken together, the data in this study demonstrate for the first time that ApoM binds to LPS with high affinity potentially contributing to endotoxin neutralization and clearance by HDL.

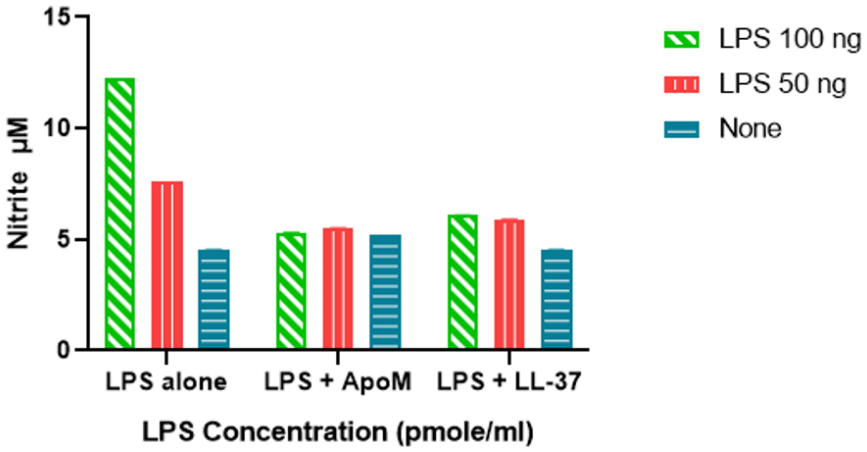

Figure 5. 4. ApoM neutralized E. coli LPS and reduced nitric oxide release from macrophages in vitro. Freshly grown RAW264 macrophages were transferred to 96-well plate ($0.25x10^6$ cell/ well) then induced with E. coli LPS (100 and 50 ng/ml) pre-incubated with or without ApoM (10 µg/ml) for 30 min. LL37 was used as a control due to its ability to neutralize endotoxin. Plates were incubated overnight, and nitrite accumulation was measured using the Greiss reaction method.

4. Discussion

HDL is known to confer anti-inflammatory effects in various conditions. For example, HDL inhibits atherogenesis formation consequently preventing cardiovascular disease progression [389, 390]. Additionally, HDL modulates innate immunity and shown to minimize the immunological cellular responses to endotoxins by inhibiting the release of inflammatory mediators such as type I interferon [374, 391]. Our data confirm that HDL neutralized endotoxins' immune stimulatory activity and inhibited TNF-α release from human macrophage-like monocytic cells THP-1 hence HDL possessed anti-inflammatory properties. In support, Levine *et. al.* investigated HDL levels in mice during endotoxemia and reported that higher HDL levels were associated with decreased plasma cytokines levels. Notably, the protective effect was observed in HDL-transgenic mice, and upon intravenous infusion of reconstituted HDL [374].

The evidence for HDL anti-inflammatory properties is well established where an alteration in HDL levels and compositions affects its functionality and protective properties [392]. Clinical trials in human showed that low HDL level was found to increase immune system activation in response to a low dose of LPS in healthy individuals [393]. In fact, HDL-associated proteins such as ApoA1 was documented to bind endotoxin and, therefore, contributed to its neutralization [394]. Although ApoA1 is the main lipoprotein among HDL-associated proteins, others such as ApoC, ApoE, ApoL, ApoM, ApoJ, SAA, PON1 and 2AM play a role in HDL remodeling and functionality including its anti-inflammatory potential. Herein, we provide evidence that ApoM binds to endotoxin and plays a critical role in augmenting HDL anti-inflammatory activity.

ApoM is found associated mainly with HDL and to some lesser extent to LDL [162]. Ninety-five percent of ApoM in circulation are bound to HDL [162]. ApoM is produced mainly in liver and kidney but the terminal signal peptide of ApoM is not cleaved prior to release to plasma which helps ApoM in anchoring to HDL particles [163]. ApoM is a lipid carrier protein and a member of the lipocalin protein family [164]. Lipocalins have a similar structure, consisting of 8 antiparallel β-strands, forming a β-barrel fold (calyx) that protects an internal binding site [158]. ApoM structure resembles that of many other lipocalins, including MD2, the co-receptor for TLR4. MD2 is composed of 2 β-strands forming a cup-like structure [164]. The high structural similarity of ApoM and MD2 folds suggest the ability to bind endotoxins. Our docking simulation studies resulted in a high-scoring ApoM -*E. coli* LPS complex that we used to gain further insight into this interaction. Based on this model, ApoM binds LPS on the surface near the calyx opening but does not mask the binding site pocket where S1P ligand is shuttled. Further investigations using ITC confirmed this interaction. A high affinity binding between ApoM and *E. coli* LPS was confirmed forming 1:1 complexes with K_d values below 1 μM. The binding process is strongly exothermic and enthalpy-driven (ΔrH = -36.5 kJ/mol), implying the formation of an extensive network of interactions between ApoM and LPS in the bound state. Our novel data suggests that ApoM contributes to endotoxin neutralization and clearance by HDL.

Functionally, ApoM is the carrier of sphingosine-1-phosphate (S1P), a bioactive lipid mediator that modulates vascular inflammation. Some studies documented that ApoM - S1P complex is important for HDL antiatherogenic and anti-inflammatory effect [142]. ApoM – S1P complexes are upregulated during inflammatory status [170]. However, Winkler *et. al*. study reported a decline in S1P level in septic shock patients and suggested that the drastic decrease in HDL level during septic shock is the reason for this decline [171]. Our

computational docking simulation showed that *E.coli* LPS does not occupy the binding site of S1P in the internal section of the calyx, however, it is not known if the ApoM bind both LPS and S1P simultaneously which warrants further investigation.

In this study, we showed that ApoM neutralized endotoxin activity and thereby decreased nitric oxide production in murine RAW264 macrophages. Endotoxins recognition or sensing is mediated by TLR4-MD2 receptor complex, mainly by the extracellular domain of TLR4 [395]. Upon LPS binding to MD2 co-receptor of TLR4, the dimerization of TLR4 ectodomains occur leading to conformational changes that initiate signal transduction through MYD88 and TRIFF signaling pathways leading to inflammatory mediators' release [384]. The signal stimulates the production of the acute-phase reactants such as SAA, TNF-α, IL-6 [396]. Based on our data, we suggest that the binding of LPS to HDL-associated protein ApoM facilitates shuttling endotoxins to liver for detoxification. The later process prevents the activation of TLR4, consequently suppressing the stimulation of downstream pathways leading to reduced production of pro-inflammatory mediators and the acute phase reactants (Figure 5).

