Original Communication

Association of vitamin D receptor polymorphisms with cardiometabolic conditions in Pakistani population

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Abstract: Apart from bone related effects, vitamin D has roles in immune modulation, hypertension, diabetes and cardiovascular diseases. Metabolic functions of vitamin D are mediated after binding with vitamin D receptor (VDR). *VDR* polymorphisms affect its physiological functions. Several *VDR* single nucleotide polymorphisms (SNPs) were reported previously. However, VDR polymorphisms causing influence on cardiovascular and metabolic disorders have not been investigated in the Pakistani population so far. Therefore, the present study was conducted to evaluate the role of *VDR* polymorphisms (rs2228570 and rs7975232) in the pathobiology of cardiometabolic disorders. In total, 400 cardiometabolic patients and 226 healthy control human adults were enrolled from Faisalabad, Pakistan. Biochemical parameters (serum glucose, liver function test, renal function test and lipid profile) were analyzed by standard kit methods. Genetic analysis was done by ARMS-PCR assay. Data was analyzed in SPSS v20. Regression analysis revealed that GG and AG genotypes of rs2228570 A>G polymorphism significantly increased the risk of hypertension in cardiovascular patients by 5.29 and 5.94 times respectively (GG: OR=5.29, 95% CI=1.63–17.2, **p=0.005**; AG: OR=5.94, 95% CI=1.70–20.7, **p=0.005**). However, rs7975232 C>A polymorphism was not correlated with cardiometabolic conditions. In conclusion, GG and AG genotypes of *VDR* SNP rs2228570 significantly contribute to hypertension in cardiovascular disease patients.

Keywords: rs7975232 *VDR* SNP, rs2228570 *VDR* SNP, Tetra ARMS-PCR, vitamin D, cardiometabolic disorders

Introduction

Cardiometabolic disorders (CMDs), a cluster of non-communicable metabolic conditions include diabetes mellitus (DM), hypertension (HTN), dyslipidemia, obesity and cardiovascular diseases (CVD). In CMDs, the co-occurrence of CVD and DM is very high [1]. Subjects with CMDs are 2-fold more likely to die from coronary heart disease. Non-communicable diseases (NCDs) account for almost 41 million (71%) annual deaths worldwide, where 31%, 13.5% and 3% of the total deaths are linked to CVD, HTN and DM respectively. In case of Pakistan, 58% deaths are due to NCDs, out of which 31% are attributed to CVD [2]. While, ∼46% Pakistani population is hypertensive [3]. According to an estimate, in Pakistan diabetes prevalence is ∼26%, however, this number exceeds 40% by including prediabetes [4]. Metabolic disorders are caused by variety of risk factors including unhealthy diet, sedentary life style and genetics. On the other hand, many biological molecules have protective roles against these diseases. One such factor is vitamin D, as its low levels are generally linked with such disorders [5] but its high levels are attributed with protective roles.

Vitamin D (Cholecalciferol) is an important micronutrient that is primarily synthesized under the skin. As a prohormone it is produced after absorbing UVB rays (290–315 nm) of sunlight by 7-dehydrocholesterol. Vitamin D is transported to the liver with the help of vitamin D binding protein where its hydroxylation leads to the formation of calcifediol (25-hydroxyvitamin D). The second hydroxylation reaction is carried out in the kidney to produce calcitriol (1,25 $(OH)_2D$), biologically active form of vitamin D [6].

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Vitamin D is well known for calcium (Ca^{+2}) and phosphorous (P) absorption, while its non-classic effects on CVD, hypertension, cancer, obesity and diabetes have also been reported. It actively participates in the regulation of cardiovascular system through Renin Angiotensin Aldosterone System (RAAS). Renin gene expression is suppressed by the activity of [1, 25$(OH)_2$D]. Studies revealed that vitamin D receptor (VDR) and 1α-hydroxylase knockout mice are at increased risk of hypertension due to a higher expression of renin and Ang II production. VDR and 1α-hydroxylase ameliorate the arterial hardening and endothelial dysfunction by the up regulation of endothelial nitric oxide synthase that produces nitric oxide [7]. Another study reported that vitamin D treated human HepG2 cell line showed marked down regulation of AT1 receptor (key component of RAAS in addition to renin) [8]. Additionally, vitamin D plays a role in the regulation of glycemic index. Vitamin D-VDR complex can directly enhance the expression of insulin receptor (INSR) gene after binding to vitamin D response elements (VDREs) present on promoter region of INSR gene, or by the activity of 1α-hydroxylase within β-cells. Vitamin D exerts indirect effect via Ca^{+2} flux to β-cells. Ca^{+2} causes the insulin release, muscle contraction and glucose uptake by increasing the activity of glucose transporter 4 (GLUT4) channels in the cells. It can also enhance the production of insulin sensitizing hormone adiponectin and maintain the energy expenditure [9].

