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Blood vitamin D levels in type- 2diabetesmellitusin Indonesia

by Elly Herwana

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Supplementary vitamin D3 and vitamin D receptor polymorphisms affect blood vitamin D levels in type-2 diabetes mellitus in Indonesia

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ABSTRACT

INTRODUCTION: There are no data on vitamin D receptor (VDR) gene single nucleotide polymorphism (SNP) influence on blood 25-hydroxy-cholecalciferol [25(OH)D] levels after supplementary vitamin D in Indonesian type 2 diabetes mellitus (T2DM) patients. This study evaluated the effects of the supplementary vitamin D3 and VDR gene SNPs rs1555410 and rs2228570 on blood 25(OH)D levels in T2DM cases.

METHODS: A randomized, double-blind placebo-controlled trial (RDPCT) was conducted at one research setting using 85 T2DM subjects divided into vitamin D group (VDG) and control group (CG) and receiving 5,000 IU/day vitamin D3 (cholecalciferol) or placebo once daily for 84 days. Levels of 25(OH)D were determined baseline and after supplementary vitamin D3 administration for 84 days. Circulatory 25(OH)D was assayed using ELISA. VDR polymorphisms were detected using sequencing.

RESULTS: Post-supplementary blood 25(OH)D rose appreciably from baseline in VDG for VDR rs1544410 genotypes G/G (p=0.001) and G/A (p=0.010), and in VDR rs2228570 genotypes T/T (p=0.012), T/C (p<0.001), and C/C (p=0.001). Post-supplementary VDG still contained 30.3% of subjects not reaching blood 25(OH)D ≥30 ng/mL.

In attaining blood 25(OH)D ≥30 ng/mL post-supplementation, VDR rs2228570 genotype T/C differed significantly from T/T (52.4% v. 100%; p=0.027), but there were no appreciable differences between genotypes C/C and T/T (78.6% v. 100%; p=0.273), as well as between VDR rs1544410 genotypes G/G and G/A (67.5% v. 100%; p=0.542).

CONCLUSION: Only 52.4% of subjects with VDR rs2228570 genotype T/C achieved sufficiently high blood 25(OH)D levels. VDR rs2228570 polymorphisms apparently influence T2DM response to supplementary vitamin D.

Keywords: Diabetes Mellitus, Vitamin D, Single Nucleotide Polymorphism, Indonesia

INTRODUCTION

Indonesia, with 10.7 million type 2 diabetes mellitus (T2DM) patients, occupies the 7th global rank, with predicted T2DM prevalence rising to 16.6 million in 2045 [1]. T2DM vitamin D deficiency prevalence is higher than in the general global population, with prevalences of 63.2- 83.2% [2,3].

The latter are higher than in Europe, with a vitamin D deficiency prevalence of 40.4% (blood 25(OH) D) <20 ng/mL) [4], while vitamin D deficiency prevalence in South Asia is 68% [5].

Vitamin D supplementation raises serum 25(OH) D concentration, influences health outcomes, and achieves maximum mortality rate reductions in developed countries from the commonest fatal diseases, such as cardiovascular disease and T2DM [6]. Gross vitamin D deficiency at 25(OH) D <12 ng/mL (or <30 nmol/L) is a health hazard [7] and should be corrected through vitamin D supplementation [8].

The utility of providing additional vitamin D to T2DM patients for glucose hemostasis and reducing insulin resistance is still debated, presumably because of the diversity of study populations for serum vitamin D status, supplementation dose and duration, methodology, gender, BMI, and ethnicity [9,10].

Vitamin D regulates signal transmission through the vitamin D receptor (VDR) [11]. The vitamin D-responsive element (VDRE) gene on chromosome 12q13.1 comprises nine exons and eight introns [11]. VDR activates and controls gene transcription via the target gene promoter VDRE. The VDR gene has over 470 polymorphisms [11], the most common being FokI (rs2228570 C to T) and BsmI (rs1544410 A to G)[11]. VDR rs1544410 at intron 8 regulates mRNA stability, thereby affecting gene expression [12]. VDR rs2228570 lies in the exon 2 start codon and changes the initiation sites [13]. VDR gene SNPs may influence VDR mRNA and protein stability and activity, resulting in a suboptimal response to supplementary vitamin D [14,15].

Multiple factors may affect post-supplementary blood 25(OH)D, such as sunshine exposure, aging, body mass index, calcium intake, supplementary vitamin D, and genetics [4,16]. It is currently uncertain whether VDR SNPs affect blood vitamin D levels after supplementary vitamin D in T2DM patients [17], due to difficulties in detecting gene involvement in the development of T2DM, because of possible minute differences in the gene and its interaction with genetic or non-genetic factors [18]. VDR SNP influence on increases in blood 25(OH)D levels that depict vitamin D condition, can be evaluated only with a randomized, double-blind placebo-controlled trial (RDBPCT).

Currently, few randomized double-blind placebo-controlled trials (RDBPCT) exist for evaluating VDR rs1544410 and rs2228570 relationships with vitamin D therapeutic response. Waterhouse et al. [14]. Studied Australian elderly aged 60 – 80 years receiving vitamin D3 supplementation (30.000 vs. 60.000 IU/month) for 12 months and found rs2228570 was not associated with increases in 25(OH)D levels after supplementary vitamin D. A 20-week Chinese RDBPCT [15] on vitamin D deficiency cases receiving vitamin D3 at 2000 IU/day or placebo, demonstrated that rs2228570 showed stronger influences on 25(OH)D levels ($p<0.04$). Post-treatment, VDR rs2228570-G, and its alleles had higher 25(OH)D levels ($p = 0.009$).

The RDBPCT of Cavalcante et al. [19]. on elderly females aged 68 ± 6 years with vitamin D insufficiency and receiving 200.000 IU vitamin D3 supplementation for 4 weeks, showed higher blood 25(OH)D concentrations in persons with BsmI genotypes BB/Bb ($p<0.001$) who responded better to supplementary vitamin D than those with bb genotype.

A prospective case-control study involving 125 T2DM patients and 125 controls, revealed that low blood 25(OH)D and gene rs2228570 correlated with T2DM risk [20]. The inconsistent study results on VDR SNP relationships were caused by variations in pre-supplementary blood vitamin D levels, study designs, small sample sizes, vitamin D dose, duration of supplementary vitamin D, dietary intake, and ethnicity.

There are currently no reports on the causal relationship of VDR SNPs rs1544410 and rs2228570 with supplementary vitamin D among Indonesian T2DM patients, for which an RDBPCT is necessary. The outcome may be useful for vitamin D3 therapeutic dose personalization in T2DM patients to reduce morbidity and mortality rates. Primary research outcomes would be responses to supplementary vitamin D by comparing post-supplementary blood 25(OH)D values. Secondary outcomes would be the impact of rs1544410 and rs2228570 polymorphisms on blood 25(OH)D changes and attainment of blood 25(OH)D of ≥30 ng/mL

METHODS

Research design

This RDBPCT was conducted from June to August 2022 at Puskesmas Mampang in South Jakarta, with subjects signing informed consent. Study protocol approval was by the Research Ethics Committee,

Faculty of Medicine, Universitas Trisakti, under No. 001/KER/FK/1/2022.

Patients and intervention

The study subjects were Mampang area residents with T2DM. Inclusion criteria: males and females ≥18 years old, T2DM, HbA1c 7-8.5%, oral antidiabetic drug monotherapy, agreeing to follow-up controls. T2DM was diagnosed using American Diabetes Association criteria [21], namely fasting blood glucose ≥126 mg/dL, or 2-hour postprandial blood glucose ≥200 mg/dL and HbA1c ≥6.5%. Exclusion criteria: previously and currently on insulin therapy, suspect kidney disease (estimated glomerular filtration rate <30 mL/min/1.73m2), abnormal liver function (SGPT 3 times normal upper limit), pregnant or lactating, allergy, hypercalcemia (plasma calcium >2.65 mmol/L), or receiving daily supplementary vitamin D in preceding 84 days. Dropout criteria: blood 25(OH) D >100 ng/mL, hypercalcemia, cholecalciferol hypersensitivity.

The study enrolled 115 subjects allocated by simple randomization to vitamin D (intervention) and control (placebo) groups at 1:1 ratio, which was conducted by personnel blinded to the intervention. VDG received daily vitamin D3 tablets containing 5,000 IU cholecalciferol, whereas controls received once-daily placebo tablets (120mg calcium), all tablets taken for 84 days. The vitamin D and placebo tablets contained in dark-colored glass bottles coded A and B were identical in visual, olfactory, and gustatory qualities. Participants and the statistician were all blinded to the origin of the tablets, whether from VDG or CG. Compliance with the intervention was determined by weekly counting the remaining tablets. VDG had 6 dropouts because of protocol non-compliance, returning to the home village, and diarrhea, while CG had 7 dropouts because of not agreeing to participate, protocol non-compliance, and nausea and vomiting. Subjects completing the study were 85 in number, consisting of 43 in VDG and 42 in CG (Figure 1).

