Vitamin D status and pathway genes in five European autoimmune Addison's disease cohorts

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Abstract

Objective: While vitamin D regulates immune cells, little is known about it in autoimmune Addison's disease (AAD). We investigated the vitamin D status in AAD patients from five European populations to assess its deficiency. In addition, we studied two case-control cohorts for vitamin D metabolism and pathway genes.

Design: Cross-sectional study.

Methods: A total of 1028 patients with AAD from Germany ($n = 239$), Italy ($n = 328$), Norway ($n = 378$), UK ($n = 44$) and Poland ($n = 39$) and 679 controls from Germany ($n = 301$) and Norway ($n = 378$) were studied for 25(OH)D₃ (primary objective). Secondary objectives (1,25(OH)₂D₃ and pathway genes) were examined in case-controls from Germany and Norway correlating 25(OH)D₃ and single nucleotide polymorphisms within genes encoding the vitamin D receptor (VDR), 1- α -hydroxylase (CYP27B1), 25-hydroxylase (CYP2R1), 24-hydroxylase (CYP24A1) and vitamin D binding protein (GC/DBP).

Results: Vitamin D deficiency (25(OH)D₃ 10–20 ng/mL) was highly prevalent in AAD patients (34–57%), 5–22% were severely deficient (<10 ng/mL), 28–38% insufficient (20–30 ng/mL) and only 7–14% sufficient (>30 ng/mL). Lower 25(OH)D₃ and 1,25(OH)₂D₃ levels were observed both in Norwegian and German AAD ($P = 0.03/0.003$ and $P = 1 \times 10^{-5}/< 1 \times 10^{-7}$, respectively) the former was associated with CYP2R1 (rs1553006) genotype G. Whereas controls achieved sufficient median 25(OH)D₃ in summers (21.4 to 21.9 ng/mL), AAD patients remained largely deficient (18.0 to 21.2 ng/mL) and synthesize less 1,25(OH)₂D₃.

Conclusion: Vitamin D deficiency and insufficiency are highly prevalent in AAD patients. The vitamin D status of AAD may be influenced by genetic factors and suggests individual vitamin D requirements throughout the year.

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Introduction

Autoimmune Addison's disease (AAD) is a rare disorder of the adrenal glands and may be a morbid component of autoimmune polyendocrine syndrome (APS) (1). Vitamin D deficiency is frequently observed in several

autoimmune diseases (2). Furthermore, in rare cases of monogenic vitamin D system disorders autoimmune diseases such as type 1 diabetes or multiple sclerosis have been described (3, 4, 5). This implies an enhanced

risk for autoimmunity in association with vitamin D deficiency. In addition, genetic variation of the vitamin D activating enzyme CYP27B1 at the population level has been found to be associated with AAD, in addition to other endocrinopathies such as type 1 diabetes, thyroid autoimmunity (6) and multiple sclerosis (7).

Vitamin D levels regulate both innate and acquired immunity through several mechanisms: vitamin D receptor (VDR) and the vitamin D biosynthetic enzymes are expressed in macrophages, monocytes, activated T-lymphocytes and B-lymphocytes (8). Experimental animal models lacking the VDR or the activating enzyme CYP27B1 show immune defects. In contrast, models with tissue specific VDR over-expression display anti-inflammatory effects as observed in airway cells (9, 10, 11, 12, 13). Vitamin D facilitates extensive immune actions as shown by genome-wide analyses of vitamin D binding to chromatin. Vitamin D acts on distinct genes where the VDR interacts with other transcription factors to induce cellular signalling (14). This chromatin targeting has identified epigenomic regulators of disease susceptibility regions in type 1 diabetes and other autoimmune disorders (15).

Since the vitamin D status can be corrected by supplementation, it represents a remediable risk factor. We therefore investigated the vitamin D status, genetic variants of its activation, binding and metabolism in order to define requirements in AAD patients.

Study participants and methods

Study design

All study participants in this cross-sectional study (16) were recruited from February 2000 to June 2014 as part of the Euradrenal Consortium (www.euradrenal.org).

One thousand and twenty-eight AAD cases, Caucasian origin were included from Germany ($n=239$),

Italy ($n=328$), Norway ($n=378$), UK ($n=44$) and Poland ($n=39$). The clinical diagnosis of all AAD patients was confirmed on the basis of a low basal serum cortisol with a high adrenocorticotrophic hormone (ACTH) level (patient characteristics are provided in Table 1).

In addition, 679 individuals without autoimmune diseases and of Caucasian origin served as a control group: 301 Germans (166 female and 135 male; median age 33 years) and 378 Norwegian (132 female and 246 male; median age 45 years).

The primary study objective $25(\text{OH})\text{D}_3$ was analysed in all participants. The secondary study objective $1,25(\text{OH})_2\text{D}_3$ was measured in German and Norwegian cases and healthy controls.