Vitamin D-VDR complex performs its physiological role after binding efficiently with retinoid X receptor (RXR) to form heterodimeric complex or with other VDR to form homodimer complex. These complexes further interact with VDREs to expose the DNA to be transcribed efficiently. Hence, modulates different genes at transcriptional level [10]. Liver disease, nephrotic syndrome and genetic variations (polymorphisms/mutations) in *VDR* gene interrupt the normal function of the vitamin D. Furthermore, due to some single nucleotide polymorphisms (SNPs), VDR is no longer able to interact with RXR effectively, hence fail to modulate expression of various genes. Such genetic variations could lead to biochemical pathologies and thus predispose subjects to cardiometabolic disorders.

To date ApaI, FokI, BsmI and TaqI are extensively studied *VDR* polymorphisms globally that span the *VDR* region between exon 2 to 9. In current study, rs2228570 (FokI):A>G and rs7975232 (ApaI):C>A polymorphisms are analyzed. rs2228570 is a start codon variant located at 5′-end of *VDR* and its translation product is one amino acid truncated as compared to normal protein, while rs7975232 is an intronic variant located at 3′ UTR region [11].Various studies have been reported for the association of these SNPs with cardiometabolic disorders. It has been found that rs7975232, rs2228570 and vitamin D deficiency are the risk factors of CVD [12]. Similarly, rs2228570 has been associated with essential hypertension [13]. Furthermore, positive correlation has been reported between rs7975232 and T2D [14]. Protective effects of rs7975232 and rs2228570 in coronary artery disease have been reported [15]. Jun et al. demonstrated a significant risk association for rs2228570 and metabolic syndrome in Henan population of China [16].

In Pakistan despite sufficient sunlight availability, people are generally vitamin D deficient and the rate of cardiovascular diseases and associated metabolic comorbidities is high. Moreover, Pakistani population has not been previously studied for impact of these polymorphisms on cardiovascular and metabolic health conditions. In this context, the present study was designed to estimate the biochemical parameters and measure genotypic and allelic frequencies of *VDR* polymorphisms amongst cardiovascular, diabetic and hypertensive patients and healthy subjects in a Pakistani Punjabi cohort.

Materials and methods

Study subjects and sample collection

Patients with Cardio-Metabolic Disorders (CMD) were recruited from the Allied Hospital and Faisalabad Institute of Cardiology (FIC), Faisalabad, Punjab, Pakistan. Total 626 human subjects were enrolled for the current study, these were divided into two groups; patients=400; controls=226. Sample size was calculated by WHO sample size calculator by giving level of confidence (95%), error margin (0.05) and prevalence of risk factor (0.46). Calculated sample size was 381, however, for current study slightly higher sample number (n=400) was collected along with 226 controls. The estimated effect size was ρ=0.14 and power of study calculated by G*power software was 0.95, which shows that the current study is adequately powered to detect the effect of the targeted polymorphism.

All the cardiac patients (with and without hypertension and type 2 diabetes) and healthy control human subjects were recruited from Faisalabad city of Pakistan. Patients who had systolic /diastolic blood pressure (SBP/DBP) higher or equal to 130/80 mmHg (as defined by AHA 2017) and/or already taking anti-hypertensive therapy were considered. Similarly, diabetic patients who were taking anti-diabetic medication were also considered. Patients and healthy control subjects were of both genders.

Exclusion criteria

Subjects younger than 35 years were not included. Moreover, individuals with any type of cancer, chronic infectious diseases and type 1 diabetes were also kept out from the study cohort.

Table 1. Primer sequences and product size for tetra-ARMS-PCR assay of rs7975232 and rs2228570 polymorphisms

SNP	Primer name	Primer sequence (5′–3′)	Product size
rs7975232 (C>A)	FO	GCCACAGGCTGTCCTAGTCAGGAG	692 bp
	FI	AAGGCACAGGAGCTCTCATCTGGACC	(control band)
	RO	GTCTGGGCTACAGGGTAGAGTGTTGC	389 bp (C allele)
	RI	GGGGTGGTGGGATTGAGCAGTGAAGT	329 bp (A allele)
rs2228570 (A>G)	FO	CACATGTAGGTGCTGAGGCTGAGGG	710 bp
	FI	GTGGCCTGCTTGCTTCTTACAGGTAC	(control band)
	RO	TGCAAGGGCTCCCCATGGAAACACCT	512 bp (A allele)
	RI	GAAGTGCTGGCCGCCATTGCCTACA	252 bp (G allele)

Ethical approval

This study was approved by the institutional (National Institute for Biotechnology and Genetic Engineering, NIBGE, Faisalabad, Pakistan) ethical review committee. Oral and/or written informed consent was obtained from all subjects. Moreover, a questionnaire regarding anthropometric and demographic details and medical history was filled for all subjects.

Blood samples (6 mL) were collected by aseptic method from all subjects and each sample was divided into two vacutainers for biochemical and genetic analysis.