In support of our conclusion, we used LL-37 to inhibit LPS immune stimulatory activity. LL-37 or cathelicidin or hCAP18 plays a similar role in inhibiting the interaction between LPS and LPS-binding protein, consequently preventing TLR4 activation and reducing TNF-α release [211-213]. Furthermore, LL-37 was found to suppress inflammation and cell death via inhibiting the IL-1β expression and caspase-1 activation [214]. Therefore, we used LL-37 as a control to study the ApoM interaction with LPS and provide supporting evidence for its role in potentiating the anti-inflammatory activity of HDL.

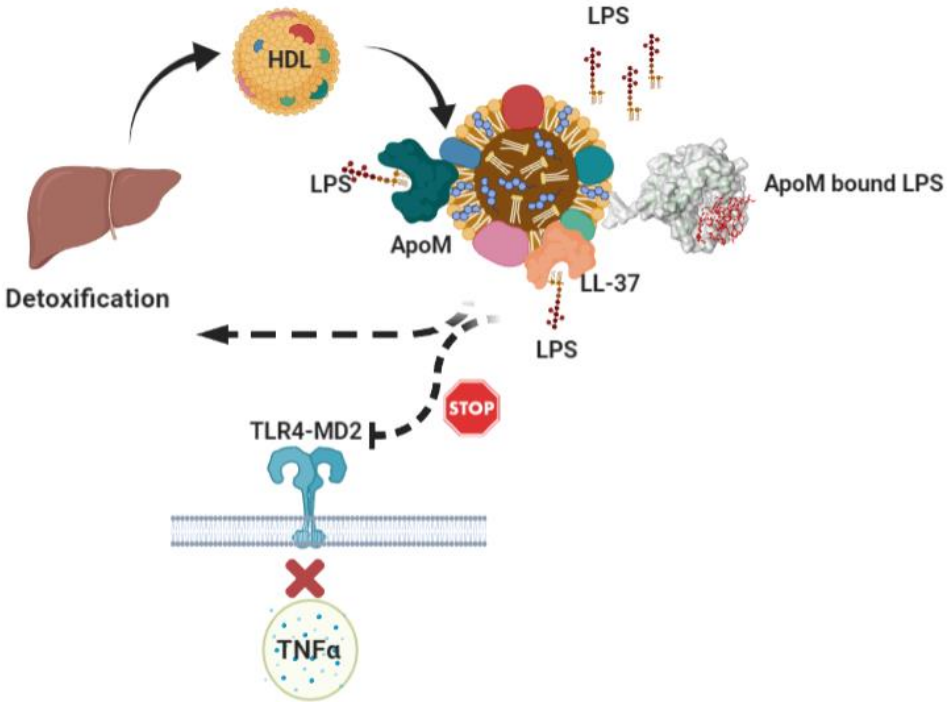

Figure 5.

5. Schematic illustration of the interaction between LPS and the HDL-associated protein ApoM. LPS binds to ApoM surface near the calyx opening on the HDL particle. The formed complex is shuttled to liver for detoxification, therefore contributing to endotoxin clearance that reduced circulating LPS binding to TLR4-MD2. This process prevents the further activation of TLR4-MD2 and this prevents TNF-α overproduction.

5. Conclusion

ApoM binds with high affinity to *E coli* LPS to facilitate endotoxin neutralization and clearance by HDL.

Author Contributions

Conceived the study: S.M.Z.; data collection: S.M.Z, AT, and H.M.; data analysis: H.M., AT, and S.M.Z. Supervision: S.M.Z. Writing manuscript draft: H.M., AT, S.M.Z. Critically reviewing and finalizing manuscript S.M.Z, AT, H.M. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 6: THE EFFECT OF VITAMIN D ON HDL ASSOCIATED PROTEINS EXPRESSIONS

Vitamin D possesses immune modulatory effects and influences the homeostatic expression of many proteins. The inverse association between vitamin D status and the protective HDL levels are well established. However, it is not clear whether Vitamin D directly affects or modulates the expression of HDL-associated proteins necessary for its biogenesis and function. Evidence showed that ApoD was increased after administration of 1,25(OH)2D3.. However vitamin D direct effect on ApoM expression is not known yet. A study by Yu, M.M., et al. demonstrated that ApoM induces the expression of vitamin D receptor (VDR) [204]. In type 2 diabetic patients, 1,25(OH)2D3 was seen to inhibit the macrophage migration and enhanced the cholesterol efflux by HDL particle and ApoA-1, leading to favorable effect reversing the atherogenic events [397]. Similarly, based on systematic review and meta-analysis of clinical trials, vitamin D supplementation was found to increase the level of ApoA-1, the major apoliporotein in HDL essential for functional HDL particle [203].

Vitamin D is known to exert anti-inflammatory effect during sepsis by attenuating the production of inflammatory mediators [398]. ApoM is critical for HDL biogenesis and studies reported that HDL and ApoM are significantly reduced during sepsis, hence diminished anti-inflammatory function of HDL [371, 379]. The direct impact of vitamin D on HDL- associated proteins expression (ApoA-1, ApoM, and ApoD) in monocytes in presence of inflammatory signal such as LPS is not fully understood. Here, we explored the effect of vitamin D active

hormone 1,25(OH)2D3 on the expression of apolipoproteins in monocytes exposed to LPS. We also examined the expression of the main anti-inflammatory cationic peptide LL-37 that associated with HDL in serum, as a positive control. LL-37 gene is known to respond to vitamin D as it contains a VDRE binding site and can be induced by vitamin D and LPS [337, 388].

Short report (In final preparation for publication)

Vitamin D effects on the expression of HDL-associated apolipoproteins in human monocytes

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Keyowrds: Vitamin D, Apolipoproteins, ApoM, monocytes, inflammation

Abstract

Introduction: Vitamin D is a prohormone with anti-inflammatory and antimicrobial properties. HDL particles and HDL-associated proteins as well as antiinflammatory proteins in addition to their role in containing the uncontrolled inflammation during sepsis. However, the effect of vitamin D on the expression of HDL-associated protein during sepsis is not fully known.

Methodology: THP1 cells were pretreated with 1,25(OH)2 D3 before inducing cells with the two LPSs Salmonella Typhimurium, and *E. coli* B55. RNA extracted from different conditions

and the gene expression were measured using RT-qPCR. ELISA has been used to detect the IL-8 and TNF-α in supernatants.

