



FokI polymorphism in vitamin D receptor gene: Differential expression of TNF α in peripheral mononuclear cells of type 2 diabetic subjects



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ABSTRACT

Introduction: FokI polymorphism has been associated with obesity and type 2 diabetes (T2D) in some populations. **Objective:** To investigate the frequencies of a genetic polymorphism of Vitamin D receptor (FokI) in patients with T2D and control subjects and investigate the role of 1,25(OH)₂D₃ in the expression of pro-inflammatory markers in peripheral blood mononuclear cells (PBMCs).

Methods: The case–control study was conducted in 160 patients with T2D and 160 control subjects, men and women (30–74 years old). The genotype and allele frequency of FokI polymorphisms were determined in these subjects. Subsequently a subgroup of 40 subjects was included from which PBMCs were removed. *In vitro*, the culture medium was supplemented with two different concentrations of 1,25(OH)₂D₃ (10^{−8} M and 10^{−10} M). The expression profiles of TNF α and mRNA were analysed by qPCR, and GAPDH and β -actin were used as housekeeping genes.

Results: The control subjects have an increased frequency of the FF genotype. In subjects with T2D, the ff genotype was associated with higher HOMA-IR values than individuals with genotype Ff ($p = 0.021$). *In vitro* study in PBMCs showed differential expression of TNF α mRNA by FokI genotype, with a lower expression of this marker of inflammation in FF genotype subjects at a concentration of 10^{−8} M of 1,25(OH)₂D₃.

Conclusion: Our data suggest that VDR FokI polymorphism is associated with T2D, and the genotypes Ff and ff of this variant show a reduced response or resistance to the anti-inflammatory action of VitD, which could indicate a functional role of FokI polymorphism of VDR.

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1. Introduction

Over the last 20 years, scientific attention has focused on the associations of vitamin D (VitD) with multiple chronic and degenerative diseases, such as metabolic disorders (Yin et al., 2012; Al-Daghri et al., 2014a; Zhao et al., 2014), cancer (Lappe et al., 2007; Keum and Giovannucci, 2014), cardiovascular (Giovannucci et al. 2008; Wang et al. 2008), infectious (Yamshchikov et al.) and autoimmune diseases (Al-Daghri et al. 2014a; Roep 2013). These effects have been attributed primarily to the immune-modulatory and anti-inflammatory actions of VitD. The cells involved in these processes (macrophages, dendritic cells, B cells and T cells) express the vitamin D receptor (VDR) and can produce and respond to its active form, 1,25-dihydroxyvitamin D₃ (1,25 (OH)₂D₃) (Sigmundsdottir et al. 2007; Overbergh et al. 2000; Liu et al. 2006; Hewison et al. 2003). In the literature, one of the more extensively reported relationships is that between VitD deficiency and

type 1 diabetes (Luong K vinh quoc et al.; Mathieu et al. 2005). Treatment with VitD has been shown to improve, and even prevent type 1 diabetes in human (Stene et al. 2000; Vitamin D supplement in early childhood and risk for Type 1 (insulin-dependent) diabetes mellitus. The EURODIAB substudy 2 study group 1999) and animal models (Gregori et al., 2002; Mathieu et al., 1994; Mathieu et al., 1992). Although mechanisms have not been elucidated, epidemiologic studies show a higher incidence and severity of T2D in VitD-deficient subjects. VitD deficiency causes a decrease in the secretion of insulin in rats and humans and its replacement improves β -cell function and tolerance to glucose (Kumar et al., 1994; Boucher et al., 1995). In addition, certain genetic polymorphisms in the vitamin D receptor binding protein of vitamin D (DBP), could influence glucose tolerance and insulin secretion (Pratley et al., 1998; Ortlepp et al., 2003), which contributes to the genetic risk for T2D.

