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Unveiling the molecular Culprit of arterial stiffness in vitamin D deficiency and obesity: Potential for novel therapeutic targets

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ABSTRACT

Cardiovascular diseases (CVDs) are highly associated with both vitamin D deficiency and obesity, two prevalent health conditions worldwide. Arterial stiffness, an independent predictor of CVDs, is particularly elevated in both conditions, yet the molecular mechanisms underlying this phenomenon remain elusive, hindering effective management of CVDs in this population. We recruited 20 middle-aged Emiratis, including 9 individuals with vitamin D deficiency (Vit D level $\leq$20 ng) and obesity (BMI $\geq$30) and 11 individuals as control with Vit D level >20 ng and BMI <30. We measured arterial stiffness using pulse wave velocity (PWV) and performed whole transcriptome sequencing to identify differentially expressed genes (DEGs) and enriched pathways. We validated these findings using qRT-PCR, Western blot, and multiplex analysis. PWV was significantly higher in the vitamin D deficient and obese group relative to controls ($p \leq 0.05$). The DEG analysis revealed that pathways related to interleukin 1 (IL-1), nitrogen metabolism, HIF-1 signaling, and MAPK signaling were over-activated in the vitamin D deficient and obese group. We found that HIF-1alpha, NOX-I, NOX-II, IL-1b, IL-8, IL-10, and VEGF were significantly upregulated in the vitamin D deficient and obese group ($p < 0.05$). Our study provides new insights into the molecular mechanisms of arterial stiffness in vitamin D deficiency and obesity, demonstrating the role of oxidative stress and inflammation in this process. Our findings suggest that these biomarkers may serve as potential therapeutic targets for early prevention of CVDs. Further studies are needed to investigate these pathways and biomarkers with larger cohort.

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1. Introduction

Vitamin D deficiency and obesity are closely associated with vascular complications, in particular endothelial dysfunction, and arterial stiffness. Both vascular damages are earlier pathological changes that usually initiate lethal cardiovascular disorders such as myocardial infarction, hypertension, atherosclerosis and stroke, which are the leading causes of morbidity and mortality worldwide [1]. Nonetheless, the cause(s) and mechanism(s) of those vascular damages especially arterial stiffness in vitamin D deficiency and obese individuals are not completely understood and consequently, treatment is not yet sufficient [2,3].

Fat-soluble vitamin D is considered a misnomer because it can be synthesized in the body upon exposure to ultraviolet rays. Thus, it needs to be recognized as a steroid hormone with a plethora of actions all over the body; although traditionally it is famously known for its role in bone development [4,5]. The endogenous source of vitamin D3 (cholecalciferol) occurs in the skin upon exposure to ultraviolet rays, while its exogenous supply is through diets (e.g. eggs, liver, etc.) and over-the-counter supplements. Vitamin D3 from both sources is carried in the blood stream via vitamin D-binding protein (DBP) or lipoproteins [6,7]. Two steps of hydroxylation occur to produce the active form. The first step is the conversion of vitamin D3 to the monohydroxy-derivative, 25(OH)D3 by 25-hydroxylase mainly in the liver. This metabolite is the one usually clinically measured to determine the level of vitamin D3 due to its stability in the blood. The second step occurs mainly in the kidney, but also in the vascular wall and monocytes because of the presence of 1-alpha--hydroxylase enzyme that is required to convert 25(OH)D3 to the active, dihydroxy-form, 1,25(OH)2D3 [8,9]. The consensus in clinical practice is that vitamin D deficiency is defined as a level of 25(OH)D3 $<$ 20 ng/mL ($<$50 nmol/L), while vitamin D insufficiency is considered with a vitamin D level is 20–30 ng/mL (50–75 nmol/L) [10].

Numerous evidence has indicated that 1,25(OH)2D plays a critical role in vascular and cardiac health and promoting their normal functions. It has been shown to have several useful genomic effects on vascular functions including a reduction in thrombogenicity, avascular dilation, reverse endothelial dysfunction, and induce vascular calcification [11,12]. Some of this evidence has been demonstrated in the vitamin D receptor (VDR) knock-out animal model, in which it showed an enhancement in thrombogenicity [13], also it results in hypertension and cardiac hypertrophy due to increasing renin and angiotensin [14,15]. Furthermore, the VDR knock-out model with a low vitamin D diet has shown an aortic calcification due to the differentiation of vascular smooth muscle cells into osteoblast-like cells [16].

Arterial stiffness is considered an independent risk factor for several cardiovascular diseases and usually this risk doubles during the aging process [17,18]. Arterial stiffness is a major factor in regulating vascular impedance which is associated with alterations in the arterial pressure and blood flow. Generally, the change in arterial stiffness could be categorized as either structural or functional type, or both can occur at the same time. The structural change is mainly linked to the arterial wall elastic and collagen fibers that determine the stretch and recoil of vessels during heart beats. A reduction of the elastic component and an increase in the collagen fibers have been reported in aging and early stages of cardiovascular disorders [19]. Functional category includes the regulators of the vascular tone such as nitric oxide and endothelin as well as vascular smooth muscle function, sympathetic tone and genetic polymorphisms [20].

Vitamin D deficiency and obesity have been linked to an increase in inflammatory markers such as interleukin-6 and nuclear factor kappa B (NF-κB), as well as an increase in oxidative stress through the activity of NOX (NADPH oxidase) enzymes. These links are not clear in contributing to the increased risk of arterial stiffness seen in individuals with vitamin D deficiency and obesity [21] There is some evidence showing that a higher level of cytokine interleukin-6 (IL-6) in vitamin D deficiency and obese individuals, may contribute to the development of arterial stiffness. NF-κB is a major inflammatory pathway consisting of many protein subunits that regulate the expression of downstream NF-κB inducible genes that in turn regulates various cellular pathways including innate immunity and inflammation [22]. In general, NF-κB enhances the production of inflammatory cytokines, chemokines, adhesion molecules, and oxidative stress, as well as regulates cell proliferation, differentiation, and apoptosis [23–25]. In the meantime, several chronic non-communicable diseases, such as cardiovascular disease, type 2 diabetes, autoimmune diseases, arthritis, and osteoporosis have been reported to have elevated NF-κB [26]. Calcitriol (1,25(OH)2D); however, was shown to inhibit the pathways related to NF-κB [27]. Thus, calcitriol could play a key role in preventing and mitigating those chronic diseases, in which inflammatory and oxidative stress processes are involved in its pathogenesis such as cardiovascular diseases, as well as an anti-cancer due to its reduction effect of cell proliferation and differentiation [28]. HIF-1 alpha (Hypoxia Inducible Factor-1 alpha) is a transcription factor that plays a vital role in the cellular response to hypoxia [29]. Recent studies have shown that HIF-1 alpha may contribute to arterial stiffness via regulating the genes expression involved in extracellular matrix remodeling and vascular smooth muscle cell proliferation. Specifically, it upregulates the expression of matrix metalloproteinases (MMPs) [30]. Also, it promotes the proliferation of vascular smooth muscle cells, which can contribute to arterial thickening and stiffness [29]. Previous study has shown that HIF-1alpha facilitates the increased expression of NADPH Oxidase (NOX) enzymes, in particular NOX-II, as result of intermittent hypoxia and consequently increase reactive oxygen species (ROS) production [31]. Vitamin D has been also reported to modulate the activity of NOX enzymes and the production of ROS. However, no study has shown a direct link between NOX enzymes and the arterial stiffness in vitamin D deficiency and obese individuals. Some studies have suggested that vitamin D deficiency may be associated with an increase in NOX activity and ROS production, which may contribute to the increased risk of cardiovascular diseases seen in individuals with vitamin D deficiency [30,31]. NOX (NADPH oxidases) family is the only *in vivo* system whose primary function is to produce ROS. From this seven-membered family, NOX-I, NOX-II, NOX-IV and NOX-V have been shown to be expressed in blood vessels and play a critical role in the pathogenesis of vascular disorders and could be potential therapeutic targets for these disorders [29]. Furthermore, NOX-I, NOX-II and NOX-V were suggested to induce endothelial dysfunction, inflammation, and apoptosis while NOX-IV has a vasoprotective effect via increasing nitric oxide bioavailability and suppressing pathways for cell death [32]. In this study, we aimed to identify the molecular mechanism of arterial stiffness in vitamin D deficiency and obese individuals of Emirati population. We