Result: Vitamin D has a stimulatory effect on HDL-associated protein ApoA-1, ApoM, and LL-37. LL-37 expression was highly stimulated with all conditions treated with vitamin D regardless of the presence of LPS. Vitamin D induces the ApoA-1 very significantly during adding Salmonella typhimurium, and E. coli B55 LPS for 5 hours. , while ApoM didn't show a significant effect with short incubation with LPS. Meanwhile, ApoM was increased significantly by vitamin D in presence of Salmonella typhimurium LPS for 24 hours. ApoD didn't show a significant increase. TNF- and IL-8 were induced by vitamin D in presence of LPSs.

Conclusion: Vitamin D induces the upregulation of HDL-associated proteins ApoA-1 and LL-37 after administration of Salmonella typhimurium, and E. coli B55 LPS for a short and long period in THP1 cells. Whereas ApoM level increased only after administrating salmonella Typhimurium LPS for a long time in cells pretreated with vitamin D. IL-8 and TNF-α were surprisingly high in presence of vitamin D and the two LPSs.

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1. Introduction

Vitamin D is a prohormone responsible mainly for bone formation by maintaining the homeostasis of calcium and phosphorus. The precursor of vitamin D is generated in the skin then undergoes various metabolism steps to yield the biologically active hormone. In the liver, 7-hydroxy cholesterol is converted to 25-hydroxyvitamin D (25(OH)D) which is a more stable metabolite than the active form. In the kidney, 25(OH)D is converted into 1α,25 dihydroxy

vitamin D (1,25(OH)2D), which is the hormonally active form [2]. The 1,25(OH)2D binds one of the transcription regulator nuclear receptors called vitamin D receptor or VDR. This interaction leads to activating or suppressing many downstream gene targets that contains vitamin D response elements (VDRE) in the promoter region [6, 7]. VDR is highly expressed in immune cells i.e. monocytes, macrophages, and dendritic cells. Therefore, vitamin D exerts immune modulatory effects in monocytes via VDR [399].

Monocytes and macrophages play a crucial role in innate immunity and in lipid homeostasis by reverse cholesterol transfer. Vitamin D enhances monocytes differentiation into macrophages and thereby plays a significant role in modulating the pathophysiology of inflammation and the release of inflammatory mediators. Upon recognition of lipopolysaccharide (LPS) by TLR4-MD-2 receptor, a downstream cascade of cytokines and pro-inflammatory mediators is activated leading to their release [119-122]. VDR is expressed in monocytes and mediates 1,25(OH)2D effect on target genes expression, for example, cathelicidin or LL-37 antimicrobial peptide that plays a critical role in defense against pathogens and possesses immune modulatory activity as well. Reports showed that vitamin D has anti-inflammatory properties and reduces the burden of inflammation and ameliorates sepsis. In presence of LPS, 1,25(OH)2D was found to downregulate the TLR2 and TLR4 in monocytes in a dose-dependent matter [266].

HDL also plays a significant role in sepsis as it neutralizes LPS / endotoxin and clears it from circulation. Thereby, HDL and associated molecules bound to its surface contribute to innate immunity [400]. Apolipoproteins associated with HDL (ApoA-1, ApoM, and ApoD) are produced mainly by hepatocytes and enterocytes and then released into the circulation [401, 402]. These apolipoproteins genes are also highly expressed in immune cells [403]. ApolipoproteinA-1(ApoA-1) the main apolipoprotein in HDL particles disrupts the formation

of the TLR-ligand complex, and recycling of TLR4 during the presence of LPS. ApoA-1 binds LPS and lipoteichoic acid (major components of the cell wall of gram-positive bacteria) and obstructs the activation and translocation of nuclear factor-κB (NF-κB). Consequently, prevents the exacerbation of the inflammatory response produced by tumor necrosis factor-α (TNF-α) and interleukin-6 (IL6) [400, 404].

In the same context, sepsis and systemic inflammatory response syndromes patients had low levels of apolipoprotein M (ApoM). The drop in ApoM level was correlated with the severity of symptoms [371]. ApoM is expressed in several immune cells but more in CD14+ monocytes [403]. In this thesis we discovered that ApoM binds LPS and reduces inflammatory mediators release, hence contributing to endotoxin neutralization and clearance by HDL. However, it is not known whether vitamin D affects the expression of HDL-associated proteins such as ApoM. In this study, we examined the alteration in the genes expression of HDL-associated proteins ApoA-1, ApoM, ApoD, and LL-37 in the presence or absence of LPS and vitamin D.

2. Methodology

Human THP-1 cells were purchased from ATCC and cultured using RPMI 1640 supplemented with 5% penicillin-streptomycin and 10 % Fetal serum bovine. Freshly grown THP-1 cells were adjusted to 1 x10^6 cells/ml and transferred to 12 well plates. Cells were preincubated with vitamin 1,25 (OH)2 D3 for overnight (Sigma- Aldrich, cat. no. 705888,) LPS derived from *Salmonella typhimurium* (Invivogen, Catalog Number: tlr1-sm lps), and *E. coli B55* (Invivogen, Catalog Number: tlrl-pb5lps) has been added for 5 hours and 24 hours. The treated cells and controls were then incubated overnight at 37°C with 5% CO2.

Total RNA was extracted from homogenized cell lysates using ISOLATE II RNA kit (Bioline Meridian BioScience, Cincinnati, Ohio, US) following the manufacturer's protocol. The concentration and purity of RNA extracts were measured using NanoDrop™ 2000/2000c Spectrophotometers (Thermo FisherScientific Inc., Waltham, MA, USA). For each sample, 1 µg of RNA was transcribed into cDNA using SensiFAST cDNA Synthesis Kit (Bioline Meridian BioScience). A final volume of 5 µL cDNA (2.5 ng), 1.6 µL of forward/reverse primers, 3.4 µL of molecular grade water, and 10 µL of SensiFASTTM SYBR No ROX kit (Bioline Meridian BioScience) was mixed for a total reaction volume of 20 µL. All samples were run in triplicates. Non-template controls (NTC) were included without reverse transcriptase to rule out contamination. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed on QuantStudio 12K Flex Real-Time PCR System (Thermo FisherScientific Inc.) using the following program: Initial denaturation for 2 minutes at 95 °C, followed by 40 cycles of 5 seconds at 95 °C, 10 seconds at 60.5 °C, and 15 seconds at 72 °C, and a melt-curve analysis. Relative mRNA expression of ApoA-1, ApoM, ApoD, and LL-37 was analyzed and normalized to the reference gene ACTB using the 2–ΔΔCt method.

