Association of vitamin D levels and polymorphisms in vitamin D receptor with type 2 diabetes mellitus

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Abstract. Type 2 diabetes mellitus (T2DM) is a leading cause of death. The prevalence of T2DM in countries of the Middle East and North Africa (MENA) region, including Jordan, is among the highest worldwide. The reason(s) behind the epidemic nature of T2DM in Jordan are unknown but warrant further exploration. Studies have indicated that T2DM could be influenced by diet and/or genetic background. Evidence suggests that numerous patients with T2DM are deficient in vitamin D. The activity of vitamin D on its target tissues may be influenced by single nucleotide polymorphisms (SNPs) in the vitamin D receptor (*VDR*) gene. It was therefore hypothesized that SNPs in *VDR* could modify the risk of T2DM. To test this hypothesis, 125 patients with T2DM were recruited along with 125 controls. The study subjects were genotyped for variations in rs2228570, rs1544410, rs7975232, and rs731236 SNPs in the *VDR*. The levels of 25-hydroxyvitamin D [25(OH)D] were measured from the serum. The analysis revealed that reduced 25(OH)D and age were associated with the risk of T2DM ($P<0.05$). Moreover, under a dominant inheritance model, the GG genotype of rs2228570 was revealed to increase the risk of T2DM in univariate and multivariate analysis ($P<0.05$). Additionally, a chromosomal block containing the GAAG haplotype of *VDR* SNPs increased the risk of T2DM (OR=1.909; CI: 1.260-2.891; P=0.0021). Collectively, the present study revealed that low levels of serum 25(OH)D and rs2228570 of the *VDR* gene are associated with the risk of T2DM.

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Introduction

The economic burden and the rapidly increasing prevalence of diabetes mellitus (DM) render it one of the major health care challenges of the 21st century. In 2015, ~415 million individuals were diagnosed with DM, with the majority of these patients living in low to middle-income regions, including countries of the Middle East and North Africa (MENA) region (1). Jordan is a developing country in the MENA region, and health statistics have revealed that DM is indeed a growing health problem in the country (2,3). The reasons behind the increase in DM prevalence in Jordan are not entirely understood. However, evidence refers to a major role played by a rapidly changing social structure in the country accompanied by Western-influenced dietary habits and a sedentary lifestyle (2). Regardless, the DM magnitude emphasizes the need for a national healthcare policy that aims to dissect the environmental and genetic causes behind the DM epidemic in Jordan and the MENA region.

DM represents a heterogeneous mixture of metabolic diseases which present themselves by a chronic elevation in blood glucose (4). The two most common types of DM include; type 1 (T1DM), caused by a near-complete absence of insulin resulting from an autoimmune attack on the β-cells of the islets of Langerhans (4); and the more frequent type 2 (T2DM), caused by failure of peripheral target tissues to elicit a response to circulating blood insulin (4). A high percentage of individuals affected with T2DM are obese (5), and an increase in total fat percentage of the body remains a major predisposing factor for insulin resistance (6). In addition to the role of diet and lifestyle, the genetic background in determining the risk of DM is receiving attention as of late (7), and several loci were found to be associated with the risk of T2DM in several patient cohorts (7-9).

Vitamin D, cholecalciferol, is a fat-soluble vitamin and a hormone (10). Vitamin D is synthesized in skin cells as a result of exposure to UV light (11). Vitamin D is mainly known for its traditional role in maintaining calcium homeostasis and bone health (12). Vitamin D deficiency is associated with

the development of metabolic bone diseases, i.e., rickets in children and osteomalacia in adults (13). In addition to its traditional role in maintaining bone health, vitamin D plays a vital role in several extra-skeletal processes (14). For example, vitamin D is increasingly recognized for its anti-proliferative (15), pro-differentiative (15), and immunomodulatory (16) activities. In the context of T2DM, several studies have revealed that vitamin D regulates the effect of insulin on target tissues (17,18).

Vitamin D elicits its action through binding to the vitamin D receptor (VDR), a member of the superfamily of transcription factors known as nuclear receptors (19). Nuclear receptors mediate their effect through transcriptional regulation of target genes (20). Generally, the activity of the VDR is governed by (i) the levels of the active form of vitamin D (1,25-dihydroxycholecalciferol) and/or (ii) the expression level of the VDR itself or its attendant co-factors (21). Moreover, several research groups have determined that VDR expression/activity is affected by genetic variation in the sequence of the *VDR* locus (22,23). In that regard, four single nucleotide polymorphisms (SNPs) in the *VDR* gene (rs2228570, rs1544410, rs7975232, and rs731236) were heavily investigated for their role in modulating the activity of VDR on its target tissues (24-26). SNPs in the *VDR* gene have been revealed to be associated with male infertility (27,28), psoriasis (29), and prostate cancer (30), all of which are conditions where vitamin D was demonstrated to lower the risk of the disease (31). Herein, it was investigated whether the serum levels of vitamin D are associated with the risk of T2DM in a Jordanian population. The same population was also used to assess whether *VDR* SNPs are associated with T2DM.