The researchers were blinded to subject allocation in all study phases (recruitment, enrolment, data collection, and group assignment). For improving verification and compliance, all empty medication bottles were returned monthly to the cadres for evaluation of subject compliance to supplementation at the completion of the study. Subjects' complaints (potential adverse events) were noted for recording. The principal investigator evaluated the complaints and their connection with the supplements. The development of all reported symptoms was monitored until the study was completed. The allocation codes stored by an independent third party were opened after the study was completed.

Measurements

On day zero (admission date), before administration of vitamin D tablets, subjects meeting recruitment criteria were interviewed to collect subject data on age, gender, and duration of diabetes mellitus, followed by blood collection at 08.00 and 9.00 a.m. local time.

Biochemical measurements

From each participant, 5 mL of venous whole blood was collected, with 3 mL for blood 25(OH) D determination and 2 mL in EDTA tubes for VDR SNP genotyping.

Blood 25(OH)D level was determined by chemiluminescent microparticle immunoassay (ARCHITECT 25-OH Vitamin D assay, Abbott), with measuring interval 8.0- 160.0 ng/mL (20.0 - 400.0 nmol/L), limit of detection (LoD) ≤ 10.0 ng/mL, limit of quantitation (LoQ) ≤ 20 ng/mL, and imprecision ≤ 10% within total CV.

Blood 25(OH)D data were presented as median ± SD, and categorized according to Endocrine Society 2011 clinical practice guideline, with <20 ng/mL defined as vitamin D deficiency, 20 – 30 ng/mL as vitamin D insufficiency, 30 – 100 ng/mL as vitamin D sufficiency [8].

Vitamin D receptor single nucleotide polymorphisms

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN) from 200 µL EDTA blood and its purity and level were determined using a NanoDrop 2000 spectrophotometer (ThermoScientific). VDR SNPs were detected by PCR, followed by sequencing. PCR used MyTaq HS Red Mix Kit (Bioline) to amplify the target sequence using specific primers, namely for rs1544410: forward primer 5'- GGG AGT ATG AAG GAC AAA GAC C-3' and reverse primer 5'- CCC GCA AGA AAC CTC AAA TAA C -3' and for rs2228570: forward primer 5'-GCA CTG ACT CTG GCT CTG-3' and reverse primer 5'-TGG ACA TTG TAA GGA AGG AGA TG-3'. PCR amplification used 2 µl DNA, 8.5 µL nuclease-free water, 2x 12.5µl My Taq HS Red Mix,

and 1.0 µl each of forward and reverse primers. Conditions: initial activation at 95oC for 5 minutes, denaturation at 95oC for 15 seconds, annealing at 58oC for 30 seconds, extension at 72oC for 30 seconds for 40 cycles, and final extension at 72oC for 3 minutes. PCR products on 2% agarose gel were visualized by electrophoresis. Purified PCR products were sequenced using BigDye Terminator Kit (Thermo Fisher Scientific) and ABI 3500 sequencer (Applied Biosystems). Sequence analysis was performed with BioEdit software to confirm mutations, which were compared to NCBI BLAST database with accession number NG_008731.1.

Sample size

In each group, 40 subjects were required to detect a treatment difference at 5% two-sided significance level (0.05) and 90% study power. µ1 − µ2 = predicted between-group mean difference, estimated at 4; α2 = expected population variance from the preliminary study, estimated at 1.02. To anticipate dropouts, the sample size became 95 for adequate power to detect outcome measure differences.

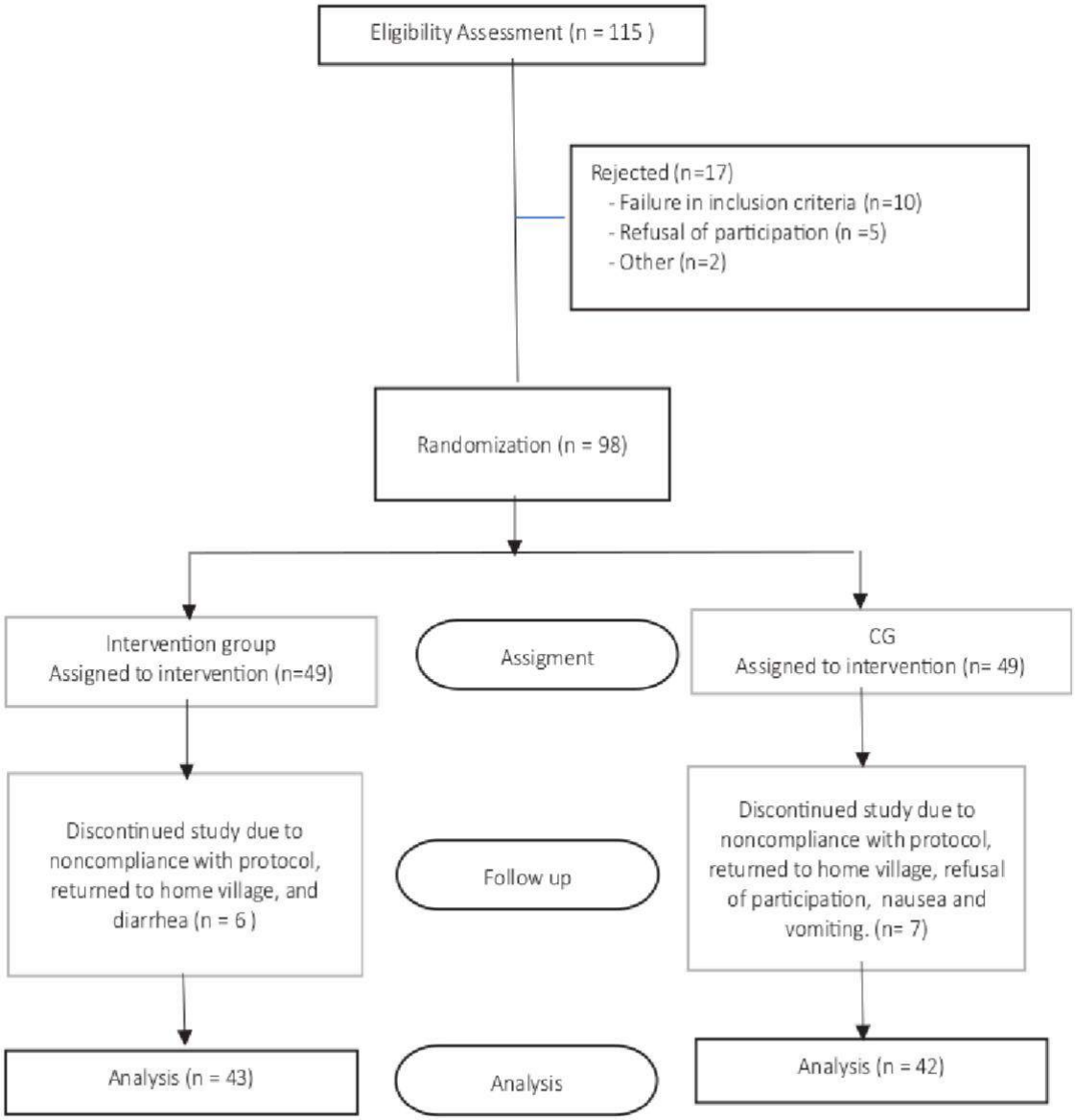

Figure 1: Flow of participants throughout trial

Statistical analysis

Continuous data of normal distribution were shown as mean and standard deviation (SD), continuous data of skewed distribution as median (minimum-maximum), and categorical data as percentages. Data were checked for normal distribution using the Kolmogorov-Smirnov test. Unpaired Student's t-test, Mann-Whitney U test, Chi-square test, and Fisher exact test were used for baseline comparisons between VDG and CG, based on the variable type and data distribution. Mann-Whitney test was used to compare blood 25(OH)D in VDG and CG at baseline and post-supplementation. Baseline and post-supplementation vitamin D status differences were evaluated using Fisher's exact test and Chi-square test. VDG baseline and post-supplementation blood 25(OH)D differences between rs1544410 genotypes were evaluated by the Mann-Whitney test. VDG baseline and post-supplementation blood 25(OH)D levels between rs2228570 genotypes were compared using the Kruskal-Wallis test, Wilcoxon signed rank test, and paired t-test at $p < 0.05$. Statistical analysis used SPSS for Windows version 23.

