Association of vitamin D receptor gene haplotypes with late-onset Alzheimer's disease in a Southeastern European Caucasian population

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Abstract. Vitamin D receptor (*VDR*) gene single nucleotide polymorphisms (SNPs) have been investigated over the past years with the aim of identifying any association with the development of Alzheimer's disease (AD). However, information regarding the potential association of *VDR* SNP haplotypes with AD is limited. The aim of the present study was to provide additional knowledge on the effects of *VDR* haplotypes on the development of late-onset AD in a cohort of Southeastern European Caucasians (SECs). The study sample included 78 patients with late-onset AD and 103 healthy subjects as the control group. *VDR* SNPs that were analyzed were TaqI (rs731236), BsmI (rs1544410) and FokI (rs2228570). The CAC (TaqI, BsmI and FokI) haplotype was found to be associated with a 53% lower risk of developing the disease (OR, 0.47; 95% CI, 0.23-0.96; P=0.04) and the TAC (TaqI, BsmI and FokI) haplotype was associated with an ~6-fold greater risk of developing AD (OR, 6.19; 95% CI, 1.91-20.13; P=0.0028). Female subjects carrying the TAC haplotype had a ~9-fold greater risk of developing AD in comparison to female control subjects (OR, 9.27; 95% CI, 1.86-46.28; P<0.05). The TaqI and BsmI polymorphisms were in high linkage disequilibrium (D'=0.9717, r=0.8467) and produced a haplotype with a statistically significant different frequency between the control and AD group. The TA (TaqI and BsmI) haplotype was associated with an ~8-fold greater risk of developing AD (OR, 8.27; 95% CI, 2.70-25.28; P<0.05). Female TA carriers had an ~14-fold greater risk of developing the disease in comparison to female control subjects (OR, 13.93; 95% CI, 2.95-65.87; P<0.05). On the whole, the present study demonstrates that in the SEC population, TAC and TA are risk haplotypes for AD, while the CAC haplotype may act protectively. SEC women carrying the TAC or TA haplotype are at a greater risk of developing AD, thus suggesting that women are markedly affected by the poor utilization of vitamin D induced by the *VDR* haplotype.

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Abbreviations: VDR, vitamin D receptor, AD, Alzheimer's disease; SECs, Southeastern European Caucasians, SNPs, single nucleotide polymorphisms

Key words: Alzheimer's disease, vitamin D receptor, polymorphism, haplotypes, single nucleotide polymorphisms

Introduction

The role of vitamin D in the development and protection of neural cells has been described in a number of studies both *in vitro* and *in vivo* (1-4). In parallel, over the past years, an increasing interest has emerged for the beneficial effects of vitamin D in neurodegenerative disorders, such as Parkinson's disease, Multiple sclerosis and Alzheimer's disease (AD) (5-13). Studies have concluded that there may be a close interaction between the molecular pathways of vitamin D mechanisms of action and the molecular pathways of AD pathology (14,15). Vitamin D receptor (VDR) is the key protein for the mediation of its functions and any alteration in the receptor's function can potentially lead to a reduced or enhanced translation of the respective genes regulated by vitamin D (14,16). Vitamin D and its association