Materials and methods

Study design. This was a prospective case-control study. The study was approved by the Institutional Review Board (approval ID 92/118/2018) of Jordan University of Science and Technology (JUST; Irbid, Jordan). Study participants were required to sign a consent form prior to their enrollment. Subject recruitment and blood sample collection was performed from December 2018 to March 2019.

Subject description. A total of 250 subjects were enrolled in this study. A total of 125 subjects were already diagnosed with T2DM according to the American Diabetes Association (ADA) guidelines (32). Subjects with diabetes were patients actively treated for T2DM at the Endocrinology clinic of King Abdullah University Hospital (KAUH). A total of 125 non-diabetic subjects were recruited during their visit to the Family Medicine clinic of KAUH. The control subjects were matched to T2DM patients according to sex and Body Mass Index (BMI). Following a short interview, it was confirmed that the non-diabetic subjects did not complain of any of the usual symptoms associated with T2DM at the time of their recruitment. Moreover, non-diabetic subjects were requested to assess their fasting blood glucose (FBG) levels on two separate occasions to confirm the absence of T2DM. Pre-diabetes individuals with a repeated FBG of 100-125 mg/dl were excluded from participating in this study. Subjects with chronic kidney or liver disease which may interfere with vitamin D metabolism were excluded from the study. Subjects with Cushing's syndrome, polycystic ovarian syndrome, thyroid dysfunction or hyperprolactinemia, and subjects who indicated receiving any of the vitamin D pharmacological preparations (dihydrotachysterol, calcitriol, ergocalciferol, cholecalciferol) by mouth or topically (calcipotriene) for supplemental or therapeutic purposes (including the treatment of chronic skin conditions such as psoriasis) were also excluded from the study. All recruited subjects were of Jordanian descent.

Anthropometric measurements. During the visit of the subjects to KAUH, the height [measured in centimeters (cm)], weight [measured in kilograms (kg)], and waist circumference (WC; measured in cm) of the subjects were recorded. The height and weight were then used to calculate the BMI according to the following equation: BMI=weight (kg)/height^2 (m^2).

Blood sampling. Following a 12-h fast, 10 ml of blood was collected into an evacuated EDTA tube (AFCO), and 5 ml of blood was collected in a plain tube with a clot activator (AFCO). Blood in the EDTA tube was stored at 4˚C and was later used for DNA extraction, as explained below. Blood samples in plain tubes were centrifuged at 4,000 x g for 5 min at room temperature to separate the serum. Serum samples were stored at -80˚C for later use to measure glucose, total cholesterol, triglycerides, and 25-hydroxyvitamin D [25(OH)D] levels.

Biochemical measurements. Measurements of serum glucose, total cholesterol, and triglycerides were performed at the laboratories of KAUH. A delayed, one-step immunoassay (ARCHITECT 25-OH Vitamin D) was used to measure serum 25(OH)D levels. The kit was purchased from Abbott Laboratories (cat. no. 3L52). Measurements were performed as per the manufacturer's guidelines (33).

DNA extraction and genotyping. Whole blood stored in EDTA tubes was used for the extraction of genomic DNA. The procedure used QIAamp DNA Blood Mini Kits (cat. no. 51104; Qiagen GmbH). Following DNA extraction, the purity of DNA was evaluated spectrophotometrically using an ND-2000 Nanodrop (Thermo Fisher Scientific, Inc.). Four SNPs in the *VDR* gene (rs2228570, rs1544410, rs7975232, and rs731236) were evaluated for their association with T2DM. Genotyping of the SNPs was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The concentrations of the reagents used in the PCR reaction and the final reaction volume were as previously described (34). Specifically, the reaction mixture contained GoTaq® Green Master Mix (Promega Corporation), 5 ng of template genomic DNA and 0.4 μM primers (forward and reverse) in a final reaction volume of 20 μl. The following thermocycling conditions were used to run the PCR reactions: Initial denaturation at 95˚C for 2 min, followed by 30 cycles of denaturation at 95˚C for 2 min, annealing at 65˚C for 30 sec and extension at 72˚C for 30 sec and a final extension at 72˚C for 5 min. The sequence of the primers used to genotype each SNP are listed in Table I. The location of the SNP on the *VDR* gene, the size of the PCR product, the restriction enzyme used for genotyping, and the size of the fragments that resulted

Table I. Genotyping strategy of *VDR* SNPs.