Biochemical analysis

Biochemical analysis of blood glucose, cholesterol, triglycerides (TG), High density lipoprotein (HDL), Low density lipoprotein (LDL), Liver function tests (LFTs) and Renal function tests (RFTs) were carried out using a semi-automated clinical chemistry analyzer (MicroLab-300, Merck, Germany) by following manufacturer's recommended protocols and conditions. Kits for biochemical analysis were obtained from commercial vendor (Merck, Germany).

Genetic analysis

For genetic analysis, blood was taken in ethylenediaminetetraacetic acid (EDTA) containing vacutainer while gel coated vacutainer was used for separation of serum to assess the level of biochemical variables. For analysis of *VDR* polymorphisms, DNA was extracted from all samples by an organic Phenol-chloroform isoamylalcohol (PCI) method.

Analysis of VDR polymorphisms

All collected samples were analyzed for polymorphisms in *VDR* [rs7975232: C>A; rs2228570: A>G]. Genotyping of these polymorphisms was done using tetra Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra-ARMS-PCR). Allele specific primers for genotyping of these SNPs were designed using freely available Primer1 software [http://primer1.soton.ac.uk/primer1.html]. Oligo Analyzer was used for the analysis of melting temperature, GC content, heterodimers, homodimers, hairpin loops etc. [https://eu.idtdna.com/pages/tools/oligoanalyzer]. *In silico* PCR analysis was carried out using *In silico* PCR of UCSC genome browser [https://genome.ucsc.edu/cgi-bin/hgPcr]. Detail of primers is provided in Table 1.

The optimized PCR reaction conditions for genotyping of the selected *VDR* polymorphisms were: one cycle of initial denaturation (95 °C/5 min) with a final extension (72 °C/7 min) along with 30 cycles of denaturation at 95 °C for 1 min, extension at 72 °C for 1 min, whereas annealing was done at 61 °C and 64 °C for rs7975232 and rs2228570, respectively, in T100^{TM} thermal cycler (Bio-Rad Laboratories, Inc. California, USA).

Total volume of each PCR reaction mixture was 30 µl that was prepared in 200 µl PCR tube. The reaction mixture included 100–200 ng/µl of genomic DNA, 3 µl 10X PCR buffer (750 mM Tris-HCl, pH 8.8), 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.2 µM of each primer, 1.6 U Taq Polymerase (Fermentas, EU), and PCR water. Agarose gel (2%) was prepared in 1X tris-acetate (TAE) buffer with the addition of 3 µl ethidium bromide (10 mg/ml). The amplified PCR product was allowed to run on gel for 40 minutes and the bands were observed under UV light in Gel Doc^{TM} EZ System (Bio-Rad Laboratories, Inc. California, USA).

Analysis of rs7975232 C>A and rs2228570 A>G *VDR* gene polymorphisms was carried out by Tetra-ARMS-PCR based assay. Amplification bands of rs7975232 and rs2228570 genotypes are given in Figure 1.

Sequence analysis

Amplified DNA products were extracted from gel and 10 µl of extracted samples along with primers used for PCR were sent to Eurofins Genomics, Germany, for validation of in-house developed Tetra-ARMS PCR via Sanger DNA sequencing. The resultant chromatograms were interpreted using sequence viewer software Chromas 2.6.6. The chromatograms are given in Figure 2.

Statistical analysis

Statistical analyses of all the data were performed by SPSS.20. Normal distribution was checked by Kolomogrov-Smirnov test. An independent t-test was applied to analyze the differences in the biochemical and clinical parameters between both study groups. All the continuous variables were expressed as mean±standard deviation, while categorical variables were written as frequencies (percentage). Influence of various genotypes of selected polymorphisms on biochemical parameters was assessed by One-way ANOVA with Tukey post hoc test. Furthermore, Chi-square test was applied to investigate and to compare the genotypic and allelic frequencies of patient and control group. Gene counting method was used to calculate the allelic frequencies. In addition to this, multinomial logistic regression analysis was carried out to check the risk of cardiometabolic diseases associated with genotypes of studied *VDR* polymorphisms. P value <0.05 was taken as significant.

Results

In this study, all 400 patients had cardiovascular disease (CVD) with concomitant conditions of hypertension (CVD-HTN; n=334) and type 2 diabetes (CVD-DM; n=140) in subsets.

Clinical and biochemical analysis

Clinical and biochemical parameters of all subjects were measured and given in the Table 2. Disease related association of these parameters was determined using independent sample t-test. Results showed significant differences between cases and controls for several variables. Blood pressure, liver enzymes (i.e. ALT, AST, and ALP), glucose, uric acid, creatinine and urea were significantly high in patients than the control subjects. Conversely, the concentrations of hemoglobin, total serum protein, LDL-cholesterol and triglycerides were significantly higher in controls as compared to patients.