with AD have been investigated over the past few years more thoroughly since the initial report of Sutherland *et al* (17) of reduced VDR mRNA levels in AD hippocampal and pyramidal cells (17-20). A number of studies have investigated the potential association of *VDR* gene single nucleotide polymorphisms (SNPs) with AD; however, the results thus far have been contradictory (21-24). The aforementioned studies were conducted in different population groups, which might be one of the reasons for the varying results between them. A recent meta-analysis concluded that only the TaqI polymorphism was associated with an increased risk of developing AD; however, the risk alleles and genotypes for each population may differ (25). The majority of the *VDR* gene polymorphisms do not result in an amino acid change in the VDR receptor and therefore have no functional effect on the receptor protein. However, to date, the reported association of *VDR* gene polymorphisms with AD appears to rely either on changes in the utilization of vitamin D, which potentially leads to lower neuroprotective effects, or on the linkage disequilibrium with other polymorphisms located in the 3'-untranslated region of the *VDR* gene (26). Alterations in the utilization of vitamin D can potentially be caused by a lower VDR expression, VDR mRNA stability or affinity to VDR receptor, DNA or retinoid X receptor (RXR) (27). As a result, the altered vitamin D utilization associated with *VDR* gene polymorphisms affects the molecular pathways and the expression of genes involved in the beneficial effects of vitamin D in the neural system, leading to decreased neuroprotection and neurodegeneration (28,29). As demonstrated by a previous study performed by the authors in this field (30), the majority of studies (21-25) investigating *VDR* SNPs and AD have mainly reported results regarding the association of specific polymorphisms with the disease and limited data have been produced on the association with the potential haplotypes. The available information thus far has not provided a definite conclusion that a specific haplotype is associated with AD. To date, the most frequent *VDR* SNPs studied include TaqI, BsmI and FokI. TaqI has been shown to be associated with AD in a previous study (25), FokI can lead to a reduced VDR protein (26) and BsmI can potentially lead to the alteration of *VDR* expression as an intronic polymorphism (31). The present study focused on the aforementioned three polymorphisms. The present study aimed to expand on initial research focusing on investigating the potential association of *VDR* SNP haplotypes produced by TaqI, BsmI and FokI polymorphisms in late-onset AD in a Southeastern European Caucasian (SEC) cohort, a population that has not been studied before as regards *VDR* haplotypes. The results of the present study were also compared with those from previous studies (22,27,32,33) in population groups other than SEC origin.

Patients and methods

Study subjects. The study sample included 78 patients with well-ascertained late-onset AD (median age, 75 years; range. 65-92 years; males, 47.4%; females, 52.6%) and 103 healthy controls (median age, 57 years; range, 51-90 years; males, 49.5%; females, 50.5%) (Table I). The study groups used for this analysis were the same as those in a previous study by the authors (30).

Table I. Demographic data of the study groups.

Parameter	Patients	Controls
Number (n)	78	103
Age, median (years)	75	57
Age, mean (years)	75	60
Max value (years)	92	90
Min value (years)	65	51
Range (difference between highest and smallest value)	27	39
Males, n (%)	37 (47.4)	51 (49.5)
Females, n (%)	41 (52.6)	52 (50.5)

The diagnosis of AD was established based on the current diagnostic criteria for the disease considering: i) A physical examination; ii) the results of the Mini-Mental State Examination (MMSE) questionnaire and Frontal Assessment Battery (FAB); iii) imaging results of brain CT scan and MRI; and iv) biomarker levels in the cerebrospinal fluid Aβ1-42, total-tau and P-tau. Patients were recruited from the Outpatient Clinic of the Cognitive Disorder-Dementia Unit of the Second Department of Neurology at the University General Hospital 'ATTIKON' (Athens, Greece). Sample collection was performed from January, 2018 to February, 2019. The present study was approved by the Scientific Council and Bioethics Committee of the University General Hospital 'ATTIKON' (Reg. no. 2812; December 21, 2017). Written informed consent for participation in the study and the use of their genetic data was obtained from all participants under Regulation (EU) 2016/679 (General Data Protection Regulation) and according to the Helsinki Declaration (64th World Medical Association, General Assembly, 2013).

DNA isolation. Blood samples of patients and controls were analyzed to determine the genotypes of the SNPs TaqI (rs731236), BsmI (rs1544410) and FokI (rs2228570) of the *VDR* gene. DNA extraction was performed from 200 μl whole blood samples using the NucleoSpin® Genomic DNA from Tissue kit (Macherey-Nagel GmbH & Co. KG).

Analysis of BsmI and FokI polymorphisms. Genotypes of BsmI (rs1544410) and FokI (rs2228570) polymorphisms were determined using the restriction fragment length polymorphism (RFLP) method. Following the initial DNA extraction, polymerase chain reaction (PCR) was performed in order to amplify the segment of the *VDR* gene that includes the two polymorphisms. For PCR the Go Taq® G2 Hot Start polymerase (Promega Corporation) and the thermal cycler DNA Engine® (Bio-Rad Laboratories, Inc.) were used. Suitable primers for each polymorphism were designed for PCR (Table II). For *BsmI* an initial polymerase activation and denaturation step at 95˚C for 5 min was followed by 35 amplification cycles for each sample. Cycles included denaturation (94˚C for 30 sec), annealing (65˚C for 40 sec) and extension (73˚C for 1 min). For *FokI* an initial polymerase activation and denaturation step at 95˚C for 5 min was

Table II. DNA sequence of forward and reverse primer for BsmI and FokI PCR amplification.