RESULTS

Baseline subject characteristics

We had 85 T2DM participants, with 68 (80%) females, mean age 55.8 ± 0.6 years, median T2DM duration 12 (1 – 36) months, and median blood 23(OH)D 11.6 (2.4 – 30.3) ng/mL. The most frequent rs1555410 genotype was G/G, comprising 79 subjects (92.9%), followed by G/A with 6 subjects (7.1%), without any T/T. Gene rs2228570 had genotypes T/C, C/C, and T/T with 38 (44.7%), 32 (37.6%), and 15 (17.6%) subjects, respectively. Vitamin D status was mostly deficient in 74 subjects (84.1%), insufficiency in 9 subjects (10.6%), and sufficiency in 2 subjects (2.4%). After group randomization, no significant differences were observed in age, gender, T2DM duration, and VDR genotype between VDG and CG (Table 1).

Baseline and post-supplementation blood 25(OH)D and vitamin D status

After vitamin D3 supplementation for 84 days, there was a much greater increase in VDG blood 25(OH)D than in CG (46(9.4-79.4) v. 14.4(6.9-38.3); ($p<0.001$) (Table 2).

Table 1: Subject characteristics at the start of the study in VDG and CG

Characteristic	Treatment group		P-value
	VDG (n=43)	CG (n=42)	
Age (years)	56 (35 – 80)	56 (35 – 69)	0.396[a]
Gender			
Male	8 (47.1)	9 (52.9)	0.745[b]
Female	35 (51.5)	33 (48.5)	
Duration of DM (months)	12 (1 – 36)	12 (1 – 36)	0.967[a]
VDR genotype (n,%)			
rs1544410			
G/G	40 (50.6)	39 (49.4)	1.000[c]
G/A	3 (50)	3 (50)	
A/A	0 (0)	0 (0)	
rs 2228570			
T/T	8 (53.3)	7 (46.7)	0.614[b]
T/C	21 (55.3)	17 (44.7)	
C/C	14 (43.8)	18 (56.2)	

Values presented as median (min-max) or n(%). Statistical analysis: aMann-Whitney test; bChi-square test; cFisher's exact test; $p<0.05$ = statistically significant. VDG = vitamin D group; CG = control group

Table 2: Blood 25 (OH)D level and vitamin D status in VDG and CG at baseline versus 84 days after supplementation with vitamin D3.

	Start of study			After supplementation		
	VDG (n=43)	CG (n=42)	p	VDG (n=43)	CG	p-value
Group			**value**		**(n=42)**	
Blood 25(OH)D level (ng/mL)						
	10.5	13.05	0.264[a]	46	14.4	0.001[a]*
	(4.7 – 30.3)	(2.4 – 26.9)		(9.4 – 79.4)	(6.9 – 38.3)	
Vitamin D status						
Deficiency	40 (50.6)	34 (45.9)		3 (8.6)	32 (91.4)	
Insufficiency	2 (22.2)	7 (77.8)	1.000[c]	10 (62.5)	6 (37.5)	0.001[b]*
Sufficiency	1 (50)	1 (50)		30 (88.2)	4 (11.8)	

Groups: VDG= vitamin D3 5,000IU/day, CG = placebo
*Vitamin D status (blood 25(OH)D level): deficiency (< 20 ng/mL); insufficiency (20 – 30ng/mL); sufficiency (30 – 100 ng/mL). Statistical analysis: aMann Whitney test; bChi-square test; cFisher's exact test; *p<0.05 =statistically significant*
VDG = vitamin D group; CG = control group

Comparison of blood 25(OH)D levels by VDR genotype after supplementary vitamin D3 administration in VDG

To find the effects of VDR genotype on post-supplementation blood 25(OH)D, 25(OH)D blood levels were compared between genotypes in VDG (Table 3). Blood 25(OH)D levels increased significantly above baseline after supplementary vitamin D3 in rs1544410 genotypes G/G [10.5 (4.7 – 30.5) v. 46.5 (9.4 – 79.4) ng/mL; p=0.001] and G/A [10.8 ± 1.3 v. 45.7 ± 7.2 ng/mL; p =0.010]. No great differences were found in 25(OH)D between rs1544410 genotypes G/G and G/A [46.5 (9.4 – 79.4) ng/mL v. 45.7 ± 7.2 ng/mL; p=0.924].

As compared to baseline, post-supplementation rs2228570 blood 25(OH)D levels increased significantly for genotypes T/T [11.4 (6.2 – 17.9) v.

Table 3: Blood 25(OH)D levels in VDG by VDR genotype after supplementary vitamin D3 administration

		Blood 25(OH)D level (ng/mL)		
VDG		**Start of study (n=43)**	**84 days (n=42)**	**p-value**
VDR genotypes				
rs1544410				
G/G (n= 40)		10.5 (4.7 – 30.5)	46.5 (9.4 – 79.4)	0.001[c]*
G/A (n= 3)		10.8 ± 1.3	45.7 ± 7.2	0.010[d]*
	P value	0.924[a]	0.924[a]	
rs2228570				
T/T (n=8)		11.4 (6.2 – 17.9)	61.2 (32.1 – 79.4)	0.012[c]*
T/C (n=21)		10.5 (7.6 – 30.3)	34.2 (9.4 – 67.4)	<0.001[c]*
C/C (n=14)		11.0 ± 4.4	43.7 ± 12.4	0.001[d]*
	P value	0.373[b]	0.024[b]*	

*Statistical analysis: aMann-Whitney test; bKruskal-Wallis test;c Wilcoxon signed rank test; dpaired t-test; *p value <0.05 = statistically significant*
VDG = vitamin D group; CG = control group

Table 4: Attainment of blood 25(OH)D levels in VDG by VDR genotype after supplementary vitamin D administration

VDR genotype	Blood 25(OH)D level		p-value
	< 30 ng/mL (n,%)	≥30 ng/mL (n,%)	
VDG			
rs 1544410			
G/G (n=40)	13 (32.5)	27 (67.5)	
G/A (n=3)	0 (0)	3 (100)	0.542
rs 2228570			
T/T (n=8)	0 (0)	8 (100)	
T/C (n= 21)	10 (47.6)	11 (52.4)	0.027*
C/C (n=14)	3 (21.4)	11 (78.6)	0.273

*Statistical analysis: logistic regression test; *p value <0.05 = statistically significant*
VDG = vitamin D group

61.2 (32.1 – 79.4); p=0.012], T/C [10.5 (7.6 – 30.3) v. 34.2 (9.4 – 67.4); p<0.001], and C/C [11.0 ± 4.4 v. 43.7 ± 12.4; p=0.001] (Table 3).

There were also significant differences in post-supplementation blood 25(OH)D between rs2228570 genotypes T/T, T/C, and C/C themselves [61.2 (32.1 – 79.4) ng/mL v. 34.2 (9.4 – 67.4) ng/mL v. 43.7 ± 12.4 ng/mL; p=0.024] (Table 3).

To determine the genotypes causing the post-supplementation differences in rs2228570, a post-hoc analysis was conducted, showing significant differences between genotypes T/T and T/C (p=0.015) and between T/T and C/C (p=0.017), but not between T/C and C/C (p=0.391).

Regarding blood 25(OH)D responses to supplementary vitamin D of rs1544410 genotypes G/G and G/A, among the 43 VDG subjects, only 30 (69.7%) attained blood 25 (OH)D levels ≥30 ng/mL. The same is true for the three rs2228570 genotypes T/T, T/C, and C/C (Table 4).

No prominent differences were found between rs1544410 G/G and G/A in attaining blood 25(OH)D ≥30 ng/mL (67.5% v. 100%; p=0.542). In rs2228570, significant differences occurred in blood 25(OH)D level attainment between T/C and T/T (52.4% v. 100%; p=0.027).

DISCUSSION

Vitamin D deficiency was found in 84.1% of subjects, with a median blood 23(OH)D level of 11.6 (2.4 – 30.3) ng/mL, which is much greater than in Europe, where 25(OH)D levels below 20 ng/mL and 12 ng/mL are observed in 40.4% and 13.0%, respectively, of the population [4]. Conversely, adult vitamin D deficiency prevalence in 5 South Asian countries was 68% [5]. Our results approximate those of earlier studies demonstrating that T2DM vitamin D deficiency prevalence is around 63.2 – 83.2% [2,3], and that vitamin D deficiency is also found in tropical countries, such as Indonesia, with abundant sunshine for cutaneous vitamin D synthesis.