Determining genetic and endocrine components involved in the development of T2D has been a difficult task. Recently it has been postulated that inflammatory and innate immune activation could be linked to the pathogenesis of T2D and its complications (Pickup, 2004). It has been suggested that low levels of vitamin D may be a risk factor for the development of T2D (Mathieu et al., 2005). At present, important

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polymorphisms have been described in the VDR that may be capable of altering the activity of this protein. Among them *BsmI* and *FokI* polymorphisms have recently been described as being related to obesity and T2D (Filus et al., 2008).

Our objective was to investigate whether *FokI* polymorphisms in the VDR gene are associated to T2D susceptibility and whether these polymorphisms are able to alter the anti-inflammatory activity of VitD.

2. Methods

2.1. Subjects

To investigate the association between the *FokI* polymorphisms in the VDR gene and T2D, a case control study was conducted from 2010 to 2011 in Santiago, Metropolitan Region, Chile. We studied 160 cases of diabetes selected from public health-care centers (PHC) and 160 gender comparable controls selected from the same PHC. All the subjects were from low and medium socioeconomic status (SES). During the study period, the 320 subjects aged 30–74 were invited to attend an appointment at the Institute of Nutrition and Food Technology, University of Chile (INTA) for an evaluation; they were interviewed with a questionnaire, then their anthropometric measurements (height, weight, waist circumference) and blood pressure were taken. Type 2 diabetes was diagnosed based on the American Diabetes Association criteria (Bloomgarden, 1998). Exclusion criteria were family autoimmune diseases and chronic kidney failure. The study protocol was approved by the Ethics committee of INTA, University of Chile. All participants signed an informed consent on enrolment after they had received written and verbal information about the study.

To investigate whether *FokI* polymorphism can alter the anti-inflammatory activity of VitD, an *in vitro* study was conducted in a sub-sample of 40 subjects, from whom peripheral mononuclear cells were extracted and stimulated with two concentrations of 1,25(OH)₂D₃. Then the expression of TNF α mRNA – as a marker of inflammation – was quantified and the results were analysed in relation to *FokI* VDR genotypes.

2.2. Anthropometric measurements

Anthropometric measurements height, weight and waist circumference were carried out by personal especially trained for the study with procedures previously described by our group (Santos et al., 2004; Angel et al., 2013). Blood pressure was measured using a sphygmomanometer after at least a 15-min rest. Two readings were taken from the right arm, systolic pressure and diastolic pressure were estimated and the average was used.

2.3. Biochemical measurements

Blood samples (10 ml) were taken in the morning between 07:00 and 09:00 h after a 12 h overnight fast. The latter collections were then centrifuged and kept at –80 °C until analysis. Serum levels of glucose, insulin, total cholesterol and HDL cholesterol, triglycerides, ultra sensitive C-reactive protein (US-CRP) and 25-hydroxyvitamin D₃ (25(OH)D₃) were measured. Blood samples were collected along the year except in February.

Plasma levels of 25(OH)D₃ were determined by radioimmunoassay (DiaSorin Stillwater, Minnesota 55082-0285, USA) with quality control materials provided by the manufacturer. The detection limits for 25(OH)D₃ were 6 nmol/l, the intra-assay CV was 10.8% and the inter-assay CV was 9.4%. Insulin was determined by RIA, DPC, INC, The Los Angeles, CA. KIT Coat-a-Count, the intra-assay CV was 5.2% and the inter-assay CV was 7.3%. Plasma US-CRP levels were determined Immunoturbidimetric Method by the Kit: Latex High sensitivity CRP Turbidimetric (Química Clínica Aplicada SA., QCA, CN-Apartado 20-E43870 Amposta/España). Serum glucose, triglycerides, total

cholesterol and HDL cholesterol were measured by enzymatic colorimetric assay. All of the intra- and inter-assay coefficients of variation were <10%.

Diabetes was defined as ≥ 126 mg/dl or use of antidiabetic agents (Grundy, 2005); fasting glucose intolerance was defined as glucose ≥ 100 mg/dl (Grundy, 2005) and insulin resistance as HOMA-IR ≥ 3 . Metabolic syndrome was identified using NCEP-ATP III (Grundy, 2005). VitD deficiency was defined as serum 25(OH)D₃ concentration < 50 nmol/l, VitD insufficiency as 50–74 nmol/l and sufficient as ≥ 75 nmol/l (Okazaki, 2007; Dawson-Hughes et al., 2005). Obesity was defined according to WHO criteria (BMI ≥ 30 kg/m (Zhao et al., 2014)).