SNP ID	Location	Forward primer 5' to 3' Reverse primer 5' to 3'	PCR Program	PCR product size (bp)	Restriction enzyme, incubation temperature and time	RFLP product (bp)
rs2228570	Exon 2	TTCTTCTCCCTCCCTTTCCA TGCAGAGGTGAACCACTAAC	95˚C, 30 sec at 61.1˚C and 1 min at 72˚C	487 bp	*Bcc*I, 25˚C for 60 min	GG: 487 bp AG: 487, 288, 199 bp AA: 288, 199 bp
rs1544410	Intron 8	TCTCCCTCTTCTCACCTCTAAC GGAAATACCTACTTTGCTGGTTTG	95˚C, 30 sec at 61.1˚C and 1 min at 72˚C	357 bp	*Bsm*I, 65˚C for 90 min	CC: 106, 230 bp AC: 336, 106, 230 bp AA: 336 bp
rs7975232	Intron 8	GGATCATCTTGGCATAGAGCAG GGATCCTAAATGCACGGAGAAG	95˚C, 30 sec at 65˚C and 1 min at 72˚C	322 bp	*Apa*I, 25˚C for 60 min	AA: 322 bp AC: 322, 178, 144 bp CC: 178, 144 bp
rs731236	Exon 9	GGCTAGCTTCTGGATCATCTT CCTAGGTCTGGATCCTAAATGC	95˚C, 30 sec at 65˚C and 1 min at 72˚C	342 bp	*Taq*αI, 65˚C for 60 min	AA: 342 bp AG: 342, 235, 107 bp GG: 235, 107 bp

VDR, vitamin D receptor; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

from restriction enzyme digestion are also listed in Table I. Restriction enzymes were all purchased from New England Biolabs. Following restriction enzyme treatment, the reaction mixture was run on a 3% agarose gel. The agarose gel was prepared directly in SYBR™ Safe DNA gel stain (cat. no. S33102; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. In brief, 10 μl of 10,000X SYBR™ Safe stain concentrate was added to 100 ml of 1XTAE buffer (Sigma-Aldrich; Merck KGaA). The aforementioned solution was then added to 3 g of powdered agarose and the mixture was heated in a microwave. Molten agarose was then used to cast gels. The gel was visualized under blue light. A representative gel image illustrating the genotyping strategy of each SNP is presented in Fig. 1.

Statistical analysis. Statistical analysis was performed using the Statistical Package for Social Studies (SPSS) software (version 23; IBM Corp.). Continuous variables were assessed using an unpaired Student's t-test. Discrete variables were evaluated using Pearson's chi-squared test. The association between allele or genotype categories of each SNP with the risk of T2DM was assessed using Pearson's chi-squared test. Differences in serum 25(OH)D levels among the genotype classes of each of the *VDR* SNPs were examined using one-way ANOVA test followed by Tukey's post hoc analysis. SHEsis software was used to run haplotype analysis (35). Multivariate regression analysis included the following variables: Age, BMI, WC, 25(OH)D, cholesterol, triglycerides, and genotype category of rs2228570 (under a dominant inheritance model). For all analyses, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Serum 25(OH)D is decreased in patients with T2DM. Baseline characteristics of the study subjects are presented in Table II. The analysis revealed that patients with T2DM were significantly older than the control subjects ($P<0.05$). However, no significant differences were revealed between patients with T2DM and controls with regard to sex distribution, BMI, or WC ($P>0.05$). Measurement of several analytes in the serum collected from the study subjects revealed that patients with T2DM had significantly higher levels of serum glucose ($P<0.0001$) but significantly lower levels of serum 25(OH)D ($P=0.0306$). Notably, in this population, both patients with T2DM and controls had a mean value of serum 25(OH)D below 20 ng/ml, a widely used cut-off value for vitamin D deficiency.

Association of rs2228570 in VDR with the risk of T2DM. Considering that serum 25(OH)D was significantly lower in patients with T2DM and that vitamin D elicits its response

Table II. Baseline characteristics of study subjects.

Variables	Controls n=125	T2DM n=125	P-value
Age (years)	49.33±7.75	58.60±9.62	<0.0001
Sex, n (%)			
Male	57 (45.6%)	57 (45.6%)	NS
Female	68 (54.4%)	68 (54.4%)	
BMI (kg/m^2)	31.95±5.40	31.35±6.02	0.4108
WC (cm)	111.10±11.34	109.08±11.82	0.1668
Triglycerides (mg/dl)	172.04±109.08	151.83±103.71	0.1347
Cholesterol (mg/dl)	196.19±61.12	196.84±56.58	0.9311
Glucose (mg/dl)	93.90±9.62	213.45±100.70	<0.0001
25(OH)D (ng/ml)	10.11±11.93	7.49±7.59	0.0306

Values for continuous variables are presented as the mean ± standard deviation. The P-values were calculated using the Student's t-test for continuous variables and Pearson's chi-squared test for discrete variable (sex). T2DM, type 2 diabetes mellitus; BMI, body mass index; WC, waist circumference; 25(OH)D, 25-hydroxyvitamin D; n, number; P-, probability; NS, not significant.