Polymorphism	Primer	DNA sequence
BsmI	Forward	5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3'
	Reverse	5'-AACCAGCGGGAAGAGGTCAAGGG-3'
FokI	Forward	5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'
	Reverse	5'-ATGGAAACACCTTGCTTCTCCTCCCTC-3'

Table III. Detection of BsmI and FokI genotypes based on DNA fragment sizes in gel electrophoresis.

	BsmI	FokI
PCR product	825 bp	265 bp
Homozygous sample	825 bp (AA)	265 bp (CC)
Homozygous sample	650 and 175 bp (GG)	196 and 69 bp (TT)
Heterozygous sample	825, 650 and 175 bp (GA)	265, 196 and 69 bp (TC)

followed by 32 amplification cycles for each sample. Cycles included denaturation (94˚C for 30 sec), annealing (60˚C for 40 sec) and extension (73˚C for 1 min). The final PCR product for BsmI and FokI had a size of 825 and 269 bp, respectively. Digestion with the appropriate endonuclease *BsmI* (New England Biolabs Inc., USA) or *FokI* (New England Biolabs Inc.) was followed by incubation of the samples at 65 and 37˚C, respectively. The *BsmI* restriction enzyme recognizes the 5'-GAATGC-3' sequence and detects a G to A substitution, while the *FokI* restriction enzyme recognizes the 5'-GGATG-3' sequence and detects a T to C substitution and the loss of the first ATG start codon of *VDR* gene mRNA. Genotypes of the patient and control samples were finally determined with electrophoresis of the DNA fragments, produced in the digestion stage, in Nusieve 2% w/v agarose gel. Detection of the result of electrophoresis was possible under an UV source at 302-366 nm. Homozygous AA samples produced one band, homozygous GG samples produced two bands and heterozygous GA samples produced three bands for the BsmI polymorphism. Accordingly, homozygous CC samples produced one band, homozygous TT samples produced two bands and heterozygous TC samples produced three bands for the FokI polymorphism following the completion of the DNA electrophoresis stage (Table III).

Analysis of TaqI polymorphism. The analysis of the Taq1 polymorphism was performed as described in a previous study by the authors on *VDR* polymorphisms and AD (30).

Statistical analysis. Data from genotype results were analyzed using SNPstats software (Catalan Institute of Oncology, 2006) (34). Logistic regression was applied to analyze the relation of the genotypes in each inheritance model with the disease and odds ratios (OR) with 95% confidence interval (CI) values were calculated. Logistic regression was also applied to analyze the relation of the possible haplotypes with the disease. The effect of sex was included in the regression analysis as a covariate. A value of $P<0.05$ was considered to indicate a statistically significant difference. All data were also tested for the Hardy-Weinberg equilibrium.

Results

Allele C in the TaqI polymorphism was found to be associated with a 46% lower risk (OR, 0.54; 95% CI, 0.30-0.99; P=0.045) of developing AD in the dominant model of inheritance TT vs. CT + CC (Table IV). In addition, the TT genotype was found to be associated with a 1.9-fold greater risk of developing the disease (OR, 1.85; 95% CI, 1.01-3.37; P=0.045) in the recessive model of inheritance CC + TC vs. TT (Table IV).

No statistically significant differences were observed between the genotype frequency of the BsmI or FokI polymorphisms in the control and AD group (Tables V and VI).