Table 6. 1. Primers used in the study

Gene Name	Primer Forward	Origin
ApoA-1	QT00015841	Qiagen
ApoD	QT00081438	Qiagen
ApoM	QT000900664	Qiagen
LL-37	P. F. GGGCACACTGTCTCCTTCAC P.R TCGGATGCTAACCTCTACCG	Qiagen
B-Actin	QT00095431	Qiagen

TNF-α and IL-8 release in the supernatants was measured using ELISA method following manufacturer instructions. Briefly, supernatnats from induced THP-1 monocytes were collected and saved at -20C till further use. ELISA kits from R&D Human (human TNF-α cat. no. DY210 and human IL-8 cat. no. DY208-05) were used. Figures were created using GraphPad Prism 8.0.0 (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical analysis was done using Sidak's multiple comparison test.

3. Result

Vitamin D effect on Apolipoproteins expression in macrophages during inflammation

To understand the immune modulatory effects of vitamin D on monocytes during inflammation, we employed THP-1 monocytes cells (1 x10^6 cell/ml) exposed to 1,25D(OH)D3 (20 nmole/ml) in the presence and absence of LPS (40 ng/ml). Apolipoproteins ApoA-1, ApoD, and partially ApoM were upregulated in THP-1 cells stimulated with *Salmonella typhimurium* and *E. coli B55* LPS for 5 hours. This gene upregulation was augmented in monocytes

preincubated with vitamin D (Figure 1). LL-37 which is known to be a vitamin D target gene and harbors VDRE consensus in its promotor, showed dramatic upregulation of more than a 60-fold increase.

ApoA-1 expression was elevated upon exposure to *Salmonella typhimurium* or *E. coli B55* LPS for 5 hours. This ApoA-1 upregulation was significantly enhanced in monocytes preincubated with vitamin D, particularly with *salmonella Typhimurium* LPS where the fold change was more than 8 folds (Figure 1A). ApoM was slightly elevated in THP-1 cells stimulated with *Salmonella typhimurium* or *E. coli B55* LPS. Vitamin D induced a slight increase in ApoM expression in presence of LPS (~1.5 fold), but ApoM was induced even in control cells without LPS (Figure 1B). Similar results were seen for ApoD expression that vitamin D increased the expression of ApoD (~1.5- fold), however, the increase was more prominent in presence of LPS (~2- fold) (Figure 1C). As expected, LL-37 expression was highly upregulated in THP-1 treated with vitamin D and LPS where the fold increase was

between 35 and 60 (Figure 1D

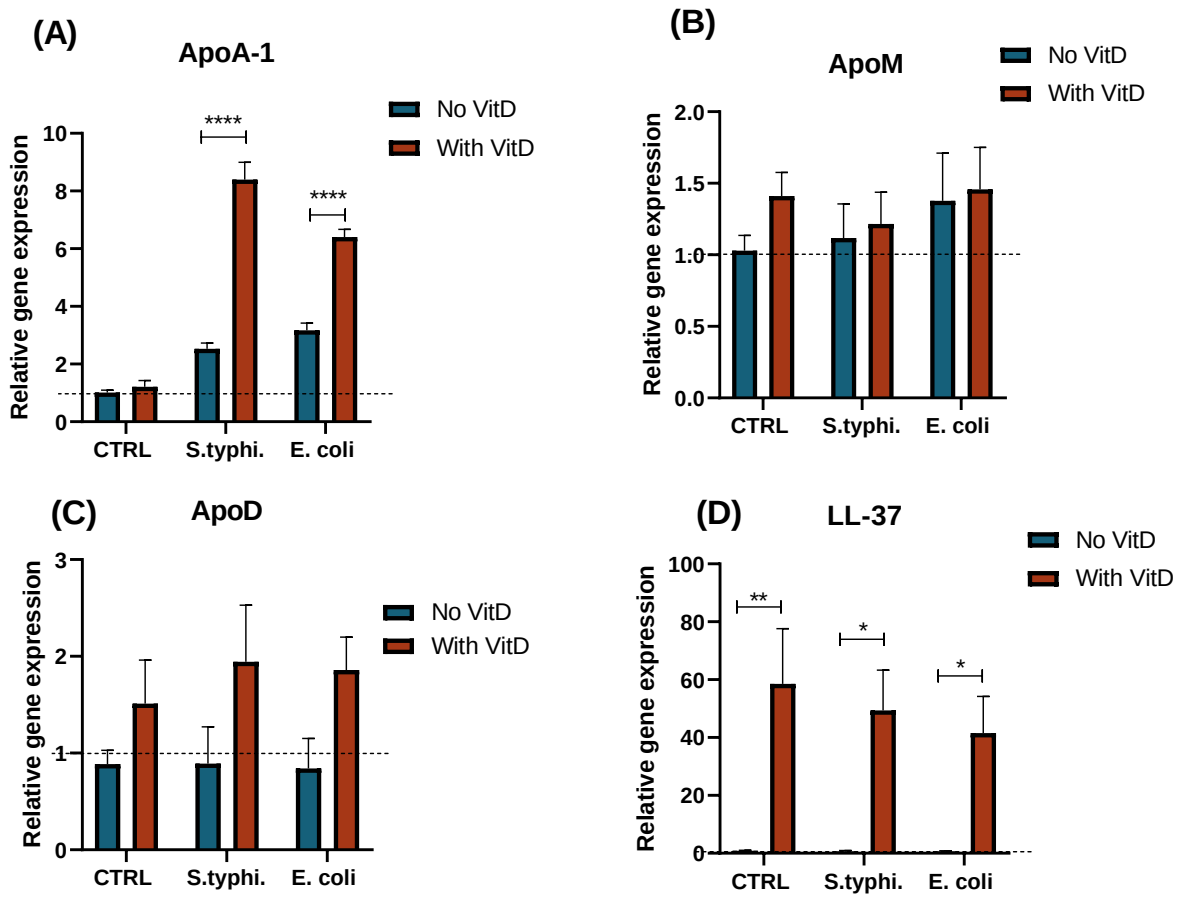

Figure 6. 1. Vitamin D effect on HDL-associated protein ApoA-1, ApoM, and ApoD gene expression. THP-1 cells (1 x10^6 cell/ml) were exposed to 1,25D(OH)D3 (20 nmole/ml) overnight prior to stimulation with Salmonella or E. coli LPS (40 ng/ml) for 5 hours. Quantitative RT-PCR was used to assess genes induction for ApoA (A), ApoM (B), ApoD (C), and LL-37 (D). Control cells without exposure to vitamin D or LPS were also used. The Error bars represent the SD of the fold change seen in three different experiments and each has a triplicate readout. *p<0.01, **p<0.001, ****p<0.0001.