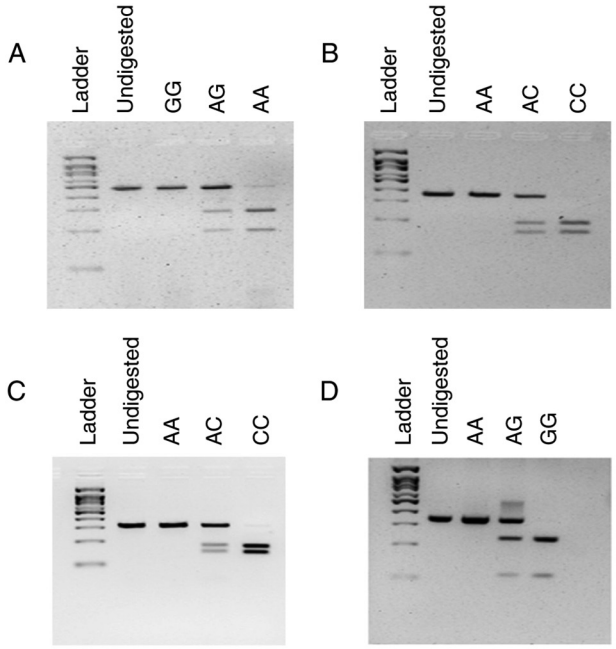

Figure 1. Genotyping strategy of VDR SNPs. A 3% agarose gel image of the different genotype classes of the (A) rs2228570, (B) rs1544410, (C) rs7975232 and (D) rs731236 SNPs of the VDR observed in the study subjects following PCR-RFLP strategy (the gel images were inverted for better clarity). VDR, vitamin D receptor; SNPs, single nucleotide polymorphisms; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

in target tissues via binding and activating VDR, the association of several SNPs in the *VDR* gene was assessed with regard to the risk of T2DM. A PCR-RFLP-based approach was used to determine the genotype of the study subjects for the following SNPs (rs2228570, rs1544410 rs7975232, and rs731236). The association of the alleles of each of the abovementioned SNPs with the risk of T2DM was first assessed. The results of this analysis are presented in Table III. The findings indicated that the frequency of the G allele of rs2228570 was higher in patients with T2DM than in controls, while the frequency of the A allele of rs2228570 was lower (P=0.0392).

Given this result, the association of genotype categories of each of the *VDR* SNPs with the risk of T2DM was then assessed. In this analysis, where only a co-dominant model of inheritance was evaluated, there was no significant (P>0.05) association between any of the genotypes of the tested SNPs with the risk of T2DM (Table IV).

The association of each of the SNPs with the risk of T2DM under three other inheritance models (dominant, recessive, and overdominant), was then examined. Using this approach, it was revealed that rs2228570 in the *VDR* gene was associated with the risk of T2DM under a dominant model of inheritance (P=0.0432; Table V). Specifically, under this inheritance model, the frequencies of AG-AA genotypes were lower in patients with T2DM compared with the control subjects (40.8% vs. 53.6%). Therefore, in this model, AG-AA genotypes reduced the risk of T2DM relative to the GG genotype (OR=0.597; CI: 0.362-0.984; P=0.0432; Table V).

In order to examine whether differences in the *VDR* genotype could be linked with differences in serum 25(OH)D, the study subjects were categorized according to their genotype class for each of the *VDR* SNPs. An ANOVA test was then performed to examine whether there were statistically significant differences in serum 25(OH)D between different genotype categories. This analysis was performed on control subjects only, case subjects only or both groups. None of the analyses revealed a significant difference in 25(OH)D between the different genotype classes for each of the *VDR* SNPs (P>0.05) (data not shown).

It was then examined whether genetic variation in rs2228570 SNP was associated with differences in serum glucose levels. To achieve this aim, study subjects were categorized according to their genotype class of rs2228570, and then it was determined whether there were significant differences in serum glucose levels between the different genotype classes. Since association of rs2228570 with T2DM was under a dominant model of inheritance, the study subjects

Table III. Allele frequencies of rs2228570, rs1544410, rs7975232, and rs731236 *VDR* SNPs in controls and patients with T2DM.