2.4. Genetic analysis of *FokI* polymorphism

Genomic DNA was extracted from leukocytes by the standardized methods (GIBCO BRL, USA). Genotypes for the polymorphic restriction sites of the VDR gene –rs2228570– were obtained by DNA amplifications with standard PCR and specific sets of primers, followed by the restriction fragment length polymorphism method (RFLP) according to previous protocols used by our group (García et al., 2007; López et al., 2008).

2.5. Cell separation and cultures

Peripheral blood mononuclear cells (PBMCs) were obtained from 40 subjects (FF = 9, Ff = 23 and ff = 8). PBMCs were isolated from heparinised blood by Ficoll-Hystopaque density gradient centrifugation (density: 1.119; Sigma Diagnostic, St. Louis MO), washed twice with RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA). PBMC contained about 90% CD3+ T lymphocytes. The PBMCs were activated extracted PHA (5 mg/ml) for 72 h. The culture medium was supplemented with two concentrations of 1,25 (OH)₂D₃ 10^{–8} M and 10^{–10} M and control without 1,25(OH)₂D₃ (only vehicle), at 37 °C in an atmosphere of 5% CO₂ in Petri dishes of 6 wells with RPMI 1640 medium, supplemented with 10% FBS and antibiotics in duplicate (concentration 1 \times 10⁶ cells by 1 ml of media culture). Cell recovery was conducted by centrifugation at low speed (3000 rpm \times 5 min) and a gentle treatment with trypsin-EDTA at 37 °C by 5 min for attached cells (monocytes). Finally, all PBMCs (lymphocytes and monocytes) were thoroughly washed in PBS and preserved at –80 °C in TRI-Reagent® solution (Molecular Research Center Inc., Cincinnati, OH, Ambion) for subsequent RNA extraction.

2.6. RNA extraction and q-PCR

The abundance of mRNA of TNF α was by quantitative PCR (q-PCR) using the primers 5'–GTT CCT CAG CCT CTT CTC CT–3' Fw and 5'–ACA ACA TGG GCT ACA GGC TT–3' Rv. Briefly, total RNA was extracted from the samples with TRI-Reagent® (Ambion) according to the manufacturer's instructions. The synthesis of first strand cDNA was performed using 3 μ g of each RNA sample using the Promega Kit (ImProm-ITM Reverse Transcriptase, Promega Corporation, Madison, USA).

qPCR for TNF α mRNA was performed using Agilent equipment Mx3005P (Agilent Technologies). The relative quantification method was used, where we compared the mRNA expression in each concentrations of 1,25 (OH)₂D₃ (10^{–8} M and 10^{–10} M) used in the culture medium with the mRNA expression in the control without 1,25(OH)₂D₃ (only vehicle). We used two endogenous control genes β -actin and GADPH. β -Actin 5'–ATT GCC GAC AGG ATG CAG AA–3' Fw and 5'–AAG CAT TTG CCG AGG ACG AT–3' Rv. GADPH: 5'–TCG GAG TCA ACG GAT TTG GT–3' Fw and 5'–GGA ATT TGC CAT GGG TGG AA–3'. The qPCR reaction was performed in a total volume of 20 μ L, with 2 μ L cDNA synthesized from 300 ng of RNA, 10 μ L master mix containing SYBR GreenII (Stratagene), MgCl₂, dNTPs, polymerase enzyme and its cofactors, 2 μ L of stock of primers (100 μ M) and 6 μ L nuclease-free water. The program used was: 10 min of initial denaturation and enzyme activation at 95 °C,

Table 1
General characteristics and components of T2D.