Vitamin D as a prohormone is available as vitamin D2 (ergoalciferol) in foods and vitamin D3 (cholecalciferol) in UV-exposed human skin. Generally, vitamin D deficiency is caused by low dietary intake and reduced cutaneous synthesis from inadequate sunlight exposure due to geographic location, skin color, age, indoor lifestyle, and cultural or religious practices[22,23]. Our greater vitamin D deficiency prevalence shows that foods and sunlight alone cannot maintain optimal vitamin D status, necessitating vitamin D supplementation.

Signal transmission in humans occurs through vitamin D binding with the vitamin D receptor (VDR) [11] expressed by insulin-sensitive tissues. Apparently, vitamin D may have a direct influence on insulin sensitivity and insulin receptor expression, thereby enhancing insulin-stimulated glucose transport. Vitamin D may also have an indirect influence [17] by reducing insulin resistance-inducing inflammatory responses [24].

A meta-analysis showed that vitamin D

supplements can raise blood 25(OH)D and reduce insulin resistance in T2DM [25]. However, systematic reviews of T2DM RCTs failed to find evidence for the efficacy of vitamin D supplements in glucose hemostasis and in decreasing insulin resistance [26,27]. According to another meta-analysis, vitamin D dosage, status, and length of supplementation affect the therapeutic response[9]. High-dose vitamin D supplementation produces greater effects in obese vitamin D-deficient Asians[9]. Irrespective of vitamin D supplementation's impact on T2DM patients, vitamin D deficiency should be corrected because vitamin D serves an essential function in calcium hemostasis and bone metabolism [22].

Our study showed that rs1555410 had genotype G/G in 79 (92.9%) subjects and no genotype A/A, whereas Chinese T2DM patients [17] had mostly rs1544410 genotype G/A (93.75%), but similarly no A/A. Our rs2228570 genotype proportions comprised T/C in 44.7%, C/C in 37.6%, and T/T in 17.6% of subjects, whereas the Chinese study [17] comprised rs2228570 genotype C/C in 59.8%, T/T in 23.2%, and C/T in 16.9% of subjects, showing that Asians vary in rs1544410 and rs2228570 gene proportions. Similarly, the research results of Sari et al. [28]. on healthy North Sumatran women differed from ours, because all subjects had heterozygous A>G for BsmI (rs1544410), and T>C for TaqI (rs2228570), showing that Indonesians also have differing VDR genotype proportions, supporting the supplementary vitamin D dosage personalization concept.

In our study, supplementary vitamin D at 5000 IU/day caused a 3.2-fold higher rise in blood 25(OH)D in VDG compared to CG [46 (9.4 – 79.4) ng/mL v. 14.4(6.9 – 38.3) ng/mL; p=0.001] (Table 2). Blood 25(OH)D may rise around 1 ng/mL (2.5 nmol/L) per 100 IU daily vitamin D3 supplement given for 56 - 84 days [29], although the supplementary dose may not be linearly correlated with blood 25(OH)D [30].

After 84 days of vitamin D supplementation at 5000 IU/day, among our 43 subjects, 13 (30.2%) subjects still did not attain blood 25(OH)D levels of ≥30 ng/mL (Table 4.) This agrees with the findings of Yao et al. [15], from a 140-day RDBPCT on 448 Chinese with vitamin D deficiency receiving 2000 IU/day vitamin D3 or placebo, where the vitamin D increased blood 25(OH)D, but could not overcome vitamin D deficiency in 25% of subjects. Al-Daghari et al. [31]. Studying T2DM patients on 2000 IU/day supplementary vitamin D for 12 months showed that 42% of subjects still could not reach target blood 25(OH)D. Hu et al. [17]. in their study on T2DM subjects receiving supplementary vitamin D at 800 IU/day for 12 months, also found that 44.6% of subjects did not attain vitamin D sufficiency.

The influence of VDR SNP on supplementary vitamin D3 results remains unclear. We found a significant increase in post-supplementary blood 25(OH)D levels above baseline values in rs1544410 genotypes G/G (p=0.001) and G/A (p=0.010) which agrees with Cavalcante et al. [19]. showing that supplementary vitamin D significantly increased blood 25(OH)D in BB/Bb (p=0.009), but not in bb subjects.

In our VDG subjects with rs2228570, large differences were found in post-supplementary 25(OH)D blood concentrations of genotypes T/T, T/C, and C/C (p= 0.024) (Table 3.). Post-hoc analysis showed differences between T/T and T/C (p=0.015) and between T/T and C/C (p=0.017), but not between T/C and C/C (p=0.391). In our study, rs2228570 apparently influenced supplementary vitamin D response in T2DM subjects. In T/C subjects, only 52.4% attained 25(OH)D ≥30 ng/mL, lower than in the other genotypes (Table 4).

One meta-analysis showed that TaqI and FokI polymorphisms may modulate supplementary vitamin D response for better results [32]. Our study confirms that the doses should be adapted ("personalized") in subjects with rs2228570 genotype T/C for optimal benefits of supplementary vitamin D.

Our study results differ from those of Al-Daghari et al. [31]. on T2DM subjects with genotype-related differences in post-supplementary blood 25(OH)D, in that genotype T/T subjects evidenced better therapeutic responses than the other genotypes. Our results also differ from those of Hu et al. [17]. showing in T2DM subjects that rs2228570 genotypes T/C and T/T had no remarkable differences in blood 25(OH)D (p=0.964). In addition, Hu's study and ours showed differences in subject characteristics regarding age, baseline blood 25(OH)D, and supplementary vitamin D dose. Hu's subjects were aged 66.3 ± 9.1 years, whereas ours were 55.8 ± 0.6 years old. Our baseline blood 25(OH)D of 11.6 (2.4 – 30.3) ng/mL exceeded that of Hu's 22.7 ± 1.9 ng/mL, presumably because Hu et al. used a lower vitamin D3 supplementation dose (800 IU) [17]. Another study found that lower baseline blood 25(OH)D levels were associated

with significantly higher blood 25(OH)D responses [15].

Opinions regarding optimal blood 25(OH)D levels in humans are inconsistent, with no uniform definition of vitamin D deficiency and insufficiency in different guidelines. The IOM recommends a minimum blood 25(OH)D concentration of 20 ng/mL (50 nmol/L) in connection with bone health [33]. However, the Endocrine Society recommends 25(OH)D levels exceeding 30 ng/mL (or 75 nmol/L) for preventing infections and obtaining other non-calcemic vitamin D benefits [8]. We showed that supplementary vitamin D3 at 5000 IU/day for 84 days still could not prevent 30% of subjects from attaining vitamin D sufficiency with blood 25(OH)D ≥ 30 ng/mL. The Endocrine Society clinical practice guideline [8] recommends supplementary vitamin D3 for increasing vitamin D levels and determining blood 25(OH)D concentrations because 25(OH)D is the most frequent circulatory vitamin D, with a half-life of 14 – 21 days, and extremely useful for monitoring vitamin D status in persons at high risk of vitamin D deficiency.

Our study confirms the need for supplementary vitamin D dose personalization and blood 25(OH)D measurement in high-risk patients in relation to VDR SNPs, apart from the contradictory relationship of vitamin D deficiency in glucose hemostasis and insulin resistance reduction [20,26,27,34]. There is a need for dosage adjustment ("personalization") in subjects with rs2228570 genotype T/C to obtain better gains from supplementary vitamin D. Our study results may provide inputs on management policies of T2DM patients susceptible to vitamin D deficiency, particularly in Indonesia.

In some populations, the interplay of genes and lifestyle may obscure the genetic component; therefore, studies on gene interactions with diet and physical activity are mandatory to confirm the relationship. Other longer-term RCTs with larger sample sizes are also necessary to better utilize the results of vitamin D supplements in patients with type 1 and type 2 diabetes.

We used an RDBPCT design that is best for measuring cause-and-effect relationships. We used an identical therapeutic procedure and supplementary vitamin D3 dosage to minimize subject variation.

One limitation of this study was that our subjects were Indonesian T2DM patients; therefore, our results may not apply to other nations. We also did not account for physical activity, diet, sunlight exposure, BMI, and parathyroid hormone as confounders.