SNP ID	Allele	Controls n (%)	T2DM n (%)	P-value
rs2228570	G	166 (66.0)	187 (75.0)	0.0392
	A	84 (34.0)	63 (25.0)	
rs1544410	C	148 (59.0)	131 (52.0)	0.1258
	A	102 (41.0)	119 (48.0)	
rs7975232	A	151 (60.0)	162 (65.0)	0.3093
	C	99 (40.0)	88 (35.0)	
rs731236	A	162 (65.0)	146 (58.0)	0.1412
	G	88 (35.0)	104 (42.0)	

P-values were calculated using the Pearson's chi-squared test of association. *VDR*, vitamin D receptor; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; n, number; P-, probability.

Table IV. Genotype frequencies of rs2228570, rs1544410, rs7975232 and rs731236 *VDR* SNPs in controls and patients with T2DM.

SNP ID	Genotype	Controls (n=125) (%)	T2DM (n=125) (%)	P-value
rs2228570	GG	58 (46.4)	74 (59.2)	0.1248
	AG	50 (40.0)	39 (31.2)	
	AA	17 (13.6)	12 (9.6)	
rs1544410	CC	41 (32.8)	36 (28.8)	0.1559
	AC	66 (52.8)	59 (47.2)	
	AA	18 (14.4)	30 (24.0)	
rs7975232	AA	47 (37.6)	56 (44.8)	0.5106
	AC	57 (45.6)	50 (40.0)	
	CC	21 (16.8)	19 (15.2)	
rs731236	AA	51 (40.8)	44 (35.2)	0.2542
	AG	60 (48.0)	58 (46.4)	
	GG	14 (11.2)	23 (18.4)	

P-values were calculated using the Pearson's chi squared test of association. *VDR*, vitamin D receptor; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; n, number; P-, probability.

were categorized according to their genotype class into two categories instead of three; AA or AG genotype in one category and the GG genotype in the second category. It was then determined whether there were significant differences in the serum glucose levels between the study subjects of each category. No significant differences were observed between the two groups aforementioned (P>0.05) (data not shown). The same analysis was performed only on patients with T2DM, or only on the control subjects. No significant differences were observed in either analysis (P>0.05) (data not shown).

Age, sex, BMI, serum cholesterol, and triglycerides are confounding variables that could modify the association of low serum 25(OH)D or rs2228570 with T2DM. To adjust for these variables, a multivariate regression analysis was performed (Table VI). The results revealed that serum 25(OH)D remained associated with T2DM and reduced its risk (OR=0.997; CI: 0.994-0.998; P=0.0390). It was also demonstrated by this analysis that the rs2228570 SNP in the *VDR* gene remained associated with T2DM, where the AG-AA genotypes reduced the risk of T2DM relative to the GG genotype (OR=0.548; CI: 0.307-0.977; P=0.0410).

Next, to determine the presence of a significant interaction between any of the following environmental variables (age, BMI and WC) with rs2228570 that could modify the risk of T2DM in the study population, three interaction terms with rs2228570 were included in the regression model (one for each variable). The analysis demonstrated the absence of any significant interaction between rs2228570 with any of the aforementioned variables (P>0.05), with only a trend for the presence of a significant role for an interaction between rs2228570 with age in determining the risk of T2DM (P=0.06) (data not shown).

Association of two haplotypes in the VDR with risk of T2DM. Finally, the genotype data of all four SNPs were examined to explore the presence of any haplotype in the *VDR* gene associated with the risk of T2DM. Herein, two haplotypes were revealed to significantly (P<0.05) modify the risk of

Table V. Association of rs2228570 with risk of T2DM under different inheritance models.

Model	Genotype	Controls (%)	T2DM (%)	OR (95% CI)	P-value
Codominant	GG	58 (46.4)	74 (59.2)	1	0.1248
	AG	50 (40.0)	39 (31.2)	0.611 (0.356-1.051)	
	AA	17 (13.6)	12 (9.6)	0.553 (0.245-1.250)	
Dominant	GG	58 (46.4)	74 (59.2)	1	0.0432
	AG-AA	67 (53.6)	51 (40.8)	0.597 (0.362-0.984)	
Recessive	GG-AG	108 (86.4)	113 (90.4)	1	0.3256
	AA	17 (13.6)	12 (9.6)	1.482 (0.676-3.249)	
Overdominant	GG-AA	75 (60.0)	86 (68.8)	1	0.1470
	AG	50 (40.0)	39 (31.2)	0.680 (0.404-1.145)	

P-values were calculated using the Pearson's chi squared test of association. T2DM, type 2 diabetes mellitus; OR, odds ratio; CI, confidence interval; P-, probability.

Table VI. Regression analysis of the effect of study variables with the risk of T2DM.