Analysis of genotypic and allelic data of rs7975232:C>A and rs2228570:A>G Polymorphisms

Genotypic and allelic frequencies of rs7975232 C>A and rs2228570 A>G polymorphisms are given in Table 3. Chi-square test was performed to estimate the association of *VDR* polymorphisms with cardiovascular diseases (CVDs), cardiovascular disease with hypertension (CVD-HTN) and cardiovascular disease with diabetes mellitus (CVD-DM). Interestingly, for genotypic frequencies of rs2228570, statistically significant difference was observed between CVD-hypertensive and non-hypertensive subset among our studied cohort (χ^2=10.2, p=0.006). Higher number of GG genotypes (70%) and G alleles (84%) were found for CVD-HTN group. Whereas, the genotypic frequencies of rs2228570 A>G were not significantly different among CVD and control groups (χ^2=5.66 p=0.06). Similarly, no significant difference was observed between CVD-diabetes and non-diabetes subsets (χ^2=0.06 p=0.97).

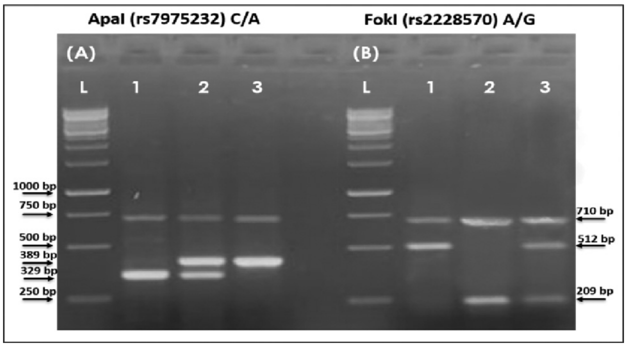

Figure 1. Tetra ARMS-PCR assay of *VDR* polymorphisms ApaI rs7975232 (C>A) & FokI rs2228570 (A>G). (A) Lane L: 1 kb DNA ladder. Lanes 1, 2, 3 show AA, CA and CC genotypes of rs7975232, respectively. (B) Lane L: 1 kb DNA ladder. Lanes 1, 2, 3 show AA, GG and AG genotypes of rs2228570, respectively. The amplification of required products was obtained following Tetra ARMS-PCR assay and the results were visualized by Gel Doc TM EZ System.

Furthermore, Chi-square results demonstrated that rs7975232 C>A polymorphism did not show any association with the pathogenesis of CVD (χ^2=0.72 p=0.39). Moreover, genotypic and allelic frequencies of rs7975232 did not find to be significantly different among CVD-HTN and CVD-DM subsets.

Logistic regression analysis of rs7975232:C>A and rs2228570:A>G Polymorphisms

Logistic regression analysis was performed for the risk assessment of rs2228570 A>G and rs7975232 C>A polymorphisms for CVD, CVD-HTN and CVD-DM. Results demonstrated that GG and AG genotypes of rs2228570 can increase the risk of hypertension by 5.29 and 5.94 times respectively (GG: OR=5.29, 95% CI=1.63–17.2, **p=0.005**; AG: OR=5.94, 95% CI=1.70–20.7, **p=0.005**) (Table 4). So, it could be inferred that rs2228570 G allele carriers have higher risk of developing hypertension as compared to A allele carriers.

Association of clinical and biochemical parameters with polymorphisms of VDR (rs7975232:C>A and rs2228570:A>G)

In order to evaluate the influence of rs7975232 C>A and rs2228570 A>G genotypes on the clinical and biochemical parameters of patient and control groups, one way-ANOVA with Tukey post hoc was performed (Table 5).

rs7975232:C>A analysis

One-way ANOVA results for rs7975232 C>A polymorphism indicated that among the control subjects, CC genotype carriers had significantly higher hemoglobin (15±2 g/dl, p=0.03). In addition to this, increased LDL-cholesterol level (75±11 mg/dl; p=0.03) was found in CA genotype