CONCLUSION

After vitamin D supplementation, blood 25(OH)D levels rose perceptibly, but a third of subjects still failed to attain blood 25(OH)D levels of ≥30 ng/mL. VDR rs2228570 genotype T/C had only 52.4% of its subjects attaining a sufficiently large 25(OH)D level, but perceptibly lower than in genotypes T/T and C/C. VDR rs2228570 polymorphisms apparently influence T2DM response to supplementary vitamin D. There is a need for personalization of vitamin D dosage and blood 25(OH)D measurement in high-risk patients due to VDR SNPs.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7

PAGE 8

PAGE 9

PAGE 10

PAGE 11

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Supplementary vitamin D3 and vitamin D receptor polymorphisms affect blood vitamin D levels in type-2 diabetes mellitus in Indonesia

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ABSTRACT

INTRODUCTION: There are no data on vitamin D receptor (VDR) gene single nucleotide polymorphism (SNP) influence on blood 25-hydroxy-cholecalciferol [25(OH)D] levels after supplementary vitamin D in Indonesian type 2 diabetes mellitus (T2DM) patients. This study evaluated the effects of the supplementary vitamin D3 and VDR gene SNPs rs1555410 and rs2228570 on blood 25(OH)D levels in T2DM cases.

METHODS: A randomized, double-blind placebo-controlled trial (RDPCT) was conducted at one research setting using 85 T2DM subjects divided into vitamin D group (VDG) and control group (CG) and receiving 5,000 IU/day vitamin D3 (cholecalciferol) or placebo once daily for 84 days. Levels of 25(OH)D were determined baseline and after supplementary vitamin D3 administration for 84 days. Circulatory 25(OH)D was assayed using ELISA. VDR polymorphisms were detected using sequencing.

RESULTS: Post-supplementary blood 25(OH)D rose appreciably from baseline in VDG for VDR rs1544410 genotypes G/G (p=0.001) and G/A (p=0.010), and in VDR rs2228570 genotypes T/T (p=0.012), T/C (p<0.001), and C/C (p=0.001). Post-supplementary VDG still contained 30.3% of subjects not reaching blood 25(OH)D ≥30 ng/mL.

In attaining blood 25(OH)D ≥30 ng/mL post-supplementation, VDR rs2228570 genotype T/C differed significantly from T/T (52.4% v. 100%; p=0.027), but there were no appreciable differences between genotypes C/C and T/T (78.6% v. 100%; p=0.273), as well as between VDR rs1544410 genotypes G/G and G/A (67.5% v. 100%; p=0.542).

CONCLUSION: Only 52.4% of subjects with VDR rs2228570 genotype T/C achieved sufficiently high blood 25(OH)D levels. VDR rs2228570 polymorphisms apparently influence T2DM response to supplementary vitamin D.

Keywords: Diabetes Mellitus, Vitamin D, Single Nucleotide Polymorphism, Indonesia

INTRODUCTION

Indonesia, with 10.7 million type 2 diabetes mellitus (T2DM) patients, occupies the 7th global rank, with predicted T2DM prevalence rising to 16.6 million in 2045 [1]. T2DM vitamin D deficiency prevalence is higher than in the general global population, with prevalences of 63.2- 83.2% [2,3].

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The latter are higher than in Europe, with a vitamin D deficiency prevalence of 40.4% (blood 25(OH) D) <20 ng/mL) [4], while vitamin D deficiency prevalence in South Asia is 68% [5].

Vitamin D supplementation raises serum 25(OH) D concentration, influences health outcomes, and achieves maximum mortality rate reductions in developed countries from the commonest fatal diseases, such as cardiovascular disease and T2DM [6]. Gross vitamin D deficiency at 25(OH) D <12 ng/mL (or <30 nmol/L) is a health hazard [7] and should be corrected through vitamin D supplementation [8].

The utility of providing additional vitamin D to T2DM patients for glucose hemostasis and reducing insulin resistance is still debated, presumably because of the diversity of study populations for serum vitamin D status, supplementation dose and duration, methodology, gender, BMI, and ethnicity [9,10].

Vitamin D regulates signal transmission through the vitamin D receptor (VDR) [11]. The vitamin D-responsive element (VDRE) gene on chromosome 12q13.1 comprises nine exons and eight introns [11]. VDR activates and controls gene transcription via the target gene promoter VDRE. The VDR gene has over 470 polymorphisms [11], the most common being FokI (rs2228570 C to T) and BsmI (rs1544410 A to G)[11]. VDR rs1544410 at intron 8 regulates mRNA stability, thereby affecting gene expression [12]. VDR rs2228570 lies in the exon 2 start codon and changes the initiation sites [13]. VDR gene SNPs may influence VDR mRNA and protein stability and activity, resulting in a suboptimal response to supplementary vitamin D [14,15].

Multiple factors may affect post-supplementary blood 25(OH)D, such as sunshine exposure, aging, body mass index, calcium intake, supplementary vitamin D, and genetics [4,16]. It is currently uncertain whether VDR SNPs affect blood vitamin D levels after supplementary vitamin D in T2DM patients [17], due to difficulties in detecting gene involvement in the development of T2DM, because of possible minute differences in the gene and its interaction with genetic or non-genetic factors [18]. VDR SNP influence on increases in blood 25(OH)D levels that depict vitamin D condition, can be evaluated only with a randomized, double-blind placebo-controlled trial (RDBPCT).

Currently, few randomized double-blind placebo-controlled trials (RDBPCT) exist for evaluating VDR rs1544410 and rs2228570 relationships with vitamin D therapeutic response. Waterhouse et al. [14]. Studied Australian elderly aged 60 – 80 years receiving vitamin D3 supplementation (30.000 vs. 60.000 IU/month) for 12 months and found rs2228570 was not associated with increases in 25(OH)D levels after supplementary vitamin D. A 20-week Chinese RDBPCT [15] on vitamin D deficiency cases receiving vitamin D3 at 2000 IU/ day or placebo, demonstrated that rs2228570 showed stronger influences on 25(OH)D levels ($p<0.04$). Post-treatment, VDR rs2228570-G, and its alleles had higher 25(OH)D levels ($p = 0.009$). The RDBPCT of Cavalcante et al. [19]. on elderly females aged 68 ± 6 years with vitamin D insufficiency and receiving 200.000 IU vitamin D3 supplementation for 4 weeks, showed higher blood 25(OH)D concentrations in persons with BsmI genotypes BB/Bb ($p<0.001$) who responded better to supplementary vitamin D than those with bb genotype.

A prospective case-control study involving 125 T2DM patients and 125 controls, revealed that low blood 25(OH)D and gene rs2228570 correlated with T2DM risk [20]. The inconsistent study results on VDR SNP relationships were caused by variations in pre-supplementary blood vitamin D levels, study designs, small sample sizes, vitamin D dose, duration of supplementary vitamin D, dietary intake, and ethnicity.

There are currently no reports on the causal relationship of VDR SNPs rs1544410 and rs2228570 with supplementary vitamin D among Indonesian T2DM patients, for which an RDBPCT is necessary. The outcome may be useful for vitamin D3 therapeutic dose personalization in T2DM patients to reduce morbidity and mortality rates. Primary research outcomes would be responses to supplementary vitamin D by comparing post-supplementary blood 25(OH)D values. Secondary outcomes would be the impact of rs1544410 and rs2228570 polymorphisms on blood 25(OH)D changes and attainment of blood 25(OH)D of ≥30 ng/mL

METHODS

Research design

This RDBPCT was conducted from June to August 2022 at Puskesmas Mampang in South Jakarta, with subjects signing informed consent. Study protocol approval was by the Research Ethics Committee,

Faculty of Medicine, Universitas Trisakti, under No. 001/KER/FK/1/2022.

Patients and intervention

The study subjects were Mampang area residents with T2DM. Inclusion criteria: males and females ≥18 years old, T2DM, HbA1c 7-8.5%, oral antidiabetic drug monotherapy, agreeing to follow-up controls. T2DM was diagnosed using American Diabetes Association criteria [21], namely fasting blood glucose ≥126 mg/dL, or 2-hour postprandial blood glucose ≥200 mg/dL and HbA1c ≥6.5%. Exclusion criteria: previously and currently on insulin therapy, suspect kidney disease (estimated glomerular filtration rate <30 mL/min/1.73m2), abnormal liver function (SGPT 3 times normal upper limit), pregnant or lactating, allergy, hypercalcemia (plasma calcium >2.65 mmol/L), or receiving daily supplementary vitamin D in preceding 84 days. Dropout criteria: blood 25(OH) D >100 ng/mL, hypercalcemia, cholecalciferol hypersensitivity.

The study enrolled 115 subjects allocated by simple randomization to vitamin D (intervention) and control (placebo) groups at 1:1 ratio, which was conducted by personnel blinded to the intervention. VDG received daily vitamin D3 tablets containing 5,000 IU cholecalciferol, whereas controls received once-daily placebo tablets (120mg calcium), all tablets taken for 84 days. The vitamin D and placebo tablets contained in dark-colored glass bottles coded A and B were identical in visual, olfactory, and gustatory qualities. Participants and the statistician were all blinded to the origin of the tablets, whether from VDG or CG. Compliance with the intervention was determined by weekly counting the remaining tablets. VDG had 6 dropouts because of protocol non-compliance, returning to the home village, and diarrhea, while CG had 7 dropouts because of not agreeing to participate, protocol non-compliance, and nausea and vomiting. Subjects completing the study were 85 in number, consisting of 43 in VDG and 42 in CG (Figure 1).