The effect of vitamin D on HDL-associated proteins expression was also examined after 24 hours of exposure to *Salmonella typhimurium* or *E. coli B55* LPS. For most genes tested

that were upregulated at 5 hours were observed to be at a basal level at 24 hours of stimulation (Figure 2). ApoA-1 was downregulated from 8-fold at 5 hours to 1.5-fold after 24 hours of exposure to LPS indicating the expression of the basal level (Figure 2A). ApoM gene expression showed significant elevation upon exposure to *Salmonella typhimurium* in presence of vitamin D compared to control cells (Figure 2B). ApoD gene expression was also downregulated after 24 hours of exposure although didn't show a significant variation with vitamin D treatment (Figure 2C). Lastly, LL-37 gene expression remained very high after 24 hours of exposure to LPS and vitamin D (Figure 2D).

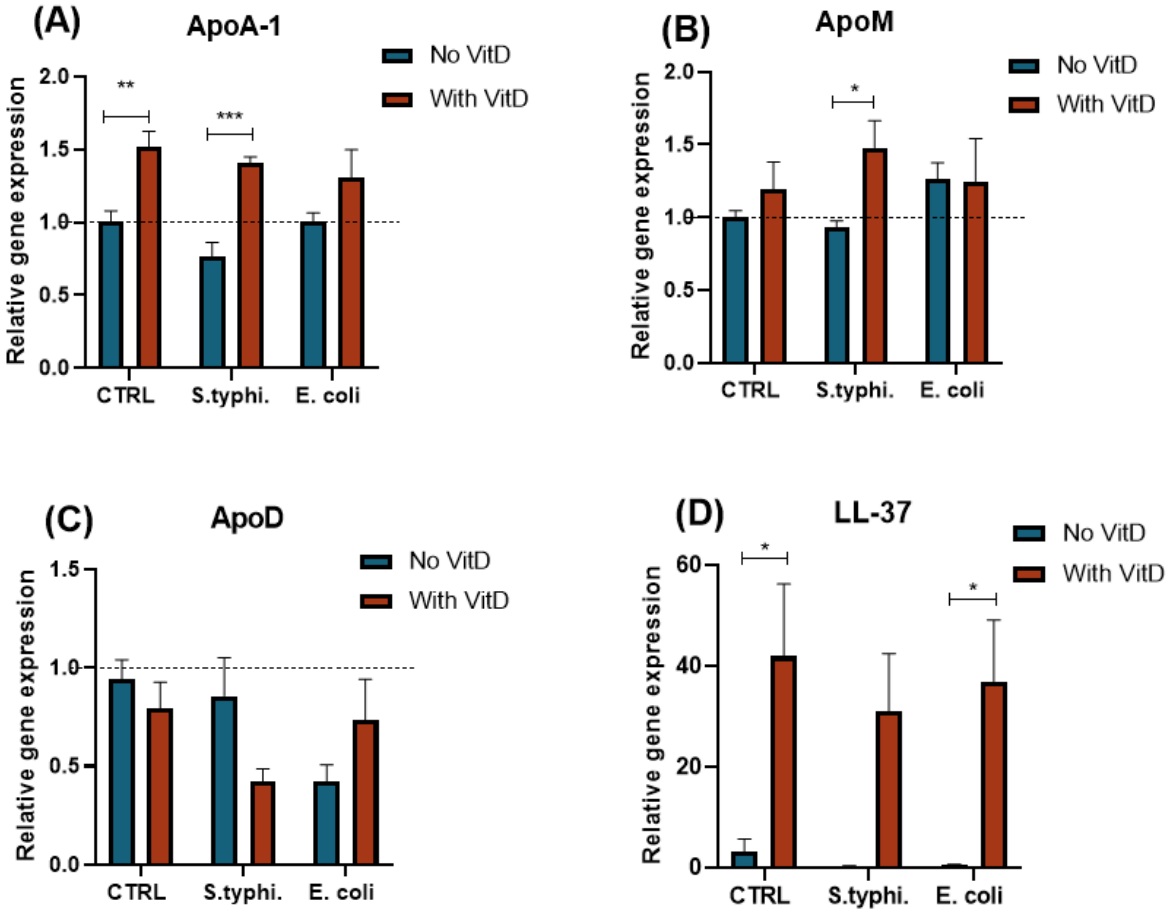

Figure 6. 2. Vitamin D effect on HDL-associated protein ApoA-1, ApoM, and ApoD gene expression after 24 hours post exposure to LPS. THP-1 cells (1 x10^6 cell/ml) were exposed to

1,25D(OH)D3 (20 ng/ml) overnight prior to stimulation with Salmonella or E. coli LPS (40 ng/ml) for 24 hours. Quantitative RT-PCR was used to assess genes induction for ApoA (A), ApoM (B), ApoD (C), and LL-37 (D). Control cells without exposure to vitamin D or LPS were also used. The Error bars represent the SD of the fold change seen in three different experiments and each has a triplicate readout. **p<0.001, ***p<0.0001.

Vitamin D effect on inflammatory mediators IL-8 and TNF-α release from mmonocytes

Vitamin D treatment induces monocyte differentiation to mature macrophages phenotype which affects their inflammatory output of inflammatory cytokines and host defense functions [405, 406].

To further assess the immune modulatory effect of vitamin D on monocytes exposed to inflammatory signals like LPS, we measured the release of chemokine IL-8 (CXCL8) and cytokine TNFα as commonly elevated during inflammation. Unlike IL-1β and IL-6, IL-8 and TNFα proteins do not contain VDRE consensus in their promoters and thereby may not be directly affected by vitamin D treatment.

Here we report that IL-8 protein was highly induced and released in supernatants of THP-1 cells treated with vitamin D and exposed to Salmonella *typhimurium* or *E. coli B55* LPS for 5 hours or 24 hours compared to THP-1 cells without vitamin D or control cells without LPS (Figure 3A and B). Noteworthy, IL-8 release from vitamin D treated THP-1 cells slightly declined after 24 hours of exposure to LPS (Figure 3B). Similarly, TNFα release from vitamin D treated THP-1 cells exposed to *Salmonella typhimurium* or *E. coli B55* LPS for 24 hours was significantly increased (Figure 3C). TNFα release was also increased upon LPS exposure

without vitamin D treatment. We measured IL-1β release from THP-1 cells treated with vitamin D and exposed to LPS but minimal or scanty amounts were detected in the supernatants that did not permit any conclusion. Previous reports showed that vitamin D treatment reduced IL-1β gene expression and protein release in a dose-dependent manner [219]. Further experiments are warranted to determine the effect of vitamin D on IL-1β expression in this particular THP-1 clone.