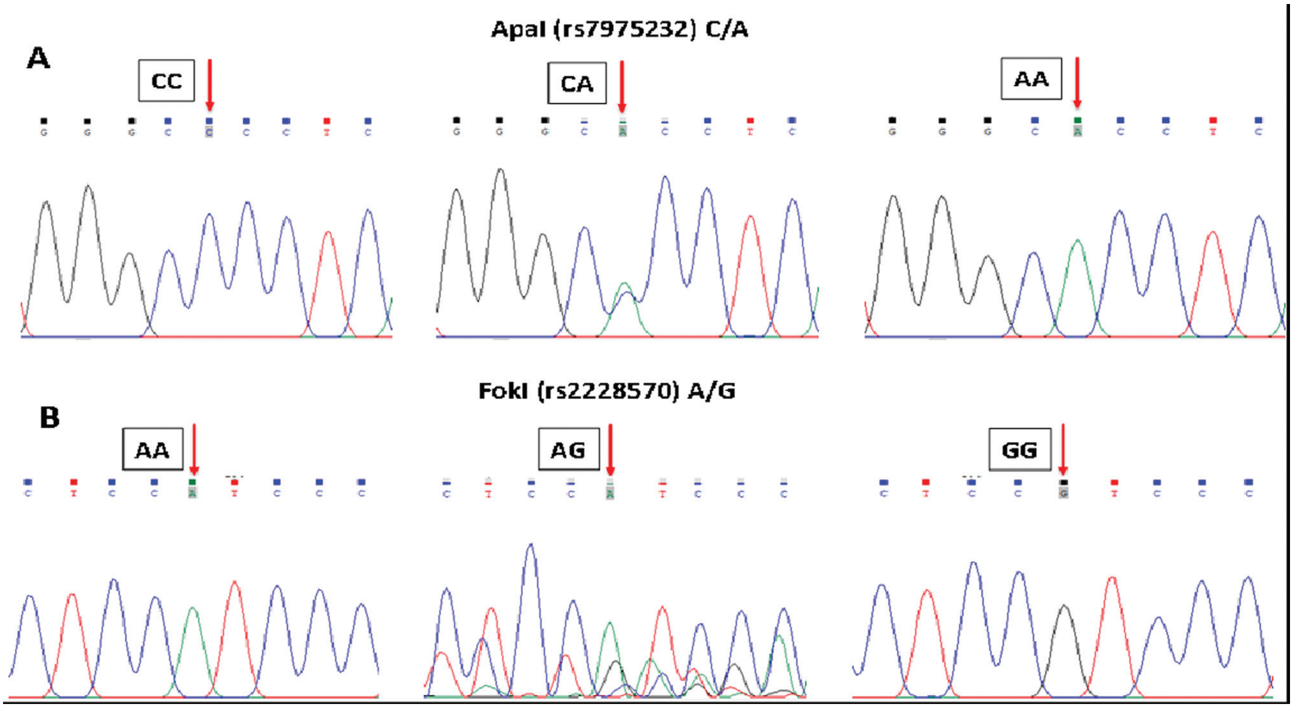

Figure 2. Electropherogram of vitamin D receptor gene polymorphisms. (A) CC shows wild type VDR genotype, CA shows heterozygous and AA shows altered VDR genotype of rs7975232. (B) AA shows wild type VDR genotype, AG shows heterozygous and GG shows altered VDR genotype of rs2228570. For a validation of in-house developed Tetra-ARMS PCR, Sanger DNA sequencing was performed through commercial vendor (Eurofins Genomics) and electropherograms were interpreted using sequence viewer software Chromas 2.6.6.

Table 2. Comparison of clinical and biochemical parameters between controls and patients

Parameters	Control (N=226)	CVD patients (N=400)	p value
Systolic BP (mmHg)	118±15	143±27	<0.001**
Diastolic BP (mmHg)	78±11	90±14	<0.001**
Hb (g/dl)	14±2	13±3	<0.001**
Glucose (mg/dl)	93±26	158±98	<0.001**
Uric acid (mg/dl)	5.9±2	7.1±3	<0.001**
ALT (U/L)	27±18	33±24	<0.001**
AST (U/L)	30±17	41±31	<0.001**
ALP (U/L)	160±59	227±89	<0.001**
Albumin (g/dl)	4.0±0.3	4.0±0.6	0.63
Protein (mg/dl)	7.2±1.0	6.2±1.1	<0.001**
Creatinine (mg/dl)	0.95±0.3	1.2±1.0	<0.001**
Urea (mg/dl)	27±10	42±28	<0.001**
Cholesterol (mg/dl)	186±45	178±58	0.07
HDL-C (mg/dl)	49±8.0	49±10	0.81
LDL-C (mg/dl)	83±11	73±11	<0.001**
Triglycerides (mg/dl)	260±132	204±86	<0.001**

Hb: Hemoglobin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; HDL-C: High density lipoprotein Cholesterol; LDL-C: Low density lipoprotein Cholesterol. *shows p value <0.05 using t test.

carriers. Moreover, patients with CC genotype have significantly higher HDL-cholesterol level (p=0.006) than the patients having CA genotypes. Furthermore, significantly higher level of creatinine (1.0±0.2 mg/dl, p=0.03) and uric acid (6.3±2 mg/dl, p=0.02) was found in healthy controls having CA genotype; however, these values fall in the normal ranges.

rs2228570:A>G analysis

Results showed that the GG genotype of rs2228570 carriers in the control group had significantly higher systolic blood pressure (SBP) as compared to AG and AA genotypes (121 ±15 vs. 113±15 and 123±6 mmHg; **p=0.007**) respectively. While for cases, AG genotype carrier subjects showed significantly elevated SBP level as compared to other genotypes (149±32 vs. 141±26 and 144±25 mmHg; **p=0.04**). Similar trend was observed for DBP, however, the difference was statistically not significant for cases. Furthermore, significantly increased level of LDL-cholesterol was observed in cardiovascular patients with GG genotype as compared to AG and AA genotype carriers (74±11 vs. 70 ±12 and 73±11 mg/dl; p=0.002), though these levels fall in the normal reference range but still showed association with the genotypes.