The researchers were blinded to subject allocation in all study phases (recruitment, enrolment, data collection, and group assignment). For improving verification and compliance, all empty medication bottles were returned monthly to the cadres for evaluation of subject compliance to supplementation at the completion of the study. Subjects' complaints (potential adverse events) were noted for recording. The principal investigator evaluated the complaints and their connection with the supplements. The development of all reported symptoms was monitored until the study was completed. The allocation codes stored by an independent third party were opened after the study was completed.

Measurements

On day zero (admission date), before administration of vitamin D tablets, subjects meeting recruitment criteria were interviewed to collect subject data on age, gender, and duration of diabetes mellitus, followed by blood collection at 08.00 and 9.00 a.m. local time.

Biochemical measurements

From each participant, 5 mL of venous whole blood was collected, with 3 mL for blood 25(OH) D determination and 2 mL in EDTA tubes for VDR SNP genotyping.

Blood 25(OH)D level was determined by chemiluminescent microparticle immunoassay (ARCHITECT 25-OH Vitamin D assay, Abbott), with measuring interval 8.0 - 160.0 ng/mL (20.0 - 400.0 nmol/L), limit of detection (LoD) ≤ 10.0 ng/mL, limit of quantitation (LoQ) ≤ 20 ng/mL, and imprecision ≤ 10% within total CV.

Blood 25(OH)D data were presented as median ± SD, and categorized according to Endocrine Society 2011 clinical practice guideline, with <20 ng/mL defined as vitamin D deficiency, 20 – 30 ng/mL as vitamin D insufficiency, 30 – 100 ng/mL as vitamin D sufficiency [8].

Vitamin D receptor single nucleotide polymorphisms

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN) from 200 µL EDTA blood and its purity and level were determined using a NanoDrop 2000 spectrophotometer (ThermoScientific). VDR SNPs were detected by PCR, followed by sequencing. PCR used MyTaq HS Red Mix Kit (Bioline) to amplify the target sequence using specific primers, namely for rs1544410: forward primer 5'- GGG AGT ATG AAG GAC AAA GAC C-3' and reverse primer 5'- CCC GCA AGA AAC CTC AAA TAA C -3' and for rs2228570: forward primer 5'-GCA CTG ACT CTG GCT CTG -3' and reverse primer 5'-TGG ACA TTG TAA GGA AGG AGA TG-3'. PCR amplification used 2 µl DNA, 8.5 µL nuclease-free water, 2x 12.5µl My Taq HS Red Mix,

and 1.0 □l each of forward and reverse primers. Conditions: initial activation at 95oC for 5 minutes, denaturation at 95oC for 15 seconds, annealing at 58oC for 30 seconds, extension at 72oC for 30 seconds for 40 cycles, and final extension at 72oC for 3 minutes. PCR products on 2% agarose gel were visualized by electrophoresis. Purified PCR products were sequenced using BigDye Terminator Kit (Thermo Fisher Scientific) and ABI 3500 sequencer (Applied Biosystems). Sequence analysis was performed with BioEdit software to confirm mutations, which were compared to NCBI BLAST database with accession number NG_008731.1.

Sample size

In each group, 40 subjects were required to detect a treatment difference at 5% two-sided significance level (0.05) and 90% study power. μ1 – μ2 = predicted between-group mean difference, estimated at 4; α2 = expected population variance from the preliminary study, estimated at 1.02. To anticipate dropouts, the sample size became 95 for adequate power to detect outcome measure differences.

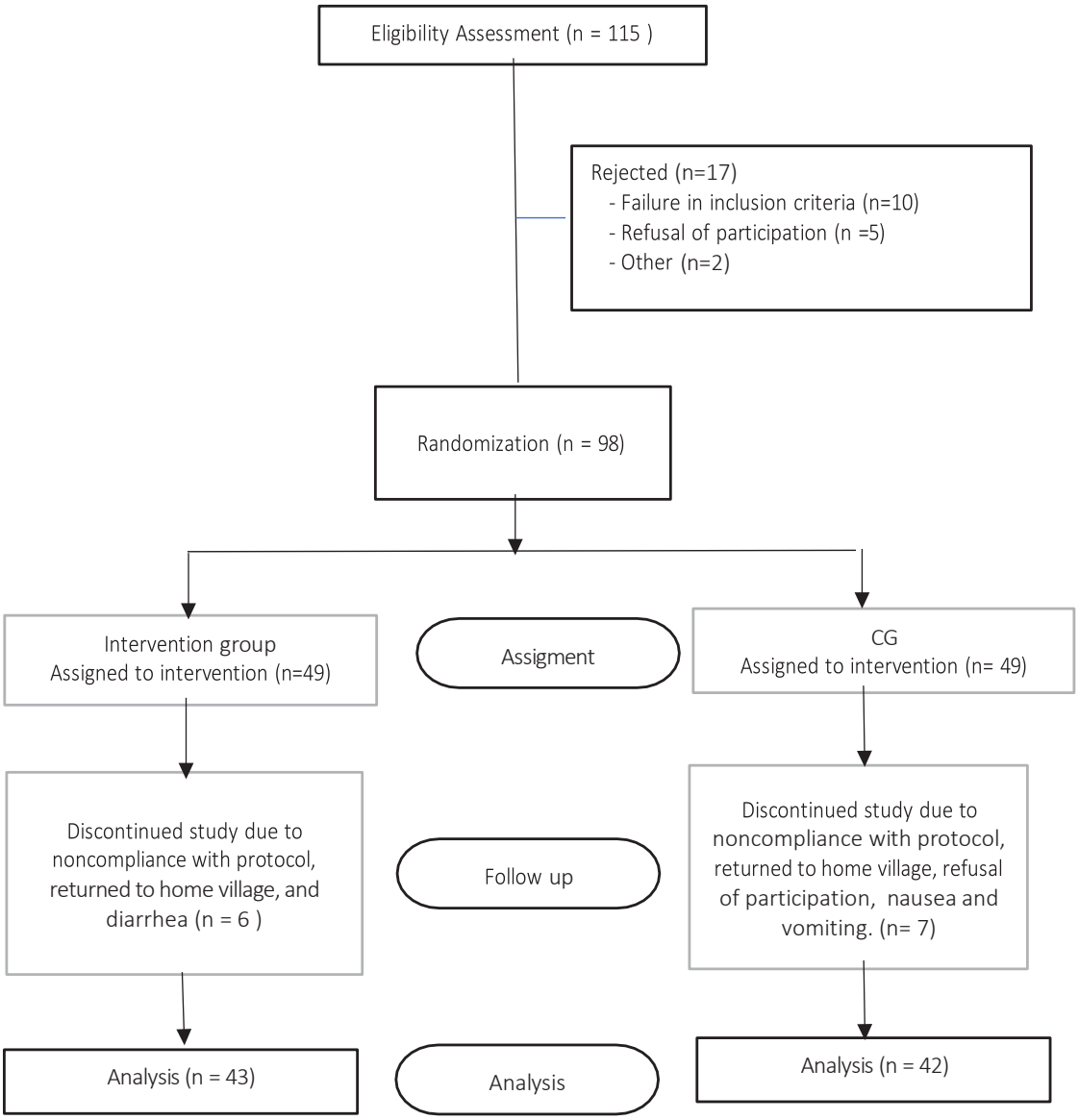

Figure 1: Flow of participants throughout trial

Statistical analysis

Continuous data of normal distribution were shown as mean and standard deviation (SD), continuous data of skewed distribution as median (minimum-maximum), and categorical data as percentages. Data were checked for normal distribution using the Kolmogorov-Smirnov test. Unpaired Student's t-test, Mann-Whitney U test, Chi-square test, and Fisher exact test were used for baseline comparisons between VDG and CG, based on the variable type and data distribution. Mann-Whitney test was used to compare blood 25(OH)D in VDG and CG at baseline and post-supplementation. Baseline and post-supplementation vitamin D status differences were evaluated using Fisher's exact test and Chi-square test. VDG baseline and post-supplementation blood 25(OH)D differences between rs1544410 genotypes were evaluated by the Mann-Whitney test. VDG baseline and post-supplementation blood 25(OH)D levels between rs2228570 genotypes were compared using the Kruskal-Wallis test, Wilcoxon signed rank test, and paired t-test at $p < 0.05$. Statistical analysis used SPSS for Windows version 23.