The frequencies of the possible haplotypes produced from the three polymorphisms (TaqI, BsmI and FokI) are presented in Table VII. The haplotype association with the disease analysis revealed two haplotypes that exhibited a statistically significant difference between the control and AD group. The CAC haplotype was associated with a 53% lower risk of developing the disease (OR, 0.47; 95% CI, 0.23-0.96; P=0.04). On the contrary, the TAC haplotype was associated with an ~6-fold greater risk of developing AD (OR, 6.19; 95% CI, 1.91-20.13; P=0.0028) (Table VIII). When haplotype association with the disease analysis included sex as covariate, it was observed that female subjects carrying the TAC haplotype had an ~9-fold greater risk of developing AD (OR, 9.27; 95% CI, 1.86-46.28; $P<0.05$) in comparison to the female control subjects (Table IX).

The TaqI and BsmI polymorphisms were in high linkage disequilibrium (D'=0.9717, r=0.8467) and haplotype analysis revealed that the TA haplotype was associated with an ~8-fold greater risk of developing AD (OR, 8.27; 95% CI, 2.70-25.28; $P<0.05$) (Table X). When sex was included in the haplotype analysis as a covariate, it was observed that female TA carriers had an ~14-fold greater risk of developing the disease (OR, 13.93; 95% CI, 2.95-65.87; $P<0.05$) in comparison to the female control subjects (Table XI).

Table IV. Frequencies of TaqI genotypes in the different inheritance models.

	TaqI rs731236 association with AD (n=181)				
Model	Genotype	Controls n (%)	AD n (%)	OR (95% CI)	P-value
Codominant	TT	35 (34%)	38 (48.7)	1.00	0.088
	TC	49 (47.6)	32 (41.0)	0.60 (0.32-1.14)	
	CC	19 (18.4)	8 (10.3)	0.39 (0.15-1.00)	
Dominant	TT	35 (34)	40 (51.3)	1.00	0.045
	TC/CC	68 (66)	46 (51.1)	0.54 (0.30-0.99)	
Recessive	TT/TC	84 (81.5)	70 (89.7)	1.00	0.12
	CC	19 (18.4)	8 (10.3)	0.51 (0.21-1.22)	
Recessive (for T allele)	CC/TC	68 (66%)	40 (51.3%)	1.00	0.045
	TT	35 (34%)	38 (48.7%)	1.85 (1.01-3.37)	

AD, Alzheimer's disease; OR, odds ratio; CI, confidence interval.

Table V. Frequencies of BsmI genotypes in the different inheritance models.

	BsmI rs1544410 association with AD (n=181)				
Model	Genotype	Controls n (%)	AD n (%)	OR (95% CI)	P-value
Codominant	GG	33 (32%)	30 (38.5%)	1.00	0.076
	GA	51 (49.5%)	26 (33.3%)	0.56 (0.28-1.11)	
	AA	19 (18.4%)	22 (28.2%)	1.27 (0.58-2.80)	
Dominant	GG	33 (32%)	30 (38.5%)	1.00	0.37
	GA/AA	70 (68%)	48 (61.5%)	0.75 (0.41-1.40)	
Recessive	GG/GA	84 (81.5%)	56 (71.8%)	1.00	0.12
	AA	19 (18.4%)	22 (28.2%)	1.74 (0.86-3.50)	

AD, Alzheimer's disease; OR, odds ratio; CI, confidence interval.

Table VI. Frequencies of FokI genotypes in the different inheritance models.

	FokI rs2228570 association with AD (n=181)				
Model	Genotype	Controls n (%)	AD n (%)	OR (95% CI)	P-value
Codominant	CC	55 (53.4%)	34 (43.6%)	1.00	0.20
	TC	38 (36.9%)	39 (50%)	1.66 (0.89-3.08)	
	TT	10 (9.7%)	5 (6.4%)	0.81 (0.25-2.57)	
Dominant	CC	55 (53.4%)	34 (43.6%)	1.00	0.19
	TC/TT	48 (46.6%)	44 (56.4%)	1.48 (0.82-2.68)	
Recessive	CC/TC	93 (90.3%)	73 (93.6%)	1.00	0.42
	TT	10 (9.7%)	5 (6.4%)	0.64 (0.21-1.95)	

AD, Alzheimer's disease; OR, odds ratio; CI, confidence interval.