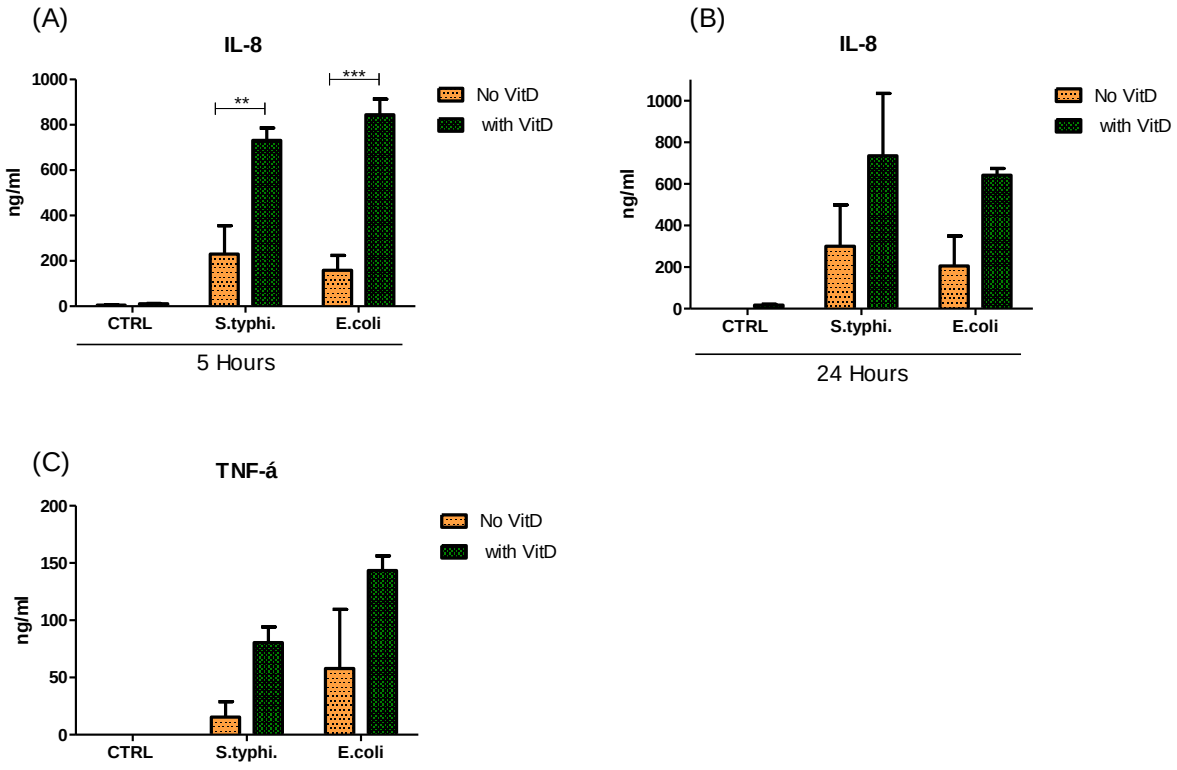

Figure 6. 3. Vitamin D effect on cytokines release from THP-1 cells exposed to LPS. THP-1 cells (1 x10^6 cell/ml) were exposed to 1,25D (OH)D3 (20 ng/ml) overnight prior to stimulation with Salmonella or E. coli LPS (40 ng/ml) for 5 hours or 24 hours. Cytokine release was measured in supernatants using ELISA method. **A**: IL-8 release at 5hours of LPS exposure, **B**: IL-8 release at 24 hours exposure, **C**: TNF-α after 24 hours of incubation with LPS. Error bars represent SD from the mean of three indepoendent experiments. **p<0.001, ***p<0.0001.

4. Discussion

Our result confirmed the effect of the hormonal vitamin D (1,25(OH)2 D3) on THP-1 monocytes and showed dramatic upregulation of LL-37 gene. Accordingly, we examined the effect of vitamin D on HDL-associated apolipoproteins and observed significant upregulation for ApoA-1, ApoD, and slight increase in ApoM when monocytes are exposed to inflammatory triggers like LPS. We selected *Salmonella* and *E. coli* LPS as triggers of inflammatory response in THP-1 monocytes. Both endotoxins share the major endotoxin structure but do vary in minor structural differences that affect LPS potency. For example, *Slamonella* LPS expresses hepta-acylated lipid A with phosphoethanolamine modification on the lipid A head group; whereas *E. coli* expresses hexa-acylated lipid A without any modifications on the head group [384]. Our data show that ApoM expression after 24 hours in vitamin D treated THP-1 cells was elevated in the presence of *Salmonella typhimurium* LPS. This *Salmonella* LPS is more virulent to the human body than *Ecoli* LPS, as Salmonella LPS has the PEA modification that prevents neutralization by host defense peptides like LL-37 and has a longer O antigen chain [407]. Vitamin D was found to enhance the TNF-α and IL-8 induction upon exposure to both *Salmonella* and *E. coli* LPS. This effect may be due to the fact that vitamin D incudes the differentiation of THP-1 monocytes to mature macrophages that secrete higher levels of proinflammatory mediators [405]. In addition, TNFα and IL-8 genes do not contain VDRE consensus in their promoter region and thus may not be directly regulated by vitamin D.

HDL levels decrease during severe inflammation and sepsis as been reported previously. The anti-inflammatory potential of HDL is also well established as it plays a critical role in endotoxin clearance and reduction of inflammation. HDL sequester LPS to form a complex that then binds to SR-BI to be internalized for cellular degradation and detoxification. HDL sequestration of LPS reduces Toll-like receptor 4 (TLR4) activation by endotoxins

consequently suppressing the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α that contribute to cytokine storm and sepsis manifestations [408]. HDL particles associate with various apolipoproteins which suggests a key role for these proteins in the anti-inflammatory potential of HDL. ApoA-1 is known to bind to LPS and other bacterial components but in this thesis we report for the first time that ApoM binds *E. coli* LPS with high affinity, thereby contributing to endotoxin neutralization by HDL which enhances the overall anti-inflammatory potential (see Chapter 5 for more details).