Discussion

Vitamin D when binds to its receptor (VDR: vitamin D receptor) in the body, it modulates multitude of

Table 3. Genotypic and allelic frequencies of VDR polymorphisms

					Patients			Patients		
Poly-morphism	Genotype/ Allele	Control N (%)	CVD Patient N (%)	Significance	Non-HTN N (%)	CVD-HTN N (%)	Significance	Non-DM N (%)	CVD-DM N (%)	Significance
rs7975232 (C>A)	Subjects	226 (100)	400 (100)	χ^2=0.89	66 (100)	334 (100)	χ^2=1.44	260 (100)	140 (100)	χ^2=0.11
	CC	69 (30.5)	128 (32)	p=0.64	25 (38)	103 (31)	p=0.49	83 (32)	45 (32)	p=0.94
	CA	88 (39)	164 (41)		26 (39)	138 (41)		108 (42)	56 (40)	
	AA	69 (30.5)	108 (27)		15 (23)	93 (28)		69 (26)	39 (28)	
	C	226 (50)	420 (53)	χ^2=0.72	76 (58)	344 (51)	χ^2=1.63	274 (53)	146 (52)	χ^2=0.02
	A	226 (50)	380 (47)	p=0.39	56 (42)	324 (49)	p=0.20	246 (47)	134 (48)	p=0.88
rs2228570 (A>G)	Subjects	226 (100)	400 (100)	χ^2=5.66	66 (100)	334 (100)	**χ^2=10.2**	260 (100)	140 (100)	χ^2=0.06
	AA	3 (1)	12 (3)	p=0.06	6 (9)	6 (2)	**p=0.006**	8 (3)	4 (3)	p=0.97
	AG	81 (36)	111 (28)		16 (24)	95 (28)		73 (28)	38 (27)	
	GG	142 (63)	277 (69)		44 (67)	233 (70)		179 (69)	98 (70)	
	A	86 (19)	135 (17)	χ^2=0.92	28 (21)	107 (16)	χ^2=2.12	89 (17)	46 (16)	χ^2=0.06
	G	366 (81)	665 (83)	p=0.34	104 (79)	561 (84)	p=0.15	431 (83)	234 (84)	p=0.81

CVD: Cardiovascular; CVD-HTN: Cardiovascular hypertensive; CVD-DM: Cardiovascular diabetics. Bold values depict the statistically significant difference between genotypic frequency among cardiovascular patient group with hypertension (CVD-HTN) and without hypertension (Non-HTN).

Table 4. Logistic regression analysis of VDR polymorphisms with disease status

		CVD	CVD-HTN	CVD-DM
Polymorphisms	Genotype	OR (95% CI), p value	OR (95% CI), p value	OR (95% CI), p value
rs7975232	CC	Reference	Reference	Reference
C>A	AA	0.84 (0.55–1.29), 0.45	1.51 (0.75–3.03), 0.25	1.04 (0.61–1.78), 0.88
	CA	1.01 (0.64–1.40), 0.78	1.29 (0.70–2.36), 0.41	0.96 (0.59, 1.55), 0.86
rs2228570	AA	Reference	Reference	Reference
A>G	GG	0.49 (0.14–1.76), 0.27	5.29 (1.63–17.2), **0.005**	0.91(0.32–2.57), 0.85
	AG	0.34 (0.09–1.25), 0.11	5.94 (1.70–20.7), **0.005**	0.86 (0.29–2.55), 0.78

CVD: Cardiovascular patients, CVD-HTN: Cardiovascular patients with hypertension, CVD-DM: Cardiovascular patients with diabetes. Bold values show that the respective genotypes are significantly associated with increased risk of hypertension.

physiological and biochemical actions [17]. Alterations in the expression or structure of VDRs due to genetic polymorphisms in the *VDR* gene can impair such vital biological processes. Several studies of *VDR* polymorphisms have been reported from different population for their association with several diseases. However, no previous data was available from Pakistan (a country with ample sunshine almost throughout the year) to show the impact of *VDR* genetic variants with cardiometabolic conditions (one of the largest causes of adult death and disability in Pakistan). Hence, the current study was conducted to investigate this association.

According to our results, the association study of serum biochemical parameters and genetic analysis of *VDR* SNPs [rs7975232 C>A and rs2228570 A>G] revealed that rs2228570 risk allele in the patient group is showing significant association with systolic blood pressure (p=0.04) and low density lipoprotein-cholesterol, LDL-C (p=0.002). Significant change in lipid profile of control subjects was also observed which is probably due to the non-fasting sampling conditions. Higher percentage of homozygous variant (GG) genotype was more prevalent among CVD-HTN patient cohort than non-HTN (70% vs. 67%). Furthermore, the difference between genotypic frequency was found to be statistically significant (χ^2=10.2, p=0.006).

Logistic regression analysis also showed that GG and AG genotype carriers had 5.29 and 5.94 folds higher risk for hypertension. This might be due to enhanced plasma renin activity. Renin needs cAMP response element binding protein-CREB binding protein (CREB-CBP/p300) complex formation for its synthesis. It is previously reported that vitamin D-VDR complex can physically bind to CREB, thus disturbing CREB-CBP/p300 complex and hindering renin synthesis [18]. Due to rs2228570 variant allele, increased biological activity of VDR has been observed that alter the binding energy of VDR for its ligand i.e. $1{,}25(OH)_2D$. This results in reduced stability of VDR and its functioning capability of maintaining blood pressure [19, 20]. In a meta-analysis it was described that almost 60% of previously reported studies showed an association of rs2228570 polymorphism with hypertension [21].