Discussion

VDR polymorphisms can potentially affect the functions of vitamin D by altering the ability of the receptor to utilize the vitamin ligand. Poor utilization of vitamin D deprives the neural system from its beneficial effects in terms of neuroprotection, neuroinflammation, calcium homeostasis, amyloid β regulation, degradation and cellular lipid

Table VII. Frequencies of possible haplotypes.

	Haplotype frequencies estimation (n=181)					
TaqI rs731236	BsmI rs1544410	FokI rs2228570	Total	Controls	AD	Cumulative frequency
T	G	C	0.4093	0.4070	0.4130	0.4093
C	A	C	0.2305	0.2819	0.1567	0.6398
T	G	T	0.1456	0.1511	0.1383	0.7854
C	A	T	0.1365	0.1305	0.1510	0.9219
T	A	C	0.0587	0.0196	0.1163	0.9806
T	A	T	0.0135	0.0000	0.0248	0.9941
C	G	C	0.0059	0.0099	0.0000	1.0000

AD, Alzheimer's disease.

Table VIII. Haplotype association with AD.

	Haplotype association with AD (n=181)				
TaqI rs731236	BsmI rs1544410	FokI rs2228570	Frequency	OR (95% CI)	P-value
T	G	C	0.4095	1.00	-
C	A	C	0.2297	0.47 (0.23-0.96)	0.04
T	G	T	0.1453	0.90 (0.43-1.88)	0.77
C	A	T	0.1373	1.10 (0.50-2.45)	0.81
T	A	C	0.0593	6.19 (1.91-20.13)	0.0028
T	A	T	0.013	-	<0.0001
C	G	C	0.006	-	-

AD, Alzheimer's disease; OR, odds ratio; CI, confidence interval.

Table IX. Haplotype analysis with covariate sex.

	Haplotype interaction with the covariate sex (n=181)		
Haplotype	Frequency	Female OR (95% CI)	Male OR (95% CI)
TGC	0.4093	1.00	1.26 (0.37-4.32)
CAC	0.2299	0.51 (0.19-1.35)	0.51 (0.16-1.59)
TGT	0.1455	0.79 (0.25-2.49)	1.12 (0.38-3.34)
CAT	0.1371	0.87 (0.25-3.03)	1.56 (0.38-6.38)
TAC	0.0592	9.27 (1.86-46.28)	4.53 (0.68-30.11)
TAT	0.013	-	-
CGC	0.006	-	-

OR, odds ratio; CI, confidence interval.

homeostasis (2,15,28,29,35-37). BsmI is an intronic polymorphism in the ligand binding site of the receptor. However, it can affect VDR expression by altering the stability of various mRNAs and may result in changes in the splicing process (31). The TaqI polymorphism is located in the last exon of *VDR* and it is a synonymous substitution. It has also been reported that it can alter the stability of mRNAs or the generation of different splicing regulatory elements (22). The FokI polymorphism is located in exon 2 and results in the loss of the first translation initiation codon of *VDR* mRNA (ATG → ACG). The VDR protein produced is three amino acids shorter; however, it has been characterized as transcriptionally more active (26).

Table X. TaqI and BsmI haplotypes association with AD.

	Haplotype association with AD (n=181)			
TaqI rs731236	BsmI rs1544410	Frequency	OR (95% CI)	P-value
T	G	0.5548	1.00	-
C	A	0.367	0.67 (0.43-1.07)	0.095
T	A	0.0722	8.27 (2.70-25.28)	<0.05
C	G	0.006	-	1.000

AD, Alzheimer's disease; OR, odds ratio; CI, confidence interval.

Table XI. TaqI and BsmI haplotype analysis with covariate sex.

	Haplotype interaction with the covariate sex (n=181)		
Haplotype	Frequency	Female OR (95% CI)	Male OR (95% CI)
TG	0.5548	1.00	1.31 (0.51-3.32)
CA	0.367	0.65 (0.32-1.29)	0.86 (0.39-1.91)
TA	0.0722	13.93 (2.95-65.87)	5.10 (0.89-29.40)
CG	0.006	-	-

OR, odds ratio; CI, confidence interval.