The majority of apolipoproteins are produced from the liver and intestine, however, some of them are highly expressed in immunological cells like ApoA-I, which are found in THP-1 monocytes, macrophages, and peripheral blood mononuclear cells (PBMCs) [409]. An indication of ApoA-1 role in immune systems that it suppressed the activation of TLR4 and TNF-α through several mechanisms. ApoA-1 upregulates the pentraxin 3 (PTX3) level which is an acute phase protein that recognizes PAMPs shed from microorganisms. This effect has been confirmed using transgenic mice overexpressing human apoA-1 where the injection of mice with HDL showed a reduction in TLR4 and TNFα expression [410]. On the other hand, TNF-α was shown to suppress ApoA-1, but increased serum amyloid A (SAA). This SAA is an acute-phase protein that dramatically increased during sepsis displacing the ApoA-1 from HDL particle to be the major protein that renders HDL particles dysfunctional i.e. reduced anti-inflammatory potential. Moreover, SAA was reported to activate inflammasome cascade in addition to TLR2, and TLR4 therefore increasing inflammation status [356].

ApoM is also found in CD14+ monocytes, CD3+ T cells, and CD19+ B cells, CD16+ and CD56+ NK cells. ApoM was observed to interact with SR-B1 on THP-1 and TNF-α, IL-6, and IL-1β. It is worth mentioning that the same effect or interaction with SR-B1 was not observed in HDL particle lacking ApoM [403]. ApoM is a lipid carrier protein that binds

sphingosine-1-phosphate (S1P). This association is documented to have many functions in the immune system. ApoM-S1P binds S1PR2 and activates PI3K/AKT pathway, which will suppress the TNF-α effect. TNF-α is known to increase the adhesion of THP-1 monocytes to endothelial cells. Consequently, more inflammatory mediators will be released and pyroptosis will take place [411]. Therefore HDL particles and the proteins associated with it undergo modulation processes during sepsis, leading to changes in the structure and function.

Apolipoprotein ApoD is produced in the brain from the glial cell, testes, breast, and B cells [412]. ApoD is overexpressed in degenerative diseases like Alzheimer's Disease [413]. ApoD upregulation is reported to protect from oxidative stress by inhibiting the accumulation of lipid peroxides, a product produced by free radicals activity [414]. Carmo et. al. study noticed that ApoD transcription and protein synthesis was overstimulated during the acute encephalitis induced by the human coronavirus OC43. The upregulation in ApoD was associated with neuroprotection and improving the survival rate of mice [190]. Clinically, proteomic profiling of patients suffering from sepsis secondary to community-acquired pneumonia showed that ApoD was downregulated [368].

Our data support the immune modulatory effect of vitamin D on HDL-associated proteins by enhancing their expression. Vitamin D enhanced the expression in apolipoproteins ApoA-1, ApoM and the immune modulatory peptide LL-37 upon exposure to LPS. Vitamin D is considered as an anti-inflammatory agent. Horiuchi et. al induced sepsis in mice using intraperitoneal injection *E. coli* LPS, accompanied by administration of 1α,25-hydroxyvitamin D3. The authors observed that the vitamin D analog was able to improve the survival rate in mice. Add to that, restoring the physiological levels of immunoreactive Thromboxane B2 and hepatic malondialdehyde (MDA) levels that are indicators of high inflammatory status [415]. Clinical studies also supported the protective role of vitamin D as an anti-inflammatory agent.

For instance, a low level of vitamin D in patients admitted to the intensive care unit was associated with a higher risk of developing infections, and also a higher mortality rate [416]. Vitamin D also possesses a direct antimicrobial activity other than containing inflammation during bacteremia [417]. Some studies such as Liu et.al. referred to the antimicrobial activity to its impact on other molecules such as cathelicidin / LL-37 which we confirmed its upregulation in our current study. In their study, they activated the TLR2/1 in monocytes using the *M. tuberculosis* H37Ra strain, and block the cathelicidin activity. They noticed a decline in vitamin D activity against bacteria [418]. Cathelicidin also called LL-37 is a small, cationic peptide, that has antimicrobial activity. The human cathelicidin/ LL37 is found associated with High-density lipoprotein (HDL) [419]. Therefore, vitamin D defeciency impacts the antiinflammatory potential of HDL-associated proteins and HDL biogenesis process as well especially during sepsis and severe inflammation.

5. Conclusion

Vitamin D induces the upregulation of HDL-associated proteins ApoA-1 and LL-37 post exposure to *Salmonella typhimurium* and *E. coli B55* LPS in THP1 cells. Whereas ApoM level increased only after exposure to *Salmonella Typhimurium* LPS pretreated with vitamin D. Therefore, vitamin D exerts immune modulatory effects that impacts HDL endotoxin neutralization and anti-inflammatory potential.

CHAPTER 7: SUMMARY

Vitamin D plays multiple roles in health and disease and impacts multi-systems from bone formation to innate immunity. Vitamin D possesses immune regulatory activity and acts as a key regulator in immunity. Therefore, disturbances in vitamin D levels such as deficiency are associated with high inflammatory status. Consequently, the cellular components of innate immunity such as monocytes and pro-inflammatory mediators e.g. TNF-α, IL6, IL 8 will be affected. Lipid profile and HDL in particular are impacted by the low vitamin D level measured as serum 25(OH) D.

The monocyte percentage to HDL ratio (MHR) has emerged as a novel biomarker in several pathological conditions e.g. ischemic stroke, and cerebral hemorrhage. Here, we investigated the relationship between serum 25(OH) D concentrations and MHR among young healthy adults in Qatar. We studied a cohort of 876 healthy participants under the age of 40 years and devoid of any co-morbidities. We observed an inverse association between vitamin D status and MHR. Our report was the first to show that vitamin D deficiency associates with increased MHR as a biomarker of subclinical inflammation among young healthy adults. The implication of this finding is that increased MHR and low HDL levels are risk factors in atherogenesis and cardiovascular events. Therefore, MHR biomarkers can predict subclinical inflammation and the risk of developing atherosclerotic plaque.

In order to understand the molecular link and identify novel biomarkers that reflect the impact of vitamin D deficiency on HDL homeostasis, we conducted metabolomics and proteomics investigations on a cohort selected from Qatar Biobank. The selected cohort was designed to include participants with or without vitamin D deficiency and dyslipidemia but

devoid of any other co-morbid conditions such as diabetes, hypertension, rheumatoid arthritis or cancer. The Metabolomic and proteomic signatures are modified in many pathological conditions including vitamin D deficiency. These modifications can be an accurate tool to depict the underlying mechanism of action. Aiming to highlight those modifications in metabolites and proteins and to reveal the cross-talk between vitamin D and dyslipidemia, we studied the metabolic and proteomic profiling for 277 healthy participants who have different vitamin D and dyslipidemia status. We categorize the cohort into four groups: Group 1 (n=64) represents participants who were vitamin D sufficient with normolipidemia (control group); Group 2 (n=26) participants who were vitamin D sufficient with dyslipidemia; Group 3 (n=85) represents participants who were vitamin D deficient with dyslipidemia; Group 4 (n=99) represents participants who exhibited vitamin D deficiency with normolipidemia. The metabolic analysis revealed that vitamin D deficiency and dyslipidemia differentially impacted certain metabolites including CMPF and Ergothioneine, as well as multiple lipidomocs pathways, particularly sphingmyelins and ceramides. The modifications were also noted on the downstream pathways including phosphatidylcholine (PC), and phosphatidylethanolamine (PE). The modifications were highest among participants with combined vitamin D deficiency and dyslipidemia.