RESULTS

Baseline subject characteristics

We had 85 T2DM participants, with 68 (80%) females, mean age 55.8 ± 0.6 years, median T2DM duration 12 (1 – 36) months, and median blood 23(OH)D 11.6 (2.4 – 30.3) ng/mL. The most frequent rs1555410 genotype was G/G, comprising 79 subjects (92.9%), followed by G/A with 6 subjects (7.1%), without any T/T. Gene rs2228570 had genotypes T/C, C/C, and T/T with 38 (44.7%), 32 (37.6%), and 15 (17.6%) subjects, respectively. Vitamin D status was mostly deficient in 74 subjects (84.1%), insufficiency in 9 subjects (10.6%), and sufficiency in 2 subjects (2.4%). After group randomization, no significant differences were observed in age, gender, T2DM duration, and VDR genotype between VDG and CG (Table 1).

Baseline and post-supplementation blood 25(OH) D and vitamin D status

After vitamin D3 supplementation for 84 days, there was a much greater increase in VDG blood 25(OH)D than in CG (46(9.4-79.4) v. 14.4(6.9-38.3); ($p<0.001$) (Table 2).

Table 1: Subject characteristics at the start of the study in VDG and CG

Characteristic	Treatment group		P-value
	VDG (n=43)	CG (n=42)	
Age (years)	56 (35 – 80)	56 (35 – 69)	0.396[a]
Gender			
Male	8 (47.1)	9 (52.9)	0.745[b]
Female	35 (51.5)	33 (48.5)	
Duration of DM (months)	12 (1 – 36)	12 (1 – 36)	0.967[a]
VDR genotype (n,%)			
rs1544410			
G/G	40 (50.6)	39 (49.4)	1.000[c]
G/A	3 (50)	3 (50)	
A/A	0 (0)	0 (0)	
rs 2228570			
T/T	8 (53.3)	7 (46.7)	0.614[b]
T/C	21 (55.3)	17 (44.7)	
C/C	14 (43.8)	18 (56.2)	

Values presented as median (min-max) or n(%). Statistical analysis: aMann-Whitney test; bChi-square test; cFisher's exact test; p<0.05 = statistically significant. VDG = vitamin D group; CG = control group

Table 2: Blood 25 (OH)D level and vitamin D status in VDG and CG at baseline versus 84 days after supplementation with vitamin D3.

	Start of study			After supplementation		
	VDG (n=43)	CG (n=42)	p	VDG (n=43)	CG	p-value
Group			**value**		**(n=42)**	
Blood 25(OH)D level (ng/mL)						
	10.5	13.05	0.264[a]	46	14.4	0.001[a]*
	(4.7 – 30.3)	(2.4 – 26.9)		(9.4 – 79.4)	(6.9 – 38.3)	
Vitamin D status						
Deficiency	40 (50.6)	34 (45.9)		3 (8.6)	32 (91.4)	
Insufficiency	2 (22.2)	7 (77.8)	1.000[c]	10 (62.5)	6 (37.5)	0.001[b]*
Sufficiency	1 (50)	1 (50)		30 (88.2)	4 (11.8)	

Groups: VDG= vitamin D3 5.000IU/day, CG = placebo
*Vitamin D status (blood 25(OH)D level): deficiency (< 20 ng/mL); insufficiency (20 – 30ng/mL); sufficiency (30 – 100 ng/mL). Statistical analysis: aMann Whitney test; bChi-square test; cFisher's exact test; *p<0.05 =statistically significant*
VDG = vitamin D group; CG = control group

Comparison of blood 25(OH)D levels by VDR genotype after supplementary vitamin D3 administration in VDG

To find the effects of VDR genotype on post-supplementation blood 25(OH)D, 25(OH)D blood levels were compared between genotypes in VDG (Table 3). Blood 25(OH)D levels increased significantly above baseline after supplementary vitamin D3 in rs1544410 genotypes G/G [10.5 (4.7 – 30.5) v. 46.5 (9.4 – 79.4) ng/mL; p=0.001] and G/A [10.8 ± 1.3 v. 45.7 ± 7.2 ng/mL; p =0.010]. No great differences were found in 25(OH)D between rs1544410 genotypes G/G and G/A [46.5 (9.4 – 79.4) ng/mL v. 45.7 ± 7.2 ng/mL; p=0.924].

As compared to baseline, post-supplementation rs2228570 blood 25(OH)D levels increased significantly for genotypes T/T [11.4 (6.2 – 17.9) v.

Table 3: Blood 25(OH)D levels in VDG by VDR genotype after supplementary vitamin D3 administration

		Blood 25(OH)D level (ng/mL)		
VDG		**Start of study (n=43)**	**84 days (n=42)**	**p-value**
VDR genotypes				
rs1544410				
G/G (n= 40)		10.5 (4.7 – 30.5)	46.5 (9.4 – 79.4)	0.001[c]*
G/A (n= 3)		10.8 ± 1.3	45.7 ± 7.2	0.010[d]*
	P value	0.924[a]	0.924[a]	
rs2228570				
T/T (n=8)		11.4 (6.2 – 17.9)	61.2 (32.1 – 79.4)	0.012[c]*
T/C (n=21)		10.5 (7.6 – 30.3)	34.2 (9.4 – 67.4)	<0.001[c]*
C/C (n=14)		11.0 ± 4.4	43.7 ± 12.4	0.001[d]*
	P value	0.373[b]	0.024[b]*	

*Statistical analysis: aMann-Whitney test; bKruskal-Wallis test;c Wilcoxon signed rank test; dpaired t-test; *p value <0.05 = statistically significant*
VDG = vitamin D group; CG = control group

Table 4: Attainment of blood 25(OH)D levels in VDG by VDR genotype after supplementary vitamin D administration

VDR genotype	Blood 25(OH)D level		p-value
	< 30 ng/mL (n,%)	≥30 ng/mL (n,%)	
VDG			
rs 1544410			
G/G (n=40)	13 (32.5)	27 (67.5)	
G/A (n=3)	0 (0)	3 (100)	0.542
rs 2228570			
T/T (n=8)	0 (0)	8 (100)	
T/C (n= 21)	10 (47.6)	11 (52.4)	0.027*
C/C (n=14)	3 (21.4)	11 (78.6)	0.273

*Statistical analysis: logistic regression test; *p value <0.05 = statistically significant*
VDG = vitamin D group

61.2 (32.1 – 79.4); p=0.012], T/C [10.5 (7.6 – 30.3) v. 34.2 (9.4 – 67.4); p<0.001], and C/C [11.0 ± 4.4 v. 43.7 ± 12.4; p=0.001] (Table 3).

There were also significant differences in post-supplementation blood 25(OH)D between rs2228570 genotypes T/T, T/C, and C/C themselves [61.2 (32.1 – 79.4) ng/mL v. 34.2 (9.4 – 67.4) ng/mL v. 43.7 ± 12.4 ng/mL; p=0.024] (Table 3).

To determine the genotypes causing the post-supplementation differences in rs2228570, a post-hoc analysis was conducted, showing significant differences between genotypes T/T and T/C (p=0.015) and between T/T and C/C (p=0.017), but not between T/C and C/C (p=0.391).
Regarding blood 25(OH)D responses to supplementary vitamin D of rs1544410 genotypes G/G and G/A, among the 43 VDG subjects, only 30 (69.7%) attained blood 25 (OH)D levels ≥30 ng/mL. The same is true for the three rs2228570 genotypes T/T, T/C, and C/C (Table 4).

No prominent differences were found between rs1544410 G/G and G/A in attaining blood 25(OH)D ≥30 ng/mL (67.5% v. 100%; p=0.542). In rs2228570, significant differences occurred in blood 25(OH)D level attainment between T/C and T/T (52.4% v. 100%; p=0.027).