Finally a secondary analysis (in Norwegian cases and healthy controls) included 25 single nucleotide polymorphisms (SNPs) within genes that encode: VDR; $1-\alpha$ -hydroxylase; 25 -hydroxylase (CYP2R1); 24 -hydroxylase and vitamin D binding protein.

Ethical approval

The study participants were locally recruited from the corresponding Endocrine Outpatient Clinic of the different centres. Study protocols were approved by the regional Ethics Committees: Italy (Padua ref. No 1583P and Perugia ref. No 1247/08), Poland (Warsaw, ref. No 18/4/2007), Norway (Bergen ref. No 047.96 and 2013_1504), UK (Newcastle, ref. No 05/Q1206/144) and Germany (Frankfurt am Main, ref. No 49/09).

All analyses were carried out in accordance with the ethical votes from the contributing countries. The Ethics committees' votes included the analysis of Addison's disease risk factors in biological samples with pseudonymized patient data from the primary study (Euradrenal). All participants gave informed, written consent.

Table 1 Characteristics of the patients with autoimmune Addison's disease.

Country	Centre	AAD, n			Age (years) at blood collection*	APS2, n (%)
		Total	F	M		
Norway	Bergen	378	234	144	51 (39–63)	193 (51)
Germany	Frankfurt am Main	239	179	60	49 (39–59)	136 (57)
Italy	Perugia/Padua	328	220	108	39 (30–50)	180 (55)
Poland	Warsaw	39	29	10	51 (39–59)	26 (67)
United Kingdom	Newcastle	44	31	13	56 (45–62)	16 (36)

*Data presented as median (IQR).

AAD, autoimmune Addison's disease; APS2, polyglandular syndrome 2; F, female; M, male; IQR, interquartile range.

Determination of vitamin D metabolites

Stored frozen plasma aliquots (-80°C) obtained from the different participating centres were analysed in the hormone laboratory in the Department of Internal Medicine I, Frankfurt am Main. Both 25-hydroxyvitamin D₃ (25(OH)D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) levels were measured using a RIA (for 25(OH)D₃ DiaSorin, Stillwater, Minnesota, USA and for 1,25(OH)₂D₃ IDS, Frankfurt am Main, Germany). The assays were performed in accordance with the manufacturer's protocols. The intra-assay variation coefficient (CV) range from 8.6 to 12.5% in the 25(OH)D₃ RIA and 8.4 to 12.9% in the 1,25(OH)₂D₃ RIA. Radioimmunoassays were standard at the time of analysis, had been applied for clinical use at the University of Frankfurt and used in numerous studies (17, 18). Routine quality control procedures were applied for both RIAs.

Analysis of single nucleotide polymorphisms

Five vitamin D pathway genes were analysed by the Euradrenal consortium: *VDR* (Chr12), *CYP2R1* (25-hydroxylase, Chr11), *CYP27B1* (Chr12), *CYP24A1* (24-hydroxylase, Chr20), *DBP* (vitamin D binding protein, Chr4). The secondary analysis included the correlation of 25(OH)D₃ and vitamin D-related gene polymorphisms within the mentioned genes. The majority of the investigated polymorphisms within these genes are located in the intron region:

rs11168275, rs10875693, rs2189480, rs2254210, rs3819545, rs10741661, rs11023374, rs1553006, rs4646536, rs703842, rs10876994, rs1570669, rs2181874, rs2209314, rs2296241, rs2762941, rs3787555, rs4809959, rs4809960, rs927650 and rs2298849. The polymorphisms *FokI* rs10735810/rs2228570, *DBP* rs4588, *DBP* rs7041 and rs2296241 are located in the exon region while rs703842 is situated in the 5' flanking region (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article, NCBI: <http://www.ncbi.nlm.nih.gov>).

SNP genotyping of Norwegian samples was performed using the Sequenom MassARRAY platform (Sequenom, San Diego) according to the manufacturer's instructions (<http://www.sequenom.com>) and explained in detail by Mitchell *et al.* (19). There was no deviation from Hardy-Weinberg equilibrium at any tested locus ($P > 0.05$).

Statistical analysis

Statistical analyses were performed using BiAS software, version 11 (epsilon-Verlag, Darmstadt, Germany).

Values for 25(OH)D₃ (ng/mL) and 1,25(OH)₂D₃ (pg/mL) (log base 10 transformed and not transformed) were checked for normal distribution using the Kolmogorov-Smirnov test.

As these values were not normally distributed, non-parametric statistical tests were applied.

We defined seasons as winter (November–March) and summer (April–October). The comparison of 25(OH)D₃ (continuous variable) between summer and winter in each centre was done using Wilcoxon-