hypothesized that the expression of the inflammatory and oxidative stress markers, especially NOX enzymes, are increased and hence they may contribute to the pathogenesis of vascular stiffness in high-risk vitamin D deficiency and obese individuals. The result of this study attempts to provide us with novel diagnostic and therapeutic targets for arterial stiffness which would prevent cardiovascular disorders before it is fully developed.

2. Methods

2.1. Study population

The study participants were recruited from family medicine and cardiology outpatients' clinics in University Hospital Sharjah during the period from December 2018 to March 2020. The participants were exclusively consisting of Emirati nationals. Inclusion and exclusion criteria were carefully defined to align with the primary research objectives. The study aimed to identify early vascular changes in middle-aged Emirati nationals with and without vitamin D (Vit D) deficiency who had not been previously diagnosed with cardiovascular disorders or other severe debilitating conditions. Specifically, the inclusion criteria included the following: 1) Participants were required to be either males over 45 years old or females over 55 years old, 2) Obesity was a qualifying factor, with a body mass index (BMI) exceeding 30 kg/m^2, 3) and Blood vitamin D, 25(OH)D3 levels had to be below 20 ng/ml. On the other hand, the exclusion criteria were as follows: 1) Individuals with a history of diagnosed coronary vascular diseases were ineligible, 2) Those with previously diagnosed peripheral vascular diseases were excluded from the study, 3) Participants exhibiting any known signs and symptoms indicative of cardiovascular diseases were not included, and 4) Chronic debilitating diseases, such as cancer, were also grounds for exclusion. In total, the study comprised three groups: 11 control participants with vitamin D levels exceeding 20 ng/ml and normal BMI, 9 participants with vitamin D deficiency (Vit D level $\leq$20 ng/ml) who were also obese (BMI $\geq$30), and a reference group for comparison.

2.2. Pulse wave velocity (PWV) measurement

Vascular stiffness assessment in the study participants was conducted using the measurement of Pulse Wave Velocity (PWV). These measurements were carried out while individuals were in the supine position, utilizing the SphygmoCor system (version 7.0, Atcor Medical, Sydney, Australia). The aortic PWV was recorded by measuring the time it took for the pulse wave to travel between the femoral and carotid arteries. To account for age-related variations, the PWV relative to age was calculated using the following formula: PWV (m/s) x 100/Age (years). The measurement procedures closely followed previously established protocols and guidelines [33].

2.3. Plasma samples collection

A total of 10 ml of whole blood was drawn from participants in EDTA tubes. After 8–10 times gentle inversions of the blood, we centrifuged the samples at 4200 RPM for 10 min. A total of 4 ml plasma was transferred to 2 ml tubes and placed right away in a −80 °C freezer. RNA was extracted from whole blood samples using TRIzol reagent (Cat. No. 15596018; Invitrogen, USA), and RNA was re-solubilized in RNase-free water. RNA was quantified using Nanodrop™ 2000/2000c Spectrophotometer (Cat. No. ND-2000; Thermo Scientific, USA).

2.4. Whole transcriptome profiling

Purified RNA samples from all patients and controls were proceeded for whole transcriptome sequencing using Ion AmpliSeq Whole Transcriptome human gene expression kit (Thermo Fisher Scientific). RNA processing and library preparation were performed as previously described (34). All library amplicons were individually quantitated using Agilent Bioanalyzer (Agilent, Santa Clara, CA), diluted to a 100 pM concentration then pooled together for further processing. The pooled libraries were next amplified using emulsion PCR using the Ion One Touch2 instruments (OT2) and enriched using Ion One Touch ES as per the manufacturer's instructions. The sequencing was performed using Ion 540 Chip on an Ion S5 XL Semiconductor sequencer (Life Technologies).

2.5. Bioinformatics analysis

RNA-seq raw data underwent processing and analysis an in-house bioinformatics pipeline, as detailed in prior descriptions [34]. The differentially expressed genes (DEGs) analysis was performed using the DESeq2 Bioconductor package in R software [35]. First, DEGs were identified by filtering low expression genes, then applying combined thresholds of fold change (FC) $>$ 2 and adjusted p-value (FDR) $<$ 0.05. Next, the significant DEGs were further annotated to identify the enriched pathways and functional ontologies using Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology (GO) databases with Benjamini-Hochberg adjusted $p <$ 0.05.

2.6. RT-PCR

cDNA was prepared from the extracted RNA using GoScript Reverse Transcription System (Cat. No. A5000; Promega, USA). C-DNA was quantified using Nanodrop™ 2000/2000c Spectrophotometer (Cat. No. ND-2000; Thermo Scientific, USA). 100 ng of cDNA was

Table 1
Sequences of the primers used for validation by quantitative PCR.

	Forward primer	Reverse Primer
HIF-1 alpha	HIF1A-S1 TGCTCATCAGTTGCCACTTC	HIF1A-AS2 TCCTCACACGCAAATAGCTG
NOX-I	HuNOX1-S1 GCCTGTGCCCGAGCGTCTGC	HuNOX1-AS2 ACCAATGCCGTGAATCCCTAAGC
NOX-II	HuNOX2-S1 GGAGTTTCAAGATGCGTGGAAACTA	HuNOX2-AS2 GCCAGACTCAGAGTTGGAGATGCT
NOX-V	NOX5-S1 ATCAAGCGGCCCCCTTTTTTCAC	NOX5-AS2 CTCATTGTCACACTCCTCGACAGC
IL-1b	CTAAACAGATGAAGTGCTCC	GGTCATTCTCCTGGAAGG
IL-8	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
IL-10	AGGCTACGGCGCTGTCATC	GGCATTCTTCAC CTGCTCCA3
VEGF	GAAGTGGTGAAGTTCATG GATGTC	CGATCGTTCTGTATCAGTCTTTCC
18S	18S–S1 TGACTCAACACGGGAAACC	18S-AS2 TCGCTCCACCAACTAAGAAC

utilized for the RT-PCR reactions, as well as Go Taq™ qPCR Master Mix (Cat. No. A6001; Promega, USA). Duplicate reactions for each sample with a final volume of 10 μl for each reaction were prepared, and real-time PCR was performed using Rotor-Gene Q Real-time PCR cycler, under the following conditions (Hold: 95 °C for 02:00 min. Cycling: 95 °C for 00:15 s, 60 °C for 00:30 s, 60 °C for 00:30 s. Melt: ramp from 60 °C to 95 °C rising by 1 °C each step, wait for 00:90 s of pre-melt conditioning on the first step, wait for 00:05 s for each step afterward). CT values were recorded and normalized into Δct, and ΔΔct based on control values, using 18S primers. The sequence of the primers is shown in Table 1.