	Controls n = 160	T2D n = 160	p Value
Women %	64.4 (57.2–71.7)	63.5 (55.8–71.3)	0.852
Age, years	69.4 ± 6.9 (68.3–70.4)	61.7 ± 11.5 (59.7–63.6)	<0.001
Waist circumference, cm	92.2 ± 10.9 (90.6–93.9)	98.1 ± 13.0 (95.9–100.3)	<0.001
Waist circumference, ≥ 102/88 cm	40.6 (33.1–48.0)	60.2 (52.2–68.3)	<0.001
BMI, kg/m ²	27.9 ± 4.7 (27.1–28.6)	31.0 ± 5.6 (30.0–31.9)	<0.001
Fasting Insulin, uUI/ml	5.2 ± 4.9 (4.5–6.0)	13.4 ± 23.6 (9.6–17.3)	<0.001
US-CRP, mg/l	2.6 ± 3.2 (2.1–3.1)	3.3 ± 3.6 (2.7–3.8)	0.099
Fasting glucose, mg/dl	89.0 ± 14.2 (86.8–91.2)	143.1 ± 88.1 (129.0–157.3)	<0.001
Serum total cholesterol, mg/dl	193.8 ± 36.8 (187.6–200.1)	195.7 ± 46.1 (186.4–205.0)	0.730
Serum HDL cholesterol, mg/dl	38.5 ± 11.1 (36.6–40.4)	33.6 ± 10.5 (31.4–35.7)	<0.001
Serum TG, mg/dl	141.8 ± 63.6 (131.0–152.5)	147.2 ± 65.2 (133.9–160.4)	0.528
Homa-IR	1.2 (1.0–1.4)	4.9 (3.4–6.5)	<0.001
Homa-β	94.8 (6.3–183.4)	97.6 (66.7–128.4)	0.956
25(OH)D, nmol/l	60.1 ± 31.5 (55.3–64.9)	54.3 ± 28.1 (49.9–59.0)	0.098
25(OH)D% (<50, nmol/l)	43.3 (35.7–50.9)	47.3 (39.2–55.3)	0.630
25(OH)D% (50–74, nmol/l)	30.1 (23.0–37.1)	34.0 (26.3–41.6)	0.043
25(OH)D% (≥75, nmol/l)	26.5 (19.7–33.2)	18.6 (12.3–24.9)	0.030

Data are mean ± SD (95%CI) or % (95%CI). P value = *t*-test.

followed by 40 cycles composed of 15 s at 95 °C, 15 s at 60 °C for annealing and 15 s at 72 °C for elongation.

2.7. Statistical methods

All statistical analyses were performed with the STATA 12.0 package (2001; Stata Corp., College Station, TX). Descriptive data were expressed by mean ± standard deviation (95%CI) or frequency (95%CI). Comparisons between any two groups were performed using independent *t*-tests. Chi-square analysis was applied to examine the variations of allele, genotype and genotype frequencies in different groups. Chi-square analysis was also used to test Hardy–Weinberg equilibrium for the genotypes in all groups of subjects. Kruskal–Wallis test was used to analyse clinical variables in different genotypes. Odds ratio and their 95% confidence interval (CI) were performed for the risk alleles from logistic regression analysis. A value of *p* < 0.05 was considered statistically significant.

3. Results

3.1. General characteristics and components of T2D

The characteristics of subjects with and without T2D are shown in Table 1. Of the total number of subjects, 64% were women. The mean age of the control group was higher than the T2D group (69.4 ± 6.9 years vs. 61.7 ± 11.5, *p* < 0.001). Type 2 diabetes subjects had higher levels of waist circumference, BMI, fasting insulin, fasting glucose, HOMA-IR and lower levels of HDL cholesterol. No difference in US-CRP, total cholesterol and triglycerides was observed. The mean of 25(OH)D₃ in the T2D group was 54.3 ± 28.1, and 60.1 ± 31.5 in control group (*p* = 0.098). The control subjects had a higher percentage of

optimum levels for this vitamin, 26.5% v/s 18.6% observed in the T2D subjects' (*p* = 0.030).