Previous research has reported the potential association of specific *VDR* polymorphisms with AD; however, the results have not been consistent (21-25). Thus far, the TaqI and ApaI polymorphisms have been reported to be associated with AD in Northwestern European Caucasians (UK) (22). In addition, the ApaI polymorphism has been shown to be associated with the disease in Northeastern European Caucasians (Poland) and in the Turkish population (21,24). Moreover, the TaqI polymorphism has been reported to be associated with the disease in the Asian population and in SECs (Greece) (25,30). In other population groups studied, such as the Iranian population or the Spanish population, the aforementioned associations were not confirmed (23,38). The available data regarding the potential association of haplotypes produced by *VDR* polymorphisms with AD have been very limited to date. A previous study on a Turkish population with late-onset AD reported that the AT (ApaI and TaqI) haplotype had a higher frequency in the control group with a statistically significant difference in comparison to the AD group, and suggested a protective role of the haplotype against AD (21). A second study again in the Turkish population, which studied all *VDR* polymorphisms (TaqI, ApaI, Tru9I, BsmI and FokI), demonstrated that the 'TaubF' or TCAGC haplotype had a statistically significantly higher frequency in the AD group and was thus associated with an increased risk of developing the disease (27). The studies from Turkey produced contradictory results as regards the effect of allele T of the TaqI polymorphism in the two respective haplotypes. In the AT haplotype, the T allele participates in a potentially protective haplotype, while in TCAGC, the same allele participates in a risk haplotype. The results from the present study, although not directly comparable, since not all VDR polymorphisms were analyzed, appear to be in agreement with those of the study from Turkey (27), which reported the risk TCAGC haplotype, for the TaqI and FokI allele, but not for the BsmI allele. In the present study, the TAC haplotype (TaqI, BsmI and FokI) was identified as risk factor for AD, since it was associated with a 6-fold greater risk of developing the disease in comparison to the control group (Table VIII). Another study from the UK on patients with AD reported that the CA haplotype (TaqI and ApaI) was associated with an increased risk of developing the disease (22); however, these findings are not in agreement with the results of the present study in terms of the presence of the C allele in TaqI.

The available data regarding the association of *VDR* SNP haplotypes with cognitive function include two more studies which however did not include patients with AD, but had large sample sizes. The first study from the Netherlands, which included participants at the age of 85 (median value) and a mean follow-up period of 4.2 years, reported that the CAA haplotype (TaqI, ApaI and BsmI) was associated with a deteriorating performance in cognitive tests (32). The second study from the USA which included 2,321 subjects, reported that the ACG haplotype (TaqI, ApaI, BsmI) was associated with a worse cognitive performance in women (33). Overall, the available studies investigating the potential association of specific *VDR* haplotypes with AD and the potential effects of these haplotypes on the mechanisms of action of vitamin D have not yet reached a solid conclusion (Table XII). It appears that the results are affected by the population group studied each time.

Table XII. *VDR* Haplotypes reported to be associated with AD or cognitive decline.

Author/(Refs.)	TaqI allele	ApaI allele	Tru9I allele	BsmI allele	FokI allele	Population
Gezen-Ak *et al* (27)	T	C	A	G	C	AD
Lehman *et al* (22)	C	A				AD
Kuningas *et al* (32)	C	A		A		General
Beydun *et al* (33)	T	C		G		General
Present study	T			A	C	AD

AD, Alzheimer's disease.

In the SEC cohort studied in the present study, the TaqI and BsmI polymorphisms were in high linkage disequilibrium and therefore, it was deemed appropriate to analyze the data excluding the FokI polymorphism. The analysis revealed that carriers of haplotype TA (TaqI and BsmI) had an ~8-fold greater risk of developing AD (Table X). Both polymorphisms can potentially alter the stability of mRNA leading to decreased translation (22,31). It may be hypothesized that the combined effect of alleles T and A in these polymorphisms can potentially affect, to a considerable degree, the utilization of vitamin D, leading to reduced neuroprotection and finally, to an increased risk of developing the disease in SECs. Moreover, the results of the present study in the specific population studied demonstrated that allele A in BsmI increased the risk of developing AD in the TaqI allele T carriers. TaqI TT carriers had a 1.8-fold greater risk of developing AD (Table IV), while TA haplotype carriers had an 8-fold greater risk of developing the disease. Overall, the findings of the present study on *VDR* haplotypes support the argument of the large genetic variability observed among European populations. *VDR* SNPs and haplotypes appear to have a differential effect in SECs in comparison to other European populations as regards the development of AD.