2.7. Western blot

Whole blood cells were lysed using TRIzol reagent (Cat. No. 15596018; Invitrogen, USA), and protein lysate was re-solubilized in 1 % SDS. Protein concentration of cell lysate was quantified using the Bradford method (Cat. No. 500-0006; Bio-Rad, USA). Electrophoresis was performed on 30 % Acrylamide and 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) using 25 μg of protein. The wet transfer was done overnight at 25V using a PVDF membrane. 5 % BSA was used to block the membrane at room temperature for 1 h. The membrane was washed with TBS-t, and incubated overnight at 4 °C with the following primary antibodies (Anti-NOX1 antibody [cat#ab131088] (from Abcam), Recombinant Anti-NOX2/gp91phox antibody [ERP6991] [cat#ab129068] (from Abcam), Anti-NOX5 antibody - C-terminal [cat#ab191010] (from Abcam), Anti–HIF–1 alpha antibody [cat#ab82832] (from Abcam), and GAPDH [cat#ab8245] (from Abcam), all at 1:1000 dilution. Secondary antibodies (Anti-Mouse IgG HRP-Linked [cat#7076S], Anti-Rabbit IgG HRP-Linked [cat#7074S]) (both from Cell Signaling Technology, USA). were incubated with the corresponding primary antibody, at 1:500 dilution for 1 h at room temperature. Chemiluminescence was performed using Clarity™ Western ECL Substrate kit [cat#170–5060] (by Bio-Rad Laboratory Inc, USA). Band detection and quantification were done using Bio-Rad Image Lab software (ChemiDoc™ Touch Gel and Western Blot Imaging System). Protein normalization was done using GAPDH.

2.8. Cytokines protein expression

Using the multiplex technique, plasma cytokines were analyzed using Luminex Human Cytokine Premixed Kit A (R&D System, Inc., Minneapolis, USA) according to the manufacturer's instructions. In brief, 50 μL of standard and plasma samples were added per well, then 50 μL of diluted Microparticle Cocktail was added to each well then incubated for 3 h at RT on a shaker at 800 rpm, followed by a washing step using (Microplate Magnetic Bead Washer). Next, 50 μL of diluted Biotin Antibody Cocktail was added to each well, covered and incubated for 1 h at RT on the shaker at 800 rpm, followed by another three washing steps, then 50 μL of diluted

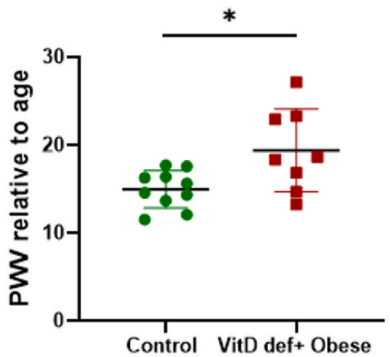


Fig. 1. Pulse wave velocity relative to age in vitamin D deficiency and obese patients (A-group), n = 9. compared to control healthy individuals with normal Vitamin D level and BMI (C-group), n = 11. Values are expressed as mean ± SEM and analyzed using unpaired two-tailed Student t-test. *P ≤ 0.05.

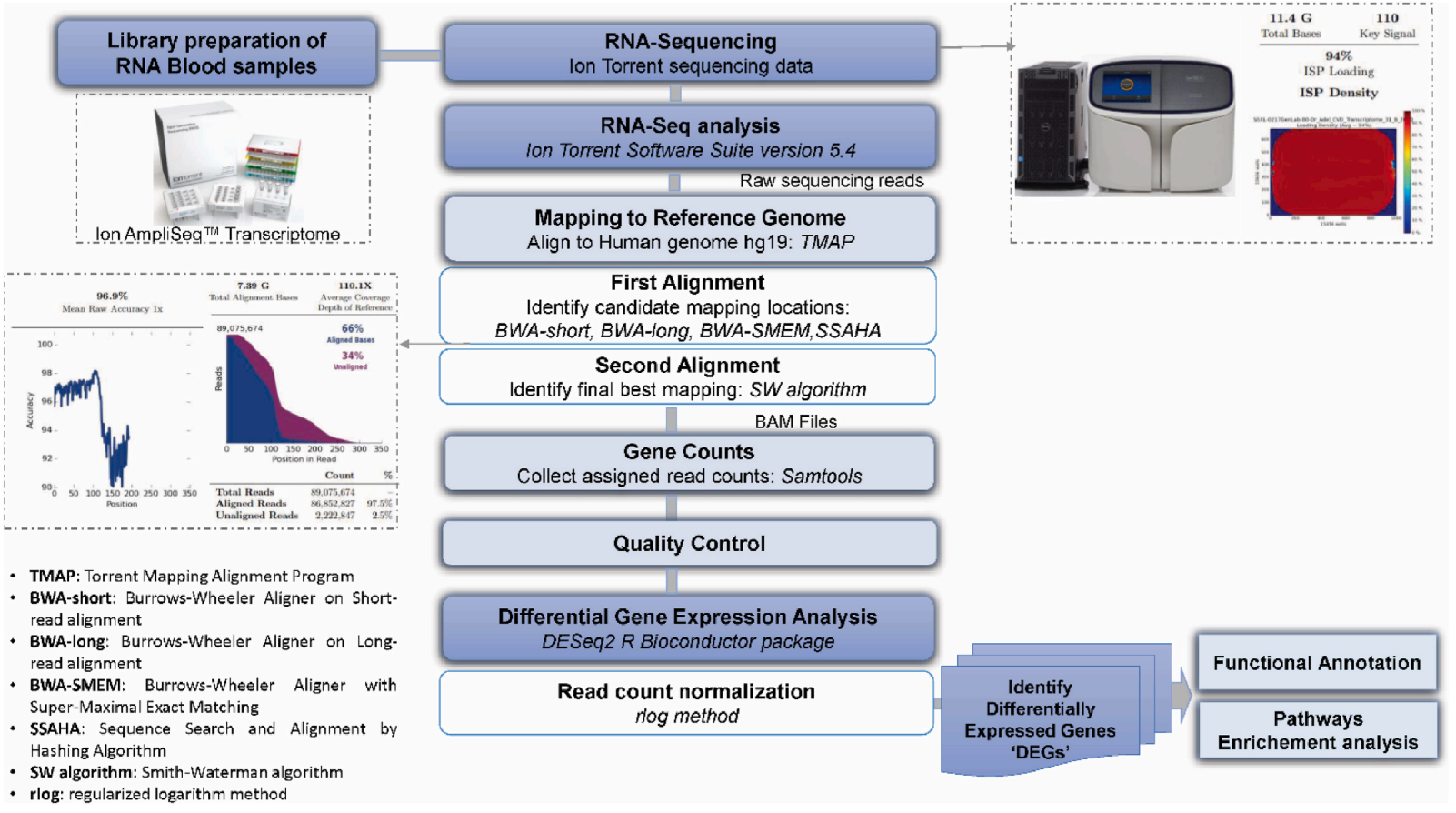


Fig. 2. Overall pipeline of RNA sequencing and bioinformatics analysis.