3.2. Distribution of VDR (*FokI*) gene polymorphism

Table 2 shows the distributions of *FokI* VDR polymorphism in T2D and control subjects, indicating that T2D subjects have a higher genotype and allelic frequency of *FokI* VDR polymorphism with a *p*-value of <0.001 and 0.003 respectively. Both cases and controls are in accordance with Hardy–Weinberg equilibrium.

The distribution of all clinical variables in relation to VDR 2228570 C > T *FokI* polymorphisms is summarized in Table 3. In subjects with T2D, the ff genotype was associated with higher HOMA-IR values than individuals with genotype Ff (*p* = 0.021). Additionally, individuals with T2D FF genotype had lower triglyceride levels than individuals with ff genotype (*p* = 0.009). In the group of control subjects, no significant associations were observed between the analysed VDR *FokI* polymorphism with metabolic variables and 25 (OH)D₃ and US-CRP.

The individual comparison between VDR SNP and components of T2D, including obesity, insulin resistance and VitD deficiency, is presented in Table 4. There was significant difference between controls and T2D subjects in the distribution of alleles [OR 1.59 (0.15–2.20), *p* = 0.003] and the *FokI* polymorphism genotypes 'Ff' [OR 2.82 (1.54–5.22), *p* = 0.0003] and 'ff' [OR 2.70 (1.34–5.70), *p* = 0.0027]. In addition, the ff genotype was associated with increased risk of obesity [OR 2.26 (1.08–4.74), *p* = 0.018]; the allele f was significantly associated with obesity [OR 1.45 (1.03–2.03), *p* = 0.023]. Analyses also revealed that the ff genotype and f allele of *FokI* were associated with an increased risk of insulin resistance [OR 2.62 (1.09–6.51), *p* = 0.017] and [OR 1.58 (1.06–2.36), *p* = 0.018], respectively. Our results also demonstrated that the association between vitamin D deficiency, high cholesterol levels and high triglycerides was not significant in subjects carrying the *FokI* Ff genotype. A logistic regression analysis assessing the association between *FokI* polymorphism and T2D adjusted by gender, age, BMI, VitD and T2D components (Table 5) showed a significant association between *FokI* (Ff + ff) and T2D (OR = 3.52, *p* = 0.003).

3.3. MRNA expression of TNFα in relation to the *FokI* genotypes

Data obtained from the subsample of 40 individuals show a differential expression of TNFα mRNA in PBMCs in relation to the concentration of 1,25(OH)₂D₃ used and the *FokI* genotype (Fig. 1). In the PBMCs' FF genotype, an expression of TNFα at a concentration of 10⁻⁸ M of 1,25(OH)₂D₃ was observed, which was lower than that of Ff or ff. This expression was increased in the f carriers (FF = 0.66, Ff = 1.22 and ff = 1.47, *ptrend* = 0.05). A similar situation was observed with a lower concentration of VitD (10⁻¹⁰ M) (FF = 0.98, Ff = 1.75 and ff = 2.18, *ptrend* = 0.19). No significant differences were observed

Table 2
Distribution of genotypes and allele frequencies for *FokI* polymorphism in VDR.a

Polymorphism VDR	T2D (n = 160)		Controls (n = 160)		p-Value
	n	Frequency	n	Frequency	
<i>FokI</i> (rs2228570 C > T)					
FF	24	0.15	53	0.33	
Ff	96	0.60	75	0.47	
ff	40	0.25	32	0.20	<0.001 ^a
F	144	0.45	181	0.57	
f	176	0.55	139	0.43	0.003 ^a
Hardy–Weinberg p-value	0.056		0.487		

^a Chi-square. "FF" Normally show dominant homozygous genotype; "Ff" Show heterozygous genotype; "ff" show recessive homozygous genotypes.

Table 3
Mean values of all variables by *FokI* genotype in both groups.