Variables	OR	95% CI	P-value
Age	1.131	1.091-1.173	<0.0001
25(OH)D	0.997	0.994-0.998	0.0390
rs2228570			
GG (Reference)	1.000	-	-
AG-AA	0.548	0.307-0.977	0.0410

P-values were calculated using logistic regression analysis. T2DM, type 2 diabetes mellitus; OR, odds ratio; CI, confidence interval; 25(OH)D, 25-hydroxyvitamin D; P-, probability.

T2DM (Table VII). The first haplotype, ACAA, was less frequent in patients with T2DM and significantly reduced its risk (OR=0.346; CI: 0.147-0.812; P=0.0112), while the second haplotype, GAAG, was more frequent in cases with T2DM and significantly increased the risk of T2DM (OR=1.909; CI: 1.260-2.891; P=0.0021).

Discussion

The present study supports the theory that serum 25(OH)D or one of its direct or indirect metabolites or an effector downstream of the VDR modifies the risk of T2DM. Additionally, the findings of the present study indicated that genetic variations in the *VDR* gene itself were associated with the risk of T2DM. These findings aid in improving comprehension of the factors that modulate the risk of T2DM, in a Jordanian population. This is particularly important considering the magnitude of the pressure that T2DM places on the health and economic sectors of this developing country and the requirement for a national health policy plan to help manage the disease and its life-threatening complications.

One of the alarming findings of the present investigation was the low level of vitamin D among the individuals recruited to participate in the study. In fact, the mean value of serum 25(OH)D in both study groups was <20 ng/ml, a widely used cut-off value for vitamin D deficiency (36). There is an ongoing debate regarding the cut-off value for vitamin D deficiency. Several groups have recommended a new definition of vitamin D deficiency, where the cut-off value is lower than the widely used value of 20 ng/ml (37-39). Nonetheless, regardless of the cut-off, the existing evidence supports a relatively high prevalence rate of vitamin D deficiency in Jordan.

Although the sample size and geographic distribution of the subjects included in the present study were not representative of the population in Jordan, the results are in agreement with other investigations that assessed the levels of 25(OH) D across different age groups. For example, in a representative sample that included 4,056 subjects aged >17 years, El-Khateeb *et al* reported an overall prevalence of vitamin D deficiency of 89.7% (40). Moreover, Abdul-Razzak *et al* demonstrated a prevalence of vitamin D deficiency of 29% in a cross-sectional sample of 275 healthy infants and toddlers between 6 to 36 months (41).

Out of a possible 4,383 h, there are 3,602 h of sunlight per year in Jordan (42). Despite this value, the findings of the present study as well as those from the study by El-Khateeb *et al* (40) refer to a high prevalence of vitamin D deficiency in this Middle Eastern country. The exact reason behind this observation is currently not understood but may be explained by several factors. The dressing style in Jordan is largely conservative with a considerable percentage of women wearing either the veil or the niqab (40), a factor that reduces the skin area exposed to the sun. Furthermore, recent epidemiological data indicates that the current rate of obesity in Jordan is alarmingly high and is increasing (43). Obesity itself is associated with low levels of vitamin D (44) and may be contributing to the high prevalence of vitamin D deficiency in Jordan.

The high prevalence rate of vitamin D deficiency in Jordan and the conclusions of the present study linking vitamin D deficiency with T2DM strongly highlight the need to address this issue by the public health authorities. In addition to the association with T2DM, vitamin D deficiency has been linked with several diseases, including cancer (45), infertility (46), and metabolic syndrome (47). There is an eminent

Table VII. Haplotype frequencies of *VDR* SNPs, rs2228570, rs1544410, rs7975232 and rs731236 in controls and patients with T2DM.

rs2228570	rs1544410	rs7975232	rs731236	Frequency in control subjects	Frequency in patients with T2DM	OR (95% CI)	P-value
A	A	A	G	0.133	0.095	0.658 (0.376-1.152)	0.1407
A	C	A	A	0.081	0.031	0.346 (0.147-0.812)	0.0112
A	C	C	A	0.117	0.112	0.925 (0.533-1.606)	0.7821
G	A	A	A	0.053	0.049	0.881 (0.397-1.955)	0.7558
G	A	A	G	0.191	0.317	1.90 (1.260-2.891)	0.0021
G	C	A	A	0.128	0.151	1.172 (0.705-1.948)	0.5400
G	C	C	A	0.248	0.225	0.845 (0.558-1.280)	0.4265

P-values were calculated using the Pearson's chi squared test of association. *VDR*, vitamin D receptor; SNP, single nucleotide polymorphism; T2DM, type 2 diabetes mellitus; OR, odds ratio; CI, confidence interval; P-, probability.

need to initiate nationwide awareness campaigns that explain the dietary and environmental sources of vitamin D, the link between vitamin D deficiency and chronic diseases, and the relative safety and cost-effectiveness of vitamin D supplementation protocols in preventing vitamin D deficiency and its numerous public health implications. In this context, it is of note that a growing body of evidence indicates that vitamin D supplementation may be of utility in achieving better glycemic control in patients with T2DM. For example, in a previous study, it was recently reported that normalizing serum vitamin D levels in patients with T2DM decreases their HbA1c and serum glucose levels (48). These results are in agreement with a study by Alqudah *et al* which reported a similar observation (49).