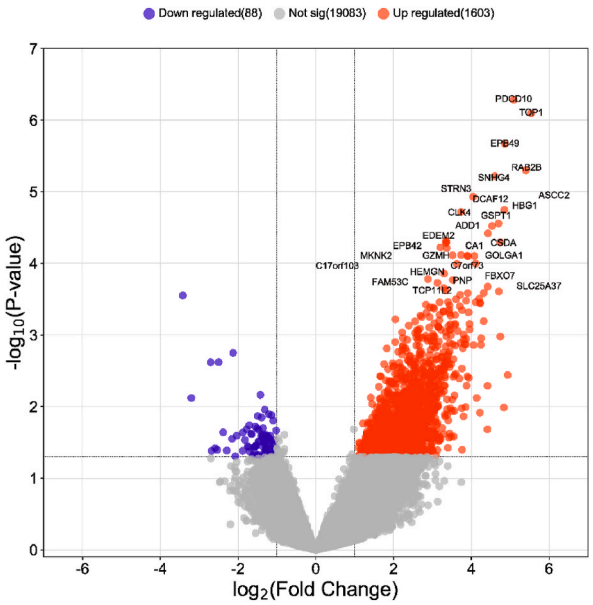


Fig. 3. Volcano plot showing the DEGs between the vitamin D deficiency and obese group compared to the healthy control group. The red scatters present the upregulated genes, bleu scatters present the downregulated genes and grey scatters indicate no significant differentially expressed genes between the vitamin D deficiency and obese group compared to the healthy control group. The top significantly expressed genes (FDR <0.05) between the vitamin D deficiency and obese group compared to the healthy control group were labeled.

Streptavidin-PE was added to each well, incubated for 30 min at RT on the shaker at 800 rpm followed by another washing step. Finally, 100 μL of Wash Buffer was added to each well and incubated for 2 min at RT on the shaker at 800 rpm (Bioplex-200 Multiplex system). The kit detected several cytokines; we are presenting the following: IL-1b, IL-8, IL-10, and VEGF. The average of three replicates from each sample was taken.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism v.5.0 (GraphPad Software, Inc. CA, USA). The unpaired *t*-test was used to compare the statistical significance between two groups (A-group vs. C-group). A two-tailed p-value (p) < 0.05 was considered statistically significant for all statistical tests.

3. Results

3.1. Clinical characteristics of study participants and vascular damage

The clinical parameters were compared between individuals with vitamin D deficiency and obesity (referred to as the A-group) and control participants with normal vitamin D levels and BMI (referred to as the C-group), building upon our previous report. The A-group had an average age of 53 ± 11.7 years, while the C-group had an average age of 58.4 ± 7.5 years. Particularly, there was a significant difference in blood vitamin D levels between the A-group and the C-group ($p < 0.0001$). In the A-group, the average vitamin D level was 12.9 ± 4.3 ng, whereas in the C-group, it was 37.4 ± 12.6 ng. Furthermore, there was a significant difference in BMI between the two groups ($p < 0.0001$). In the A-group, the average BMI was 33.6 ± 3.1, while in the C-group, it was 26.4 ± 2.6. An important finding was the significant increase in vascular stiffness, as measured by pulse wave velocity (PWV) relative to the participants' age, observed in the A-group when compared to the control group. The A-group exhibited a PWV of 19.4 ± 4.7 m/s, whereas the C-group showed a lower PWV of 14.7 ± 2.1 m/s ($P < 0.05$) as shown in Fig. 1. A similar trend was observed in other parameters such as in augmentation pressure (AP) and ALX% (AP/pulse wave pressure) [36].

3.2. Differentially expressed genes in the plasma of high-risk patients for vascular damage

The workflow of RNA sequencing and bioinformatics analysis is summarized in Fig. 2. After filtering genes with low read counts and applying standard normalization, the differential gene expression analysis in the plasma of the vitamin D deficiency and obese group (A-group) compared to the healthy control group (C-group) showed distinct gene expression profiles as shown in the volcano plot figure (Fig. 3). Importantly, the top DEGs in the A-group compared to the C-group (FDR <0.05) resulted a total of 27 highly expressed genes (log2FC > 2.8) in the D deficiency and obese patients, Table 2. Furthermore, the gene annotation of the top DEGs list

Table 2
Differentially expressed genes between individuals with Vitamin D deficiency and Obesity (A-group) and healthy individuals with normal Vit. D level and BMI as control group (C-group).

#	Genes ID	Description	log2FoldChange	p-value	FDR
1	TOP1	DNA topoisomerase 1; Releases the supercoiling and torsional tension of DNA introduced during the DNA replication and transcription by transiently cleaving and rejoining one strand of the DNA duplex	5.54	7.99E-07	0.0032
2	RAB2B	Ras-related protein Rab-2B; Required for protein transport from the endoplasmic reticulum to the Golgi complex	5.40	5.00E-06	0.0096
3	PDCD10	Programmed cell death protein 10; Promotes cell proliferation. Modulates apoptotic pathways. Increases mitogen-activated protein kinase activity and STK26 activity. Important for cell migration, and for normal structure and assembly of the Golgi complex. Increases the stability of KDR/VEGFR2 and prevents its breakdown. Required for normal cardiovascular development. Required for normal angiogenesis, vasculogenesis and hematopoiesis during embryonic development	5.09	5.18E-07	0.0032
4	EPB49	Dematin; Membrane-cytoskeleton-associated protein with F-actin- binding activity that induces F-actin bundles formation and stabilization	4.87	2.15E-06	0.0057
5	DCAF12	DDB1- and CUL4-associated factor 12; May function as a substrate receptor for CUL4-DDB1 E3 ubiquitin-protein ligase complex	4.85	1.79E-05	0.0188
6	CSDA	Y-box binding protein 3	4.75	5.10E-05	0.0274
7	ASCC2	Activating signal cointegrator 1 complex subunit 2; Activating signal cointegrator 1 complex subunit 2; Enhances NF-kappa-B, SRF and AP1 transactivation	4.70	2.78E-05	0.0237
8	SNHG4	Small Nucleolar RNA Host Gene 4, is affiliated with the lncRNA class	4.60	6.05E-06	0.0096
9	HBG1	Hemoglobin subunit gamma 1	4.53	3.00E-05	0.0237
10	GSPT1	Eukaryotic peptide chain release factor GTP-binding subunit ERF3A; Involved in translation termination in response to the termination codons UAA, UAG and UGA. Stimulates the activity of ERF1. Involved in regulation of mammalian cell growth. Component of the transient SURF complex which recruits UPF1 to stalled ribosomes in the context of nonsense-mediated decay (NMD) of mRNAs containing premature stop codons; Belongs to the TRAFAC class translation factor GTPase superfamily. Classic translation factor GTPase family. ERF3 subfamily (637 aa)	4.43	3.80E-05	0.0273
11	SLC25A37	solute carrier family 25 member 37	4.11	1.00E-04	0.0335
12	GOLGA1	golgin A1	4.08	7.88E-05	0.0284
13	STRN3	Striatin-3; Binds calmodulin in a calcium dependent manner. May function as scaffolding or signaling protein	4.05	1.17E-05	0.0154
14	FBXO7	F-box protein 7	3.92	7.91E-05	0.0284
15	C7orf73	short transmembrane mitochondrial protein 1	3.90	7.85E-05	0.0284
16	CLK4	Dual specificity protein kinase CLK4; Dual specificity kinase acting on both serine/threonine and tyrosine-containing substrates	3.74	1.91E-05	0.0188
17	CA1	Carbonic anhydrase 1; Reversible hydration of carbon dioxide. Can hydrates cyanamide to urea	3.74	7.64E-05	0.0284
18	HEMGN	hemogen	3.63	1.02E-04	0.0335
19	TCP11L2	t-complex 11 like 2	3.53	1.70E-04	0.0498
20	GZMH	Granzyme H; Cytotoxic chymotrypsin-like serine protease with preference for bulky and aromatic residues at the P1 position and acidic residues at the P3' and P4' sites. Probably necessary for target cell lysis in cell-mediated immune responses	3.52	7.70E-05	0.0284
21	EPB42	Erythrocyte membrane protein band 4.2; Belongs to the transglutaminase superfamily, probably plays an important role in the regulation of erythrocyte shape and mechanical properties	3.37	6.13E-05	0.0284
22	EDEM2	ER degradation-enhancing alpha-mannosidase-like protein 2; Initiates the endoplasmic reticulum-associated degradation (ERAD) that targets misfolded glycoproteins for degradation in an N-glycan-dependent manner	3.36	5.03E-05	0.0274
23	ADD1	Alpha-adducin; Membrane-cytoskeleton-associated protein that promotes the assembly of the spectrin-actin network	3.35	4.82E-05	0.0274
24	MKNK2	MAP kinase-interacting serine/threonine-protein kinase 2; Serine/threonine-protein kinase that phosphorylates SFPQ/PSF, HNRNPA1 and EIF4E. May play a role in the response to environmental stress and cytokines	3.35	5.21E-05	0.0274
25	PNP	purine nucleoside phosphorylase	3.30	1.39E-04	0.0438
26	C17orf103	N-acetyltransferase domain containing 1	3.20	5.95E-05	0.0284
27	FAM53C	family with sequence similarity 53 member C	2.89	1.66E-04	0.0498