DISCUSSION

Vitamin D deficiency was found in 84.1% of subjects, with a median blood 23(OH)D level of 11.6 (2.4 – 30.3) ng/mL, which is much greater than in Europe, where 25(OH)D levels below 20 ng/mL and 12 ng/mL are observed in 40.4% and 13.0%, respectively, of the population [4]. Conversely, adult vitamin D deficiency prevalence in 5 South Asian countries was 68% [5]. Our results approximate those of earlier studies demonstrating that T2DM vitamin D deficiency prevalence is around 63.2 – 83.2% [2,3], and that vitamin D deficiency is also found in tropical countries, such as Indonesia, with abundant sunshine for cutaneous vitamin D synthesis.
Vitamin D as a prohormone is available as vitamin D2 (ergoalciferol) in foods and vitamin D3 (cholecalciferol) in UV-exposed human skin. Generally, vitamin D deficiency is caused by low dietary intake and reduced cutaneous synthesis from inadequate sunlight exposure due to geographic location, skin color, age, indoor lifestyle, and cultural or religious practices[22,23]. Our greater vitamin D deficiency prevalence shows that foods and sunlight alone cannot maintain optimal vitamin D status, necessitating vitamin D supplementation.
Signal transmission in humans occurs through vitamin D binding with the vitamin D receptor (VDR) [11] expressed by insulin-sensitive tissues. Apparently, vitamin D may have a direct influence on insulin sensitivity and insulin receptor expression, thereby enhancing insulin-stimulated glucose transport. Vitamin D may also have an indirect influence [17] by reducing insulin resistance-inducing inflammatory responses [24]. A meta-analysis showed that vitamin D

supplements can raise blood 25(OH)D and reduce insulin resistance in T2DM [25]. However, systematic reviews of T2DM RCTs failed to find evidence for the efficacy of vitamin D supplements in glucose hemostasis and in decreasing insulin resistance [26,27]. According to another meta-analysis, vitamin D dosage, status, and length of supplementation affect the therapeutic response[9]. High-dose vitamin D supplementation produces greater effects in obese vitamin D-deficient Asians[9]. Irrespective of vitamin D supplementation's impact on T2DM patients, vitamin D deficiency should be corrected because vitamin D serves an essential function in calcium hemostasis and bone metabolism [22].

Our study showed that rs1555410 had genotype G/G in 79 (92.9%) subjects and no genotype A/A, whereas Chinese T2DM patients [17] had mostly rs1544410 genotype G/A (93.75%), but similarly no A/A. Our rs2228570 genotype proportions comprised T/C in 44.7%, C/C in 37.6%, and T/T in 17.6% of subjects, whereas the Chinese study [17] comprised rs2228570 genotype C/C in 59.8%, T/T in 23.2%, and C/T in 16.9% of subjects, showing that Asians vary in rs1544410 and rs2228570 gene proportions. Similarly, the research results of Sari et al. [28]. on healthy North Sumatran women differed from ours, because all subjects had heterozygous A>G for BsmI (rs1544410), and T>C for TaqI (rs2228570), showing that Indonesians also have differing VDR genotype proportions, supporting the supplementary vitamin D dosage personalization concept.

In our study, supplementary vitamin D at 5000 IU/day caused a 3.2-fold higher rise in blood 25(OH)D in VDG compared to CG [46 (9.4 – 79.4) ng/mL v. 14.4(6.9 – 38.3) ng/mL; p=0.001] (Table 2). Blood 25(OH)D may rise around 1 ng/mL (2.5 nmol/L) per 100 IU daily vitamin D3 supplement given for 56 - 84 days [29], although the supplementary dose may not be linearly correlated with blood 25(OH)D [30].

After 84 days of vitamin D supplementation at 5000 IU/day, among our 43 subjects, 13 (30.2%) subjects still did not attain blood 25 (OH)D levels of ≥30 ng/mL (Table 4.) This agrees with the findings of Yao et al. [15], from a 140-day RDBPCT on 448 Chinese with vitamin D deficiency receiving 2000 IU/day vitamin D3 or placebo, where the vitamin D increased blood 25(OH)D, but could not overcome vitamin D deficiency in 25% of subjects. Al-Daghari et al. [31]. Studying T2DM patients on 2000 IU/day supplementary vitamin D for 12 months showed that 42% of subjects still could not reach target blood 25(OH)D. Hu et al. [17]. in their study on T2DM subjects receiving supplementary vitamin D at 800 IU/day for 12 months, also found that 44.6% of subjects did not attain vitamin D sufficiency.

The influence of VDR SNP on supplementary vitamin D3 results remains unclear. We found a significant increase in post-supplementary blood 25(OH)D levels above baseline values in rs1544410 genotypes G/G (p=0.001) and G/A (p=0.010) which agrees with Cavalcante et al. [19]. showing that supplementary vitamin D significantly increased blood 25(OH)D in BB/Bb (p=0.009), but not in bb subjects.

In our VDG subjects with rs2228570, large differences were found in post-supplementary 25(OH)D blood concentrations of genotypes T/T, T/C, and C/C (p= 0.024) (Table 3.). Post-hoc analysis showed differences between T/T and T/C (p=0.015) and between T/T and C/C (p=0.017), but not between T/C and C/C (p=0.391). In our study, rs2228570 apparently influenced supplementary vitamin D response in T2DM subjects. In T/C subjects, only 52.4% attained 25(OH)D ≥30 ng/mL, lower than in the other genotypes (Table 4).

One meta-analysis showed that TaqI and FokI polymorphisms may modulate supplementary vitamin D response for better results [32]. Our study confirms that the doses should be adapted ("personalized") in subjects with rs2228570 genotype T/C for optimal benefits of supplementary vitamin D.

Our study results differ from those of Al-Daghari et al. [31]. on T2DM subjects with genotype-related differences in post-supplementary blood 25(OH)D, in that genotype T/T subjects evidenced better therapeutic responses than the other genotypes. Our results also differ from those of Hu et al. [17]. showing in T2DM subjects that rs2228570 genotypes T/C and T/T had no remarkable differences in blood 25(OH)D (p=0.964). In addition, Hu's study and ours showed differences in subject characteristics regarding age, baseline blood 25(OH)D, and supplementary vitamin D dose. Hu's subjects were aged 66.3 ± 9.1 years, whereas ours were 55.8 ± 0.6 years old. Our baseline blood 25(OH)D of 11.6 (2.4 – 30.3) ng/mL exceeded that of Hu's 22.7 ± 1.9 ng/mL, presumably because Hu et al. used a lower vitamin D3 supplementation dose (800 IU) [17]. Another study found that lower baseline blood 25(OH)D levels were associated

with significantly higher blood 25(OH)D responses [15].

Opinions regarding optimal blood 25(OH)D levels in humans are inconsistent, with no uniform definition of vitamin D deficiency and insufficiency in different guidelines. The IOM recommends a minimum blood 25(OH)D concentration of 20 ng/mL (50 nmol/L), in connection with bone health [33]. However, the Endocrine Society recommends 25(OH)D levels exceeding 30 ng/mL (or 75 nmol/L) for preventing infections and obtaining other non-calcemic vitamin D benefits [8]. We showed that supplementary vitamin D3 at 5000 IU/day for 84 days still could not prevent 30% of subjects from attaining vitamin D sufficiency with blood 25(OH)D ≥ 30 ng/mL. The Endocrine Society clinical practice guideline [8] recommends supplementary vitamin D3 for increasing vitamin D levels and determining blood 25(OH)D concentrations because 25(OH)D is the most frequent circulatory vitamin D, with a half-life of 14 – 21 days, and extremely useful for monitoring vitamin D status in persons at high risk of vitamin D deficiency.

Our study confirms the need for supplementary vitamin D dose personalization and blood 25(OH) D measurement in high-risk patients in relation to VDR SNPs, apart from the contradictory relationship of vitamin D deficiency in glucose hemostasis and insulin resistance reduction [20,26,27,34]. There is a need for dosage adjustment ("personalization") in subjects with rs2228570 genotype T/C to obtain better gains from supplementary vitamin D. Our study results may provide inputs on management policies of T2DM patients susceptible to vitamin D deficiency, particularly in Indonesia.

In some populations, the interplay of genes and lifestyle may obscure the genetic component; therefore, studies on gene interactions with diet and physical activity are mandatory to confirm the relationship. Other longer-term RCTs with larger sample sizes are also necessary to better utilize the results of vitamin D supplements in patients with type 1 and type 2 diabetes.

We used an RDBPCT design that is best for measuring cause-and-effect relationships. We used an identical therapeutic procedure and supplementary vitamin D3 dosage to minimize subject variation.

One limitation of this study was that our subjects were Indonesian T2DM patients; therefore, our results may not apply to other nations. We also did not account for physical activity, diet, sunlight exposure, BMI, and parathyroid hormone as confounders.

CONCLUSION

After vitamin D supplementation, blood 25(OH)D levels rose perceptibly, but a third of subjects still failed to attain blood 25(OH)D levels of ≥30 ng/mL. VDR rs2228570 genotype T/C had only 52.4% of its subjects attaining a sufficiently large 25(OH)D level, but perceptibly lower than in genotypes T/T and C/C. VDR rs2228570 polymorphisms apparently influence T2DM response to supplementary vitamin D. There is a need for personalization of vitamin D dosage and blood 25(OH)D measurement in high-risk patients due to VDR SNPs.

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