Table 5. Association of biochemical parameters with the *VDR* polymorphisms

		rs7975232 (C>A)				rs2228570 (A>G)			
Parameters	Group	CC	CA	AA	P value	AA	AG	GG	P value
SBP (mmHg)	Control	119±14	117±14	118±16	0.74	123±6	113±15	121±15	**0.007**
	Patient	143±27	143±28	144±25	0.93	144±25	149±32	141±26	**0.04**
DBP (mmHg)	Control	79±10	77±12	79±12	0.50	80±0.00	74±12	81±11	**0.003**
	Patient	90±15	91±15	89±11	0.63	88±9	93±15	89±15	0.17
Hb (g/dl)	Control	15±2	14±2	14±2	0.05	14±2	14±2	14±2	0.66
	Patient	13±3	13±2	13±3	0.72	13±2	13±3	13±3	0.93
Glucose (mg/dl)	Control	100±33	90±23	91±19	**0.03**	109±29	104±56	101±50	0.89
	Patient	155±90	161±104	159±97	0.87	180±132	162±109	156±91	0.64
Uric Acid (mg/dl)	Control	5.8±2	6.3±2	5.5±2	**0.04**	6.3±0.6	5.9±2.0	6.0±1.7	0.87
	Patient	7.4±2.5	6.9±2.5	7.1±2.9	0.29	6.4±1.4	7.4±2.4	7.1±2.7	0.27
ALAT (U/L)	Control	28±20	27±18	25±16	0.61	30±18	28±17	26±18	0.71
	Patient	32±23	34±24	33±23	0.84	24±12	32±21	34±25	0.25
ASAT (U/L)	Control	32±17	30±19	29±16	0.59	35±12	31±18	30±17	0.56
	Patient	41±30	42±34	41±30	0.86	31±17	40±32	43±33	0.41
ALP (U/L)	Control	169±66	151±49	161±62	0.15	154±35	155±53	166±63	0.45
	Patient	227±93	225±79	229±100	0.93	219±64	215±80	236±116	0.26
Albumin (g/dl)	Control	4.0±0.3	4.1±0.3	4.0±0.4	0.70	4.0±0.0	4.0±0.3	4.1±0.3	0.87
	Patient	3.9±0.6	4.0±0.6	4.1±0.7	0.12	4.2±0.7	4.0±0.6	4.0±0.7	0.36
Protein (mg/dl)	Control	7.2±0.8	7.3±1.0	7.1±1	0.68	8.3±1.2	7.3±0.9	7.2±1.0	0.11
	Patient	6.2±1.3	6.3±1.0	6.3±1.0	0.83	6.4±1.0	6.2±1.1	6.3±1.1	0.73
Creatinine (mg/dl)	Control	0.9±0.3	1.0±0.2	0.9±0.2	**0.03**	1.0±0.0	0.97±0.2	0.97±0.3	0.83
	Patient	1.3±1.3	1.2±0.7	1.1±0.5	0.21	1.0±0.6	1.2±0.8	1.2±1.0	0.71
Urea (mg/dl)	Control	26±10	27±11	27±9	0.69	25±9	28±10	26±10	0.47
	Patient	47±35	41±27	41±23	0.12	37±17	45±35	42±27	0.57
Cholesterol (mg/dl)	Control	182±45	188±44	187±48	0.71	176±55	183±44	191±48	0.41
	Patient	176±54	182±60	174±57	0.49	164±42	175±44	180±60	0.54
HDL-C (mg/dl)	Control	48±8	50±8	48±9	0.18	44±7	49±9	49±8	0.54
	Patient	51±10	47±10	49±11	**0.006**	53±9	50±11	48±10	0.21
LDL-C (mg/dl)	Control	85±12	83±9	81±12	0.13	87±8	84±11	84±11	0.89
	Patient	72±11	75±11	72±12	**0.03**	73±11	70±12	74±11	**0.002**
Triglycerides (mg/dl)	Control	271±137	260±139	247±118	0.55	483±383	274±179	270±131	0.06
	Patient	187±80	212±91	211±83	**0.03**	199±93	206±103	207±93	0.96

Bold values represent that the respective biochemical parameters are significantly different among the genotype carriers for the studied polymorphisms.