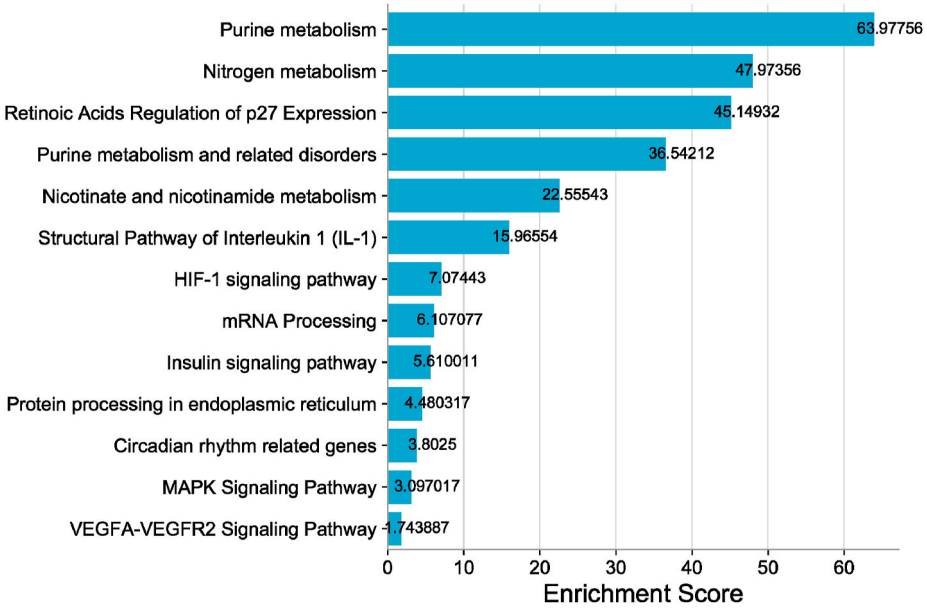


Fig. 4. Functional annotation and enrichment analysis of the top differentially expressed genes resulting in Vit D deficiency and obese group compared to the controls group. The top significant enriched pathways are displayed (Benjamini-Hochberg adjusted $p < 0.05$).

was performed to show important candidate genes for vascular damage risk such as ASCC2 (activating signal cointegrator 1 complex subunit 2) which enhances NF-kappa-BSRP and AP1 transactivation especially during inflammatory process as recently reported [37].); MKNK2 (MAP kinase-interacting serine/threonine-protein kinase 2) is critical in response to stress and inflammatory cytokines; PDCD10 (Programmed cell death protein 10) which is important in cell apoptosis and proliferation and required for normal angiogenesis and vasculogenesis; RAB2B (Ras-related protein Rab-2B), EPB49 (Dematin), ADD1 (adducin1) and STRN3 (Striatin-3) which are important in cellular protein formation, transport and signaling; also, GZMH and PNP among other in cell-mediated immune responses.

3.3. Inflammatory and oxidative stress pathways are significantly altered in high-risk patients for vascular damage

In order to understand the potential mechanism underlying the vascular changes in vitamin D deficiency and obese patients at the transcriptome level, clustering and functional annotation of the DEGs were performed. In the vitamin D deficiency and obese group, the significantly over-activated pathways ($p < 0.05$) are mainly related to the Structural Pathway of Interleukin 1 (IL-1) WP2637, MAPK Signaling Pathway WP382, Nitrogen metabolism, HIF-1 signaling pathway, MAPK signaling pathway as shown in Fig. 4. Besides validating the HIF-1 alpha, we select the NADPH oxidases (NOX) family to test in our samples since is the only *in vivo* system whose primary function is to produce ROS. To our knowledge, no previous study has examined NOX expression in arterial stiffness of vitamin D deficiency and obese patients. Furthermore, we explored the expression of various cytokines inflammatory markers, therefore our focus was mainly on those two major pathways in the rest of the study which were the oxidative stress makers of HIF-1, NOX-I, NOX-II, and NOX-V, as well as inflammatory makers such as IL-1b (FC = 2.18, p = 0.04), IL-8 (FC = 2.45, p = 0.01), IL-10 (ns), and VEGF (ns).

3.4. Oxidative stress pathways in vitamin D deficiency and obese patients

To evaluate the involvement of oxidative stress pathways in high-risk patients for vascular damage, gene and protein expression profiles of key related oxidative biomarkers including NOX-I, NOX-II, and NOX-V were assessed in the A-group and the C-group. As shown in Fig. 5, significant over-expression levels of HIF-1alpha (Fig. 5A), as well as NADPH Oxidase genes were reported in NOX-I (Fig. 5B), NOX-II (Fig. 5C) ($p < 0.05$), but not NOX-V (Fig. 5D) in the A-group compared to the C-group using RT-qPCR. Furthermore, Fig. 6 shows the protein expression of HIF-1 alpha (Fig. 6A), and NOX-I (Fig. 6B) were higher in A-group compared to C-group using Western blot analysis However, NOX-II (Fig. 6C) and NOX-V (Fig. 6D) was not statically significant ($p > 0.05$) but showed a higher expression trend. Representative immunoblots depicting protein levels of HIF-1alpha, NOX-I, NOX-II, NOX-V, and GAPDH in 5 blood samples from each individual or patient. In addition, Heme Oxygenase 1 and catalase protein expression did not show significant difference between the two groups.