Another interesting finding of the present study was revealed when the data were analyzed with regards to the sex of the participants. Female carriers of haplotypes TAC and TA had an ~9- and ~14-fold greater risk of developing AD, respectively in comparison to the control female subjects. In general, data from meta-analysis studies have demonstrated that the prevalence of AD is higher in females in comparison to males, and that this difference will continue for the ensuing decades, mainly due to the longer life span of females (39-41). The incidence of AD has also been reported by studies to be higher in women; however, data regarding the incidence of AD among males and females appear to differ according to the geographical region of each study (42-44). However, the higher risk of developing the disease in TAC/TA female carriers observed in the present study, indicates that *VDR* haplotypes may contribute to the increased risk of AD in females and that the decreased utilization of vitamin D may be more detrimental for females in comparison to males. Since the clinical manifestations of late-onset AD occur at post-menopausal ages in women, one of the factors that has been detected to contribute to the risk of developing the disease is the low level of estrogens. A number of studies have reported that the neuroprotective effects of estrogens in neural cells against amyloid β-induced neurotoxicity are based on amyloid β regulation or degradation or other molecular mechanisms (45-51). Low vitamin D levels or the poor utilization of vitamin D due to *VDR* SNPs in post-menopausal women elevates the risk of developing AD. In addition, vitamin D is involved in the regulation and has been proposed to play a crucial role in estradiol synthesis (52,53). What is generally considered as a fact is that both levels of vitamin D and estrogens are very low at the age of 65, from which the diagnosis of late-onset AD is possible when dementia symptoms are present.

The present study had certain limitations which need to be stated. Two main limitations have to be reported for the present study. The first is the statistically significant difference between average ages in the control and patient group (t-test, $P<0.01$; only the average ages were statistically analyzed; no other patient characteristics were statistically analyzed). The control subjects were younger than the patients. The second limitation was the small study sample, which affects the statistical power and safety of the conclusions. In addition, vitamin D and estrogen levels of the study subjects were not analyzed.

The present study aimed to investigate potential *VDR* SNP haplotypes associated with late-onset AD in a population who has presented a high genetic variability and has not previously studied as regards *VDR* haplotypes. The results from the present study indicate that the TAC (TaqI, BsmI, FokI) and TA (TaqI, BsmI) haplotypes may increase the risk of developing late-onset AD in SECs. Moreover, it was noted that women in the SEC population who are TAC/TC carriers face a greater risk of developing the disease, leading to the assumption that TAC/TA haplotypes potentially affect in a higher degree female subjects in terms of low vitamin D utilization. In females in the SEC population, the overall risk is possibly multiplied due to other conditions present, such as low estrogen levels. However, the findings of the present study need to be confirmed in a larger sample size of the same or other population in order to reach more definitive conclusions.

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Availability of data and materials

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

Authors' contributions

ND conceived the study and provided the control samples and data. MSK and ML performed the sample analysis. ED obtained the ethics committee study approval, performed the literature review, and the statistical and data analyses, and was responsible for the manuscript composition under the supervision and assistance of ND, CK and KA. DAS and AT contributed to the editing of the final manuscript. KA and CK reviewed and analyzed the results of the statistical analysis. DAS, AT, SP and VP contributed to the collection of the clinical data and patient scores. SP, PM and CK also provided the patient samples. PM contributed to the design and optimization of the RFLP analytical method used in this study and in the editing of the final manuscript. All authors discussed the results and agreed on the conclusions of the study and all authors have read and approved the final manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Scientific Council and Bioethics Committee of the University General Hospital 'ATTIKON' (Reg. no. 2812; December 21, 2017). Written informed consent for participation in the study and the use of their genetic data was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. All the other authors declare that they have no competing interests.

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