	VDR rs2228570 C > T (<i>FokI</i>)			p-Value	T2D			p-Value
	Control group				FF	Ff	ff	
	FF n = 53	Ff n = 75	ff n = 32		n = 24	n = 96	n = 40	
BMI (kg/m ²)	27.2 (25.7–28.7)	27.8 (27.0–28.6)	29.1 (27.0–31.3)	0.509	31.3 (28.3–34.4)	30.6 (29.4–31.8)	31.7 (29.7–33.6)	0.434
WC (cm)	90.6 (87.5–93.7)	93.2 (91.2–95.2)	93.0 (88.0–97.4)	0.394	100.0 (92.5–107.2)	98.0 (95.3–100.3)	97.4 (93.3–101.5)	0.781
Total cholesterol (mg/dl)	193.4 (183.0–203.8)	195.2 (185.4–205.0)	191.6 (179.1–204.0)	0.710	181.4 (162.0–201.0)	197.1 (185.4–209.0)	198.0 (178.0–217.9)	0.437
HDL cholesterol (mg/dl)	38.2 (34.6–42.0)	39.1 (36.3–42.0)	37.5 (34.2–40.8)	0.995	39.1 (29.5–48.7)	33.0 (30.8–35.3)	32.8 (28.1–37.5)	0.317
Triglycerides (mg/dl)	140.0 (120.4–159.5)	146.7 (129.3–164.0)	133.8 (117.0–150.6)	0.974	110.3 (83.2–137.3)	153.4 (135.8–171.0)	146.4* (123.5–169.4)	0.214
Fasting glucose (mg/dl)	86.7 (82.8–90.6)	90.1 (86.9–93.3)	90.0 (85.7–94.4)	0.426	140.1 (121.5–158.7)	133.3 (123.1–143.5)	168.3 (119.0–217.5)	0.495
Fasting insulin (uUI/ml)	5.1 (3.6–6.6)	5.2 (4.2–6.2)	5.6 (4.0–7.2)	0.222	11.3 (6.9–16.1)	14.3 (8.0–20.5)	12.8 (9.1–16.5)	0.173
HOMA-IR	1.3 (0.9–1.7)	1.2 (0.9–1.5)	1.3 (0.9–1.7)	0.173	3.05 (1.4–4.7)	2.93 (2.2–3.6)	4.55** (3.0–6.0)	0.021
US-CRP (mg/dl)	2.4 (1.5–3.3)	2.8 (2.2–3.4)	2.6 (1.5–3.8)	0.461	2.7 (1.7–3.6)	3.9 (3.0–4.8)	3.8 (2.4–5.0)	0.805
25(OH)D ₃ (nmol/l)	59.4 (52.3–66.5)	61.9 (53.7–70.1)	57.3 (48.0–66.6)	0.697	57.0 (48.9–65.1)	56.0 (49.1–62.4)	50.0 (42.7–57.3)	0.210

Data are mean (95%CI). p value = Kruskal–Wallis test.

** Multiple comparisons between groups, Ff v/s ff, p = 0.0031.

* Significant for the genotype FF v/s ff.

when comparing the expression of TNF α between different concentrations in both groups and individual genotype differences.

4. Discussion

Our results show that the polymorphism analysed in this study – the *FokI* VDR gene (rs2228570 C > T) – was associated with T2D. Significant associations between f allele and insulin resistance, obesity and T2D were observed.

Vitamin D is an interesting nutritional candidate in the pathogenesis and development of T2D. Given the evidence that VitD has immunoregulatory and anti-inflammatory properties, key in the development and persistence of T2D, it is necessary to assess the impact of VDR genotypes in susceptibility to T2D.

The determination of genetic and endocrine components involved in the development of T2D has been a difficult task to achieve. It has been postulated that low levels of VitD may be a risk factor for T2D (Knekt et al., 2008; Study, 2007; Kirii et al., 2009; Arora & Haddad, 2014). Recently it has been suggested that inflammatory processes and activation of the innate immune system may be linked to the pathogenesis and complications of T2D (Al-Daghri et al., 2014a; Marcotorchino et al.,

2012; Mezza et al., 2012). Those cells involved in the inflammatory processes and activation of the immune system (macrophages, dendritic cells, B cells and T cells) express VDR and can produce and respond to its active form 1,25-(OH)₂D₃ (Overbergh et al., 2000; Deluca & Cantorna, 2001).