3.5. Inflammatory biomarkers pathways in vitamin D deficiency and obese patients

To further validate the DEGs of the inflammatory pathway, the relative gene expression using qRT-PCR analysis of the

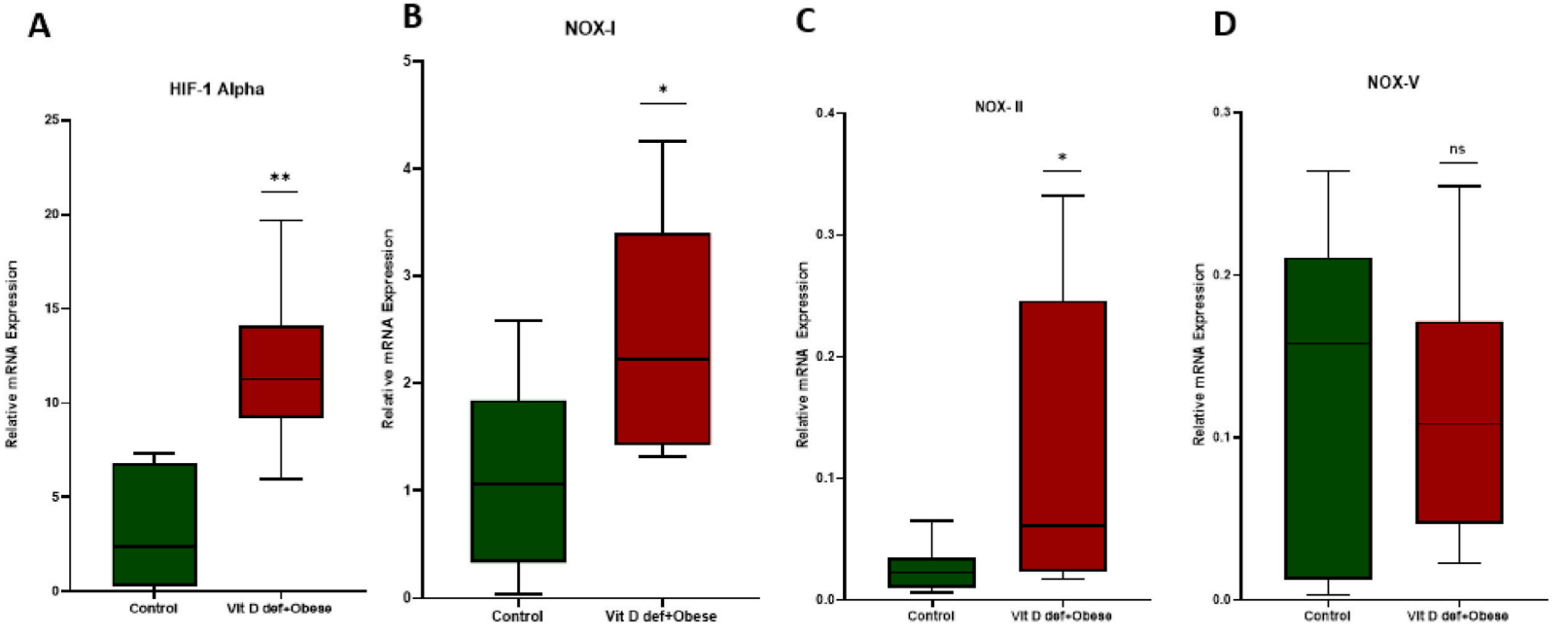
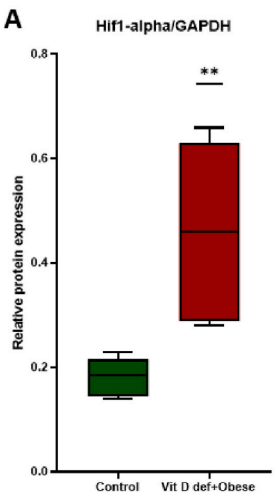
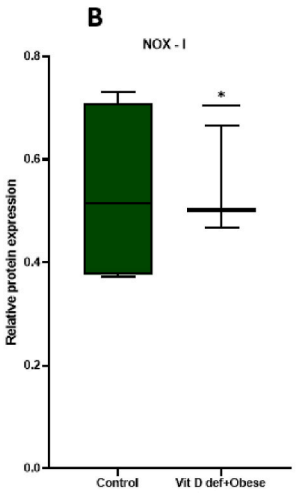
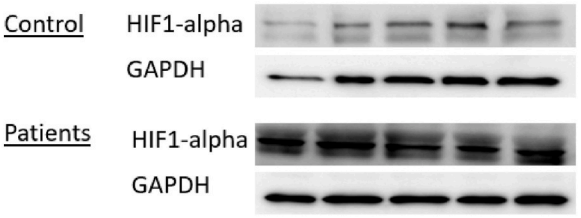
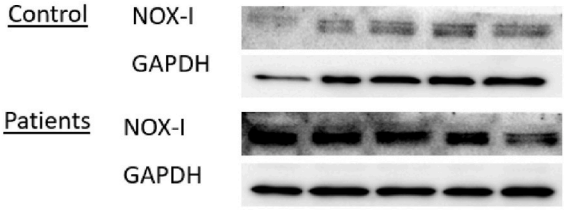


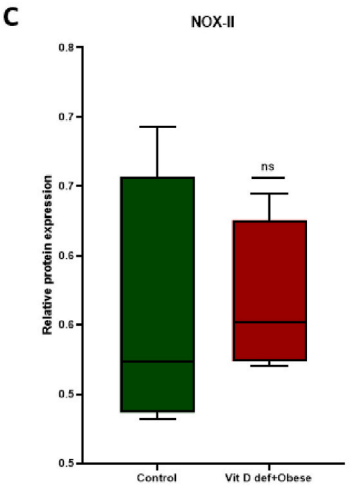
Fig. 5. Gene expression levels of HIF-1 alpha and NADPH Oxidase: HIF-1 alpha (A), NOX-I (B), NOX-II (C), and NOX-V (D) using qRT-PCR analysis in patients with vitamin D deficiency and obese (A-group) compared to healthy individuals with normal level of Vit. D and BMI, control group (C-group). Calculated mean ± SD fold change in gene expression levels in patient's plasma based on two separate experiments. *P $\leq$ 0.05, **P $\leq$ 0.01, determined using unpaired two-tailed Student t-test.