The proteomic profiling reflected changes in apolipoproteins associated with HDL such as ApoA-1, ApoM, and ApoD. Apolipoproteins are a group of small proteins usually found associated with Lipoproteins (HDL, LDL, VLDL, and chylomicrons), and have several functions including antioxidant and anti-inflammation. In this study, a reduction in ApoA-1, the main apolipoprotein associated with HDL formation, level was observed during vitamin D deficiency. ApoM and ApoD were also downregulated during combined dyslipidemia and vitamin D deficiency. ApoM is critical for HDL biogenesis, therefore, our data provide

evidence that vitamin D deficiency is affecting HDL biogenesis by suppressing the expression of apolipoproteins associated with it. Additionally, vitamin D deficiency affects the anti-inflammatory potential of HDL since apolipoproteins have a role in suppressing inflammation. This could explain the subclinical inflammation seen in vitamin D deficiency. In this study, proteins such as SAA1 which associated with HDL remodeling in case of inflammation by replacing ApoA-1, was very elevated level during vitamin D deficiency indicating the changes in HDL function and structure observed. In general, our proteomic profiling revealed the enrichment of high inflammatory status among participants with combined vitamin D deficiency and dyslipidemia. Proteomics enrichment of inflammation and cancer pathways were observed and include MAPK pathway, JAK-STAT signaling pathway, Ras signaling pathway, cytokine-cytokine receptor interaction, AGE-RAGE and ErbB signaling.

The classical function of HDL is transferring the excess cholesterol from tissues to the liver to be recycled. In clinical studies, HDL was reported to be declined during sepsis and global system inflammation. Many studies demonstrated the role of HDL in endotoxin/ LPS neutralization and clearance. LPS is part of the outer membrane of gram-negative bacteria that causes activation of TLR4, which lead to an activation cascade of downstream targets, and ends up stimulating a storm of proinflammatory mediators such as TNF-α and IL-6. The mechanism by which HDL neutralizes LPS is not fully elucidated. To determine the anti-inflammatory potential of HDL we tested its ability to neutralize endotoxins in vitro. Monocytic THP-1 cells were stimulated with different LPSs from *S. typhimurium*, *E. coli B55*, *S. minnesota*, and *V. cholera* in the presence and absence of purified HDL and LDL fractions. HDL showed more potent activity in neutralizing the mentioned endotoxins more than LDL, where it suppressed TNF-α release. We also investigated the role of apolipoproteins ApoM in such an effect. ApoM is a lipocalin formed of β-strands forming a barrel-like pocket. It has a similar 3D structure to

MD2, the co-receptor for TLR4 responsible for LPS recognition. We studied ApoM physical structure and function using computational docking simulations and isothermal titration calorimetry (ITC). The docking simulations revealed that *E. coli* LPS docks on the surface near the calyx of ApoM but does not block the S1P binding site inside the calyx. The binding of ApoM and LPS is confirmed by ITC studies, where we found that ApoM has a high affinity for *E. coli* LPS, forming a 1:1 complex with a dissociation constant below 1 μM. To further confirm the biological impact of ApoM binding to endotoxin, RAW264 macrophages were stimulated with *E. coli* LPS in presence and absence of ApoM. the data showed that ApoM decreased the nitric oxide production, indicating of neutralization of endotoxin by ApoM. This finding was highly novel and shed light on the mechanism by which HDL neutralizes and clear endotoxin from circulation i.e. confer the anti-inflammatory effect.

Vitamin D has a protective role during the endotoxemia, by stimulating many proteins which has VDR-responsive element (VDRE) such as the antimicrobial cationic papetide cathelicidin or LL-37. This antimicrobial protein LL-37 was found to be associated with HDL particles as revealed by proteomics analysis. Vitamin D deficiency leads to a reduction in LL-37 during severe inflammation and sepsis. It is not known whether apolipoproteins associated with HDL ApoA-1, ApoM, and ApoD would be regulated directly by vitamin D and whether they contain a VDRE. To this end, we employed THP-1 cells preincubated with vitamin D prior to exposure to LPSs from *S. typhimurium* or *E. coli B55*. The gene expression of apolipoproteins ApoA-1, ApoM, ApoD, and LL-37 was investigated using quantitative RT-PCR. Vitamin D enhanced the expression of ApoA-1 and ApoM, while the effect on ApoD was not significant. Vitamin D treatment enhanced monocyte differentiation and modulated inflammatory TNFα and IL-8 release post-exposure to LPS. Taken together, the data suggest

that vitamin D modulated HDL-associated proteins expression with implications to effect on the anti-inflammatory potential.

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Thesis Scholarly Outcomes

Published papers:

- Serum 25-Hydroxyvitamin D Is Inversely Associated with Monocyte Percentage to HDL Cholesterol Ratio among Young Healthy Adults in Qatar. Hanaa Mousa et al. December 2020. https://pubmed.ncbi.nlm.nih.gov/33396346/

- Metabolomics Profiling of Vitamin D Status in Relation to Dyslipidemia. Hanaa Mousa et. al. August 2022. https://pubmed.ncbi.nlm.nih.gov/36005643/

Submitted paper

- ApoM binds endotoxin and contribute to clearance by HDL. Submitted to Biochemical and Biophysical Research Communications. Hanaa Mousa et al. October 2022

Papers in final preparation for submission

- Vitamin D status affects proteomic profile of HDL associated proteins and inflammatory mediators in dyslipidemia

- Vitamin D effects on the expression of HDL-associated apolipoproteins in human monocytes. Hanaa Mousa et al.

Conferences

- International Innate Immunity Society Conference. Kobe, Japan, October 2021
  Oral presentation of "ApoM binds LPS and contribute to neutralization and clearance by HDL".

- International Biobanking Conference. Doha, Qatar, February 2021. Oral poster presentation "Vitamin D deficiency inversely associate with monocyte to HDL ratio (MHR).

- Qatar University Annual Research Forum. Doha, Qatar, October 2022. Two posters presentations:

  Metabolomics profiling of vitamin D deficiency and dyslipidemia
  ApoM binds endotoxin and contribute to neutralization and clearance by HDL