Vitamin D elicits its response in target tissues through binding and transcriptional activation of its receptor (VDR). Genetic variation in the sequence of the *VDR* gene was reported to influence its activity. Considering the findings of the present study, associating vitamin D levels with the risk of T2DM, the association of several SNPs in the *VDR* gene with the risk of T2DM was investigated. The results of the genetic analysis revealed that the frequency of the major G allele of rs2228570 was higher in patients with T2DM than in control subjects. Furthermore, it was determined that the GG genotype of rs2228570 increased the risk of T2DM in a dominant model of inheritance in both univariate and multivariate analyses. Finally, the haplotype frequencies of all four *VDR* SNPs genotyped in the present investigation revealed that a specific haplotype containing the G allele of rs2228570 was more frequent in patients with T2DM and increased its risk. These results indicate that the major G allele of rs2228570 may be a high-risk allele for T2DM, in Jordan. This is consistent with the conclusion of a study by Angel *et al*, which also demonstrated using a case-control design, that the G allele of rs2228570 was a high-risk allele for T2DM in a Chilean population (50). However, the above-mentioned study only included older adults with an age range of 60-79 years. This finding is also comparable to a study by Safar *et al*, which revealed that the G allele of rs2228570 was significantly associated with the risk of T2DM in a population of the UAE, a Middle Eastern country with T2DM trends similar to Jordan (51).

Rs2228570 is a genetic variant found in the coding sequence of the *VDR* gene (22,52). Upon the translation of the resulting cDNA, this polymorphism alters the length of the VDR (22). Specifically, the presence of the G allele abolishes a translation initiation codon causing translation to start 9 bp downstream from the original initiation site (22). This results in a 424 amino acid protein instead of the 427 VDR (22). Previous *in vitro* data using cell reporter assays in transfected HeLa or COS-7 cells indicated that the shorter VDR protein, containing the G allele, has higher transcriptional activity (22). Notably, these findings were never replicated in any other cell line system. Additionally, there is no conclusive literature describing whether there are differences in the affinity of the shorter protein to its ligand [1,25(OH)D] (50).

The present investigation indicated that the G allele of rs2228570 is a high-risk allele of T2DM. This is in agreement with observations in an Emirati (51) and a Chilean (50) population as well as in a meta-analysis of 14 studies on Asian populations (53). However, the role of the G allele as a high-risk allele appears counterintuitive to the previously published data above, demonstrating the enhanced transcriptional activity of