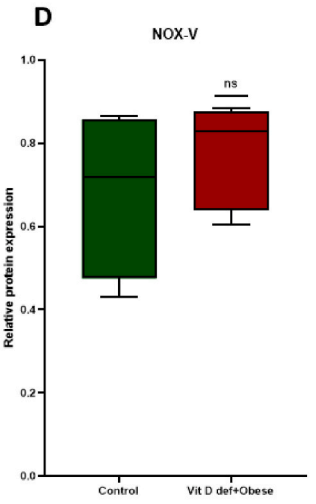


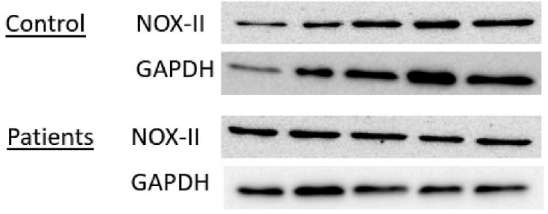












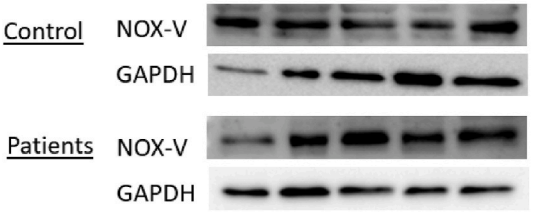


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Fig. 6. Western blot analysis showing HIF-1 alpha and NADPH Oxidase protein expression: HIF-1 alpha (A), NOX-I (B), NOX-II (C), and NOX-V (D) in patients with vitamin D deficiency and obese (A-group) compared to healthy individuals with normal level of Vit. D and BMI, control group (C-group). Calculated mean ± SD fold change in protein-expression levels in patient's plasma based on two separate experiments. *P ≤ 0.05, **P ≤ 0.01, determined using unpaired two-tailed Student t-test. Note that full uncropped images are provided in the supplementary materials.

inflammatory and angiogenesis markers were shown in Fig. 7: IL-1b ($p = 0.0002$) (Fig. 7A), IL-8 ($p = <0.0001$) (Fig. 7B), and VEGF ($p = 0.0015$) (Fig. 7D) were elevated in the A-group compared to the C-group. While the expression of IL-10 with much lower in the A-group compared to the C-group (Fig. 7C). Their corresponding proteins using the Luminex Human Cytokine Premixed Kit A showed the same pattern as shown in Fig. 8, with higher protein expression in IL-1b ($p = 0.0078$) (Fig. 8A), IL-8 ($p = 0.0119$) (Fig. 8B), and VEGF ($p = 0.0494$) (Fig. 8D), but lower in IL-10 ($p = 0.0210$) (Fig. 8C).

4. Discussion

The effect of vitamin D in cardiovascular diseases (CVDs) has been controversial in numerous studies using *in-vitro*, animal, or human models. Moreover, the exact mechanisms underlying the early vascular damages, which usually initiate CVDs, in vitamin D deficiency and obese individuals are not known [11,32]. In the current study, we provide, for the first time, some evidence of the arterial stiffness mechanisms in vitamin D deficiency and obese individuals (A-group) and compare them to healthy control individuals (C-group) with normal vitamin D and BMI levels.

The top differentially expressed genes and their pathway analysis between A-group and C-group using GO category and KEGG were as follows: Structural Pathway of Interleukin 1 (IL-1) WP2637, MAPK Signaling Pathway WP382, Nitrogen metabolism, HIF-1 signaling pathway, MAPK signaling pathway. Subsequently, we focused on the inflammatory and oxidative stress biomarkers in those individuals. NADPH oxidase (NOX) family is known as the only *in-vivo* system with a primary function to produce ROS. Thus, we have decided to test its relative members along with HIF-1 alpha and others. There was a significance increased in the expression levels of HIF-1 alpha, NOX-I, and NOX-II, but not NOX-V, catalase, and Heme Oxygenase 1 in A-group compared to C-group. Furthermore, we demonstrated an elevation of gene and protein expression of some cytokine inflammatory markers such as IL-1b, IL-8, and VEGF in the A-group compared to the C-group.

Our results are consistent with a recent report by Mirza et al. which showed the link between vitamin D deficiency and obesity with the inflammatory cytokines and vascular dysfunction [38]. Specifically, it demonstrated the association of the severity of vitamin D deficiency with lower DNA methylation that is inversely related to an increase in the expression of inflammatory adipokines such as B-cell lymphoma 6, C-X-C Motif Chemokine Ligand 8, histone deacetylase 5, interleukin 12A, and nuclear factor κB in the adipose tissues. Another recent study of a 12-month follow-up of lifestyle change showed a correlation between vitamin D deficiency and atherosclerotic parameters with the inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8, and IL-17A in heart failure patients [39]. In contrast, a recent randomized clinical trial on a younger overweight and obese individual did not show a significant effect of vitamin D supplements on arterial stiffness and endothelial function or systemic inflammation. However, it lowered BP and fasting glucose levels and improve insulin sensitivity [40]. The participant's age factor and the small correction of their vitamin D levels in this study need to be considered when comparing it with our results and others.

Furthermore, the earlier large VITAL study trial has reported the effect of vitamin D3 and omega-3 supplements for five years in middle-aged individuals aged ≥50 years old and showed no beneficial prevention result on cardiovascular events (i.e. myocardial infarction, stroke, and cardiovascular mortality) in comparison to placebo [41]. Despite the multiple strength of the VITAL trial, it used only one dosage of vitamin D (2000 IU/day) for 5 years' duration and included healthy individuals with normal serum levels of vitamin D (25(OH)D) or at the higher side of vitamin D deficiency, as well as some overweight participants which may have a different response to vitamin D supplements. A recent randomized cohort study has demonstrated a blunted vitamin D metabolism and internal dose-response following a vitamin D supplement of 2000 IU/d in overweight or obese participants [42]. This study suggests that the reduced efficacy of vitamin D supplementation for cardiovascular and cancer in VITAL trial and others could be in subgroups of the participants with higher body mass index.

The role of oxidative stress as a mechanism of arterial stiffness in vitamin D and obesity populations is also not clear in several studies. A short-term supplement of vitamin D in overweight and obese adult African Americans who suffered from vitamin D deficiency resulted in a significant reduction of oxidative stress and inflammatory mediators, urinary isoprostane and adipocyte cytokine expression respectively. In this study, the augmentation index, an indirect measure of arterial stiffness, was reduced but not the pulse wave velocity [43]. Furthermore, our results are consistent with previous reports indicating that NOX-I and NOX-II as NADPH oxidases that produce ROS and have been implicated in mediating blood pressure and arterial stiffness in knockout mice. In this study, the normalization of blood pressure was accomplished by reducing NOX-I medicated vascular ROS production which highlights the therapeutic target of NOX-I for vascular elastin insufficiency and blood pressure [44]. Other studies also provide evidence in the mice model indicating the importance of NOX-IV in aging related cardiovascular diseases through mitochondrial oxidative stress [45]. In addition, an earlier report has demonstrated that NOX2 facilitates vitamin D induced vascular regeneration in knockout mice models. The mechanism was attributed to the activation of MAPKs which is known to support the cytokine stem cell derived factor (SDF1) induction [46]. Furthermore, Hypoxia-inducible factor-1 (HIF-1) was reported as an inducer for vascular smooth muscle cell calcification which is a common complication of chronic kidney disease [47]. HIF-1 in our result was in the top five several differential pathways, and the validation at both gene and protein expression levels were significantly higher in vitamin D deficiency and obese individuals compared to the control healthy group.