In recent years the association between VDR polymorphisms, involving mainly *FokI* and *BsmI*, and different metabolic diseases, such as metabolic syndrome, obesity and inflammatory activity, diabetes and vitamin D deficiency, has been investigated (Al-Daghri et al., 2014a; Zhao et al., 2014; Al-Daghri et al., 2014b). Although the individual contribution of these polymorphisms in the pathogenesis of T2D still needs to be confirmed, the analysis of this association in our population produced some interesting results. A few studies in Chilean subjects that analyse levels of vitamin D have shown a high prevalence of vitamin D deficiency in the elderly, and these low levels have been associated with obesity and metabolic disorders (Mezza et al., 2012; Bid et al., 2009; Malecki et al., 2003; Angel et al., 2013).

It has been reported that the VDR gene is not the defining factor in the variation in circulating levels of 25 (OH)D₃ (Ahn et al., 2010; Wang et al., 2010). Its most important role is to activate the target cells against the action of 1,25-(OH)₂D₃ (Goltzman, 2010) in the synthesis and

Table 4
Comparison among *FokI* polymorphism of the VDR gen with T2D and its components.

	T2D	Obesity	Insulin resistance	Vitamin D deficiency	Triglycerides >150 mg/dl	Cholesterol >200 mg/dl
	Odds ratio (95% CI) p	Odds ratio (95% CI) p	Odds ratio (95% CI) p	Odds ratio (95% CI) p	Odds ratio (95% CI) p	Odds ratio (95% CI) p
rs2228570 C > T (<i>FokI</i>)						
FF	Reference	Reference	Reference	Reference	Reference	Reference
Ff	2.82 (1.54–5.22) 0.0003	1.58 (0.84–2.94) 0.126	1.59 (0.72–3.70) 0.216	0.96 (0.54–1.72) 0.899	1.05 (0.52–2.16) 0.869	0.94 (0.46–1.91) 0.866
ff	2.70 (1.34–5.70) 0.0027	2.26 (1.08–4.74) 0.018	2.62 (1.09–6.51) 0.017	0.94 (0.47–1.88) 0.857	0.88 (0.37–2.06) 0.752	0.53 (0.22–1.25) 0.114
F	Reference	Reference	Reference	Reference	Reference	Reference
f	1.59 (1.15–2.20) 0.0034	1.45 (1.03–2.03) 0.023	1.58 (1.06–2.36) 0.018	0.97 (0.70–1.34) 0.861	0.94 (0.64–1.38) 0.756	0.75 (0.51–1.10) 0.128

Table 5Logistic regression for T2D according *FokI* polymorphism and T2D components.

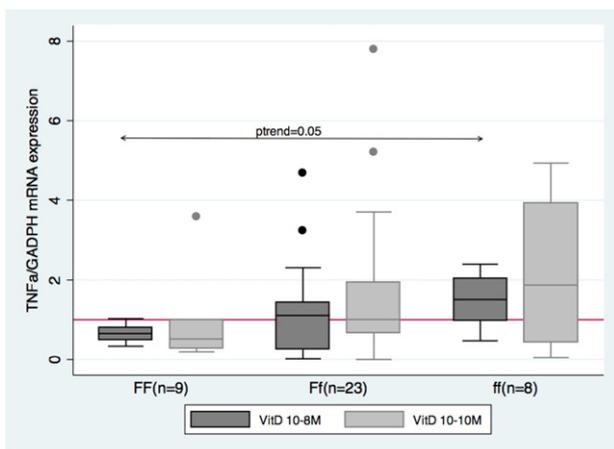
T2D	OR (95%CI)	p Value
<i>FokI</i> carriers (Ff + ff)	3.52 (1.53–8.09)	0.003
Sex	0.79 (0.39–1.56)	0.501
Age (years)	0.96 (0.91–1.01)	0.186
BMI (kg/m ²)	1.13 (1.06–1.21)	<0.001
VitD (nmol/l)	0.99 (0.98–1.00)	0.085
US-CRP (mg/l)	1.00 (0.92–1.10)	0.850
Serum TG (mg/dl)	0.99 (0.98–1.00)	0.079
Serum HDL cholesterol (mg/dl)	0.95 (0.91–0.98)	0.012
Serum total cholesterol (mg/dl)	1.00 (0.99–1.00)	0.848