In case of rs7975232, no significant difference of genotype and allelic frequencies was found between control and cases, showing no association with CVD, HTN or DM in the investigated cohort. This finding is in agreement with some earlier studies. However, controversies are always there that require further research in this area. Such as, Jiao and colleagues conducted a meta-analysis that included 1671 subjects (636 cases and 1035 control). They found a protective role of rs2228570 (GG genotype) against diabetic retinopathy among Chinese population. On the other hand, their study did not find any association of TaqI, BsmI and rs7975232 polymorphisms with diabetic retinopathy [22]. Whereas, in Han Chinese population, increased percentage of GG genotype was linked with lower risk of coronary heart disease [23]. Another research group performed a meta-analysis and reviewed that rs7975232 and rs2228570 did not show any association with coronary artery diseases [24]. In a study performed by Vimaleswaran et al. on 5160 British birth cohort, no effect of rs2228570 on 25(OH)D and cardiometabolic risk was observed [25]. Another study showed the protective effect of AG and increased risk effect of GG genotype of rs2228570 polymorphic variant against lumbar degenerative disc disease in Turkish case-control study [26]. Hajj and coworkers demonstrated the association of rs2228570 with triglycerides and high density lipoprotein-cholesterol (HDL-C) [27].

In accordance with our study outcomes, the case-control study described a significantly increased Diastolic BP in patients with altered rs2228570 polymorphism (p=0.002). Furthermore, this study examined negative correlation between 25(OH)D and Systolic BP (p=0.04), as well as lower 25(OH)D was associated with Ff genotype of rs2228570 (p=0.03) and Bb genotype of BsmI (p=0.02). Though, an inhibitory action of vitamin D on blood renin production is well documented. However, they did not observe any significant association between 25(OH)D and plasma renin activity and overall hypertension. Moreover, this study has a limitation of smaller sample number [28]. The administration of high dose of vitamin D resulted in significant increase in serum vitamin D level and beneficial effects on lipid profile, while other biochemical parameters did not show any significant improvement [29].

Another study conducted in North Indian population described positive role of rs7975232 and BsmI for prostate cancer while rs2228570 showed no association [30]. It is also confirmed the involvement of altered VDR expression in the progression of breast cancer and rs2228570 is ascertained to be linked with higher expression of cytoplasmic VDR [31]. A small study of 50 Mediterranean individuals tried to characterize the association of five *VDR* SNPs (BsmI, rs7975232, Cdx2, TaqI, rs2228570) with CVD patients having at least one risk factor; however, they did not assess any association for BsmI, rs7975232, Cdx2, TaqI polymorphisms with plasma 25(OH)D level and CVD. Though, rs2228570 variant was more frequently observed in subjects with CVD risk factors. However this study is not strong enough for follow up due to its smaller sample size along with tedious and relatively expensive genotyping assay [Restriction fragment length polymorphism (RFLP)] [32] as compared to Tetra ARMS-PCR based technique. Liu et al. carried out a study and they recruited maintenance hemodialysis (MHD) patients and they did not find a significant difference for Fok I, Apa I and Taq I polymorphisms among MHD with and without left ventricular hypertrophy, while they predicted the association of Bsm I genotype with cardiovascular disease [33]. In a recent preliminary research study the association of rs2228570 TT genotype has been found to be linked with higher risk of hypertension and heart failure [34]. An earlier study did not associate rs7975232 and TaqI polymorphisms with the pathogenesis of coronary artery disease in Egyptian male [35].

For Pakistani population, data that correlates the genetics of vitamin D receptor with cardiometabolic disorders is very scarce. However, in a recent study Ashraf and coworkers investigated a relation of rs7975232 and TaqI with type 2 diabetes but failed to find any significant association [36]. This result is in accordance with the present study outcomes that show no association of rs7975232 with cardiovascular disease, hypertension and diabetes. Recent studies revealed that rs7975232 polymorphic variation showed no association with the diabetes mellitus that is consistent to our results [37].

Apart from reasonably good results, there are some limitations of this study. Firstly, this is a small scale study conducted on Pakistani Punjabi population. Secondly, serum vitamin D level could not be estimated due to limited resources. Therefore, further large scale study is needed to determine the association of vitamin D receptor polymorphisms with serum vitamin D level. Moreover, the impact of vitamin D deficiency on pathogenesis of cardiometabolic disorders needs to be investigated. Thus it is hoped that ongoing research efforts would help to elucidate the impact of vitamin D in the management of non-communicable diseases such as cardiovascular problems, hypertension, obesity and type 2 diabetes mellitus.

Conclusions

This is one of the first studies conducted on a Pakistani cohort to evaluate the association of *VDR* polymorphisms (rs7975232 and rs2228570) with clinical and biochemical parameters accompanying cardiovascular diseases and associated complications such as hypertension and diabetes mellitus. The findings of our study conclude that GG and AG genotype of (rs2228570 A>G) increase the risk of hypertension by 5.29 and 5.94 times respectively.

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History

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Conflict of interest

The authors declare that there are no conflicts of interest.

Authors' contribution

H.F. and F.R.A. conceived this study. A.R.K. was also involved in the study design and manuscript drafting. A.B. and S.B. helped with patient sampling. H.F. M.H. and H.N.K. performed the data collection, experiments and analysis. H.F. prepared initial draft of manuscript. F.R.A. revised the manuscript critically for important intellectual content and all the co-authors finally approved the manuscript.

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