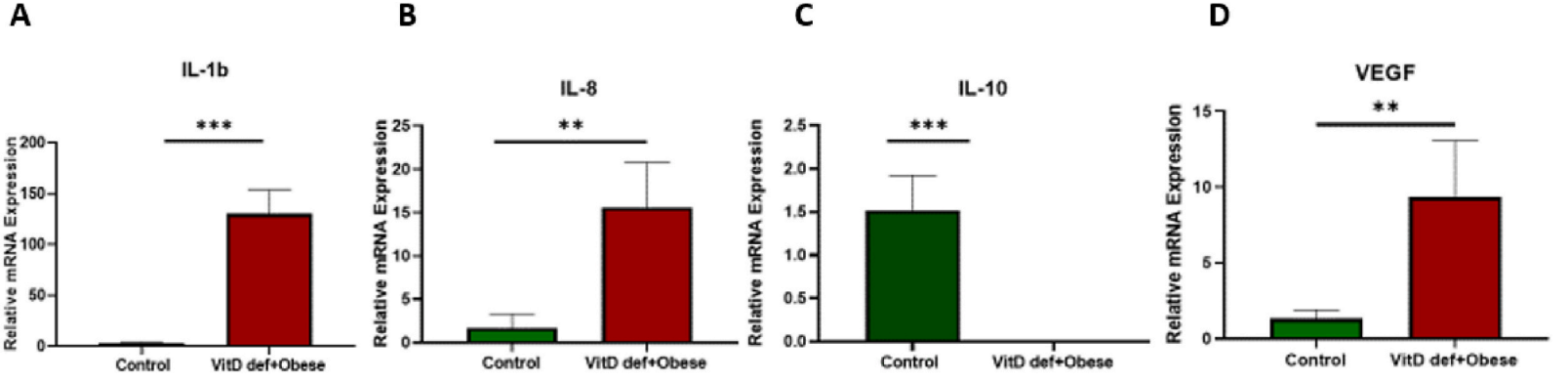

Fig. 7. Relative gene expressions of inflammatory and angiogenesis markers A-D (Il-1b, IL-8, IL-10, and VEGF) in patients with vitamin D deficiency and obese (A-group) compared to healthy individuals with normal level of Vit. D and BMI, control group (C-group). Values are expressed as mean ± SEM; (n = 9). Analyzed using unpaired two-tailed Student t-test. *P ≤ 0.05, **P ≤ 0.01 ***P ≤ 0.001.

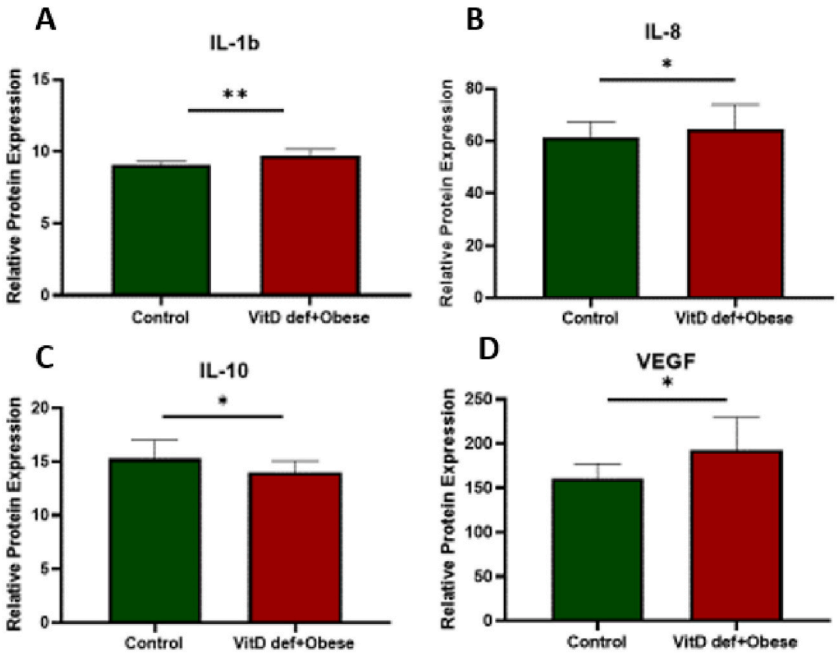

Fig. 8. Relative proteins expressions of inflammatory and angiogenesis markers A-D (Il-1b, IL-8, IL-10, and VEGF) in patients with vitamin D deficiency and obese (A-group) compared to healthy individuals with normal level of Vit. D and BMI, control group (C-group). Values are expressed as mean ± SEM; (n = 9–11). Analyzed using unpaired two-tailed Student t-test. *P ≤ 0.05, **P ≤ 0.01.

Our results have some strengths and limitations. It is the first study, to the best of our knowledge, that directly links the mechanism of arterial stiffness in vitamin D deficiency and obese Emirati individuals to oxidative stress makers especially HIF-1alpha, NOX-I, and NOX-II. Also, the link to inflammatory markers such as IL-1b, IL-8, IL-10, and VEGF was demonstrated. One of the main limitations of our study is the relatively small sample size, which can be attributed to the narrow criteria used for participant inclusion and exclusion. To establish a more comprehensive understanding of the direct correlation and evidence between arterial stiffness and the inflammatory and oxidative stress pathways in these conditions, further studies are necessary. Additionally, future research should consider investigating several other oxidative stress and inflammatory biomarkers that were not included in this study. Furthermore, it is important to explore other markers associated with arterial stiffness, such as MMPS, collagen, anti-fibrin, and endothelin, in future studies. Moreover, the contribution of other risk factors, including coagulation, various vitamins, and minerals, to arterial dysfunction should also be investigated.

Overall, our results revealed several differentially expressed genes and two specific signaling pathways that contribute to the development of arterial stiffness in individuals with both vitamin D deficiency and obesity. These findings suggest that oxidative stress and inflammatory biomarkers may hold promise as potential targets for both diagnostic and therapeutic interventions aimed at mitigating early vascular damage and the subsequent risk of cardiovascular diseases in this high-risk population. Nevertheless, further, and larger studies are warranted to study the oxidative stress and inflammatory pathways and biomarkers associated with arterial stiffness in these highly risk individuals.

5. Ethics statement

This study underwent a comprehensive review and obtained approval from both the University of Sharjah Research Ethics Committee (Reference number REC 16-11-12) and the ethics committee of University Hospital Sharjah (UHS). The study procedures strictly adhered to the principles outlined in the Declaration of Helsinki. Prior to participating in the study, all patients/participants provided written informed consent.

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Data availability

The raw RNA-seq data are deposited to an online repository and can be accessed from DOI: 10.6084/m9.figshare.22,067,042.

CRediT authorship contribution statement

Adel B. Elmoselhi: Conceptualization, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **Amal Bouzid:** Data curation, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. **Mohamed Seif Allah:** Conceptualization, Formal analysis, Methodology, Validation. **Zeinab Ibrahim:** Data curation, Formal analysis, Methodology, Writing – original draft. **Khuloud Bajbouj:** Formal analysis, Methodology, Validation, Writing – original draft. **Rebal S. Abou Assaleh:** Data curation, Formal analysis, Methodology. **Thenmozhi Venkatachalam:** Data curation, Formal analysis, Methodology, Software. **Mohamed Madkour:** Data curation, Formal analysis, Methodology, Software. **Ruqaiyyah Siddiqui:** Formal analysis, Investigation, Validation, Writing – review & editing. **Naveed Ahmed Khan:** Formal analysis, Investigation, Validation, Writing – review & editing. **Rifat A. Hamoudi:** Conceptualization, Formal analysis, Investigation, Software, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22067.

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