secretion of insulin (Arora & Haddad, 2014). Although in our sample T2D subjects had higher frequencies of vitamin D insufficiency than the control group (34 v/s 31.1%, $p = 0.043$), this difference can be attributed to the higher frequency of obesity in the diabetic group. However a significant association with insulin resistance in diabetic carriers of *FokI* polymorphisms of VDR gene was observed reflecting the *in-situ* action of vitD.

The VDR is expressed in multiple tissues and various cell types; studies in animal and human models have demonstrated that the administration of VitD can prevent or delay the onset of certain autoimmune diseases, such as type 1 diabetes (Mathieu et al., 2005). Although the mechanisms by which VitD might perform this immunosuppressive role are unknown, a model of monocytes from T2D postulates that it may be by TNF α , IL-6, IL-1 and IL-8 (Giulietti et al., 2007).

In our study we demonstrated that after two different treatments with two concentrations – *in vitro* – of 1,25(OH)₂D₃, the TNF α mRNA expression in PBMCs is expressed differently in relation to the genotype variant of the VDR gene *FokI*, which may suggest that the immune property of VitD is exerted more efficiently in subjects not carrying the *FokI* variant (FF). Furthermore, different response to the concentration of VitD used in the culture medium was observed, with a more effective suppression of the TNF α expression with the high concentration of 1,25-(OH)₂D₃ (10^{-8} M) than with the lowest concentration (10^{-10} M).

There is no clear information in the literature regarding the physiological role of VDR polymorphism and varying concentrations of 25(OH)D₃, or its relationship to the expression of TNF α in peripheral mononuclear cells. We know there is evidence in other autoimmune models, such as type 1 diabetes, regarding the association between



mRNA expression of TNF α following 72-hour 1,25(OH)₂D₃ (VitD) treatments with two concentrations: 10^{-8} M (black box) and 10^{-10} M (grey box), in the peripheral blood mononuclear cells (PBMCs) of 40 subject (FF n=9; Ff n=23; ff n=8). p -trend=0.05 for 10^{-8} M of 1,25(OH)₂D₃. Fold change expression of the vehicle, control was set as 1 (solid line).

Fig. 1. mRNA expression of TNF α relative to the *FokI* genotypes. mRNA expression of TNF α following 72-h 1,25(OH)₂D₃ (VitD) treatments with two concentrations: 10^{-8} M (black box) and 10^{-10} M (grey box), in the peripheral blood mononuclear cells (PBMCs) of 40 subject (FF n = 9; Ff n = 23; ff n = 8). p -trend = 0.05 for 10^{-8} M of 1,25(OH)₂D₃. Fold change expression of the vehicle, control was set as 1 (solid line).

VDR polymorphisms and HLA class II alleles and this interaction in terms of response to cytokines, circulating levels of 25(OH)D₃ and more or less aggressive beta pancreatic pattern (Okazaki, 2007; Dawson-Hughes et al., 2005).

One of the limitations of our study is the difference in mean age between cases and control subjects although this fact wouldn't affect the results considering the controls are older than the subjects with T2D. The main strength of the study is that the results obtained with the case and control design correlate with the *in-vitro* study.

In conclusion, our data supports that VDR *FokI* polymorphism is associated with T2D, and the genotypes Ff and ff of this variant show a reduced response or resistance to the anti-inflammatory action of VitD, suggesting a pathophysiological mechanism for this association.

Disclosure

The authors declare no conflict of interest.

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