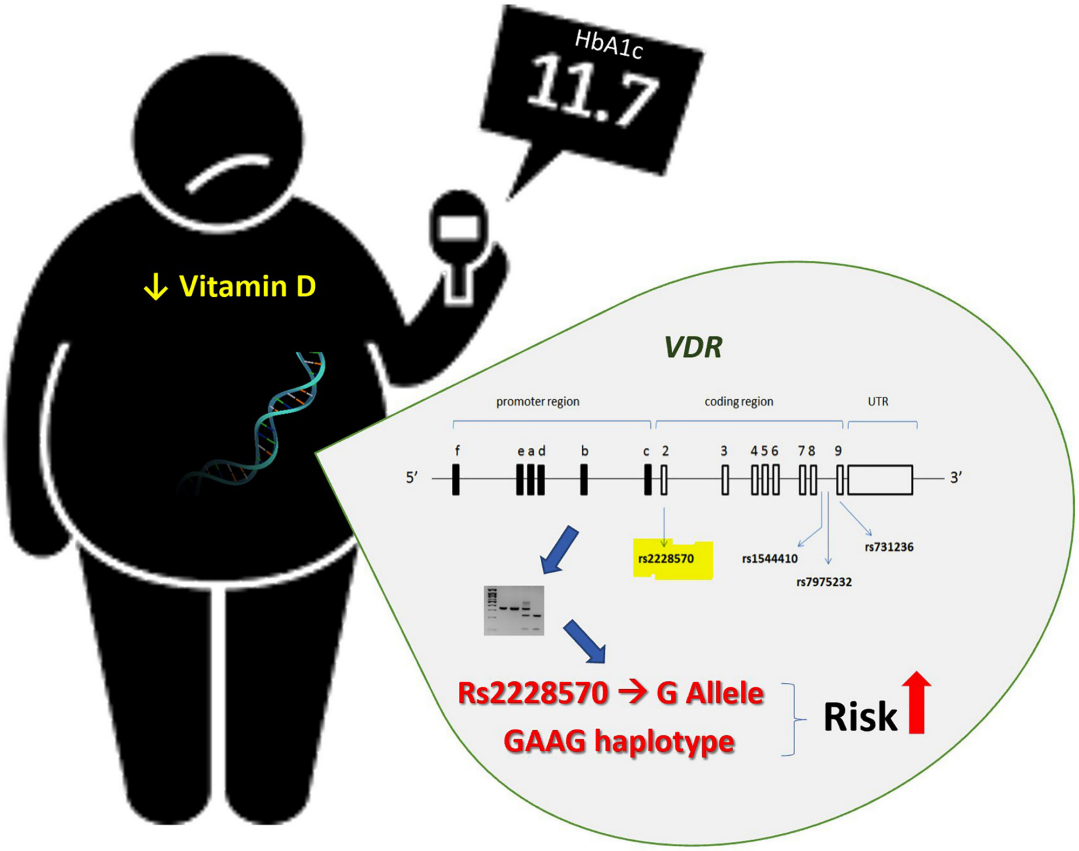

Figure 2. Graphical schematic summarizing a tentative role of genetic variations in the *VDR* in determining the risk of type 2 diabetes mellitus. *VDR*, vitamin D receptor.

the resulting VDR upon the presence of the G allele. This may be explained by the presence of another genetic variant in linkage disequilibrium with the G allele which modulates its effect specially in Asian populations. This should also be an invitation to evaluate the activity of the shorter 424 amino acid VDR in relevant pancreatic cell line models or conclusively determine its affinity to 1,25(OH)D.

The results of the present investigation as well as those of others clearly demonstrate that multiple factors play a role in determining the risk of T2DM. The risk of T2DM appears to be influenced by the complex interaction of a group of environmental (54), behavioral (55) and/or genetic factors (56). Consequently, the prevention of developing this disease extends beyond the simple recommendation of a better diet and lifestyle. Accordingly, given the complicated nature of this issue and the magnitude of the T2DM problem, public health policy decision-makers should adopt a well-rounded, holistic approach to reduce the risk of T2DM, including dietary, behavioral, and genetic counseling components.

In the present study, subjects recruited to the control group were matched to patients with T2DM as regards age and BMI. Considering that obesity is a well-established risk factor for T2DM (57), this resulted in subjects of the control group having a mean BMI value of 31 which indicates that numerous control subjects were obese. Recognizing that obesity itself is linked with lower vitamin D levels (44), this may explain the low levels of vitamin D observed in the control group. In fact, failure to include more control subjects with a normal BMI is a limitation of the present study.

The serum levels of vitamin D are affected by a plethora of factors, including age, dress style, latitude, activity of metabolizing enzymes, body fat distribution, and eating behavior (54,58). Data which reflect these factors were not collected. For example, another limitation of the present study was the failure to collect parameters that correspond to body fat distribution, such as waist-hip or visceral fat ratios. A third limitation was the inability to collect information on the eating behavior of the participants. These factors were demonstrated in previous studies to affect vitamin D serum levels (58,59).

In conclusion, the present case-control study demonstrated that decreased levels of vitamin D and genetic variation in the *VDR* gene were associated with the risk of T2DM (Fig. 2). Although several investigations across multiple populations have explored the association of rs2228570 with T2DM with inconsistent results, the present study is of significance as it reinforces the growing body of evidence of a possible ethnic variation of the role of rs2228570 or the VDR itself in the pathophysiology of T2DM. This is of particular interest considering that rs2228570 is a functional variant of the *VDR* gene with an established effect on VDR activity. Given the high prevalence of vitamin D deficiency in Jordan, the initiation of awareness campaigns that explain the sources of vitamin D and the implications of its deficiency on health and disease are strongly recommended.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAA and OAS conceived the study. MAA, AA and MK contributed to the methodology of the study. MAA and AA contributed to the validation of the results. MAA, AA and RS performed the formal analysis. MAA, AA and MK performed the experiments. MAA, ZA, RS and OAS interpreted the data. MAA, ZA and MZA obtained the resources required for the study. MAA, AA and MK performed the data curation. MAA, AA and MZA wrote the original draft of the manuscript. MAA and MZA wrote, reviewed and edited the manuscript. MAA, OAS and MZA supervised the study. MAA contributed to the project administration. MAA and MZA contributed to the funding acquisition. MAA, OAS and MZA confirm the authenticity of the raw data. All authors have read and approve the published version of the manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of Jordan University of Science and Technology and King Abdullah University Hospital Institutional Review Board (approval ID 92/118/2018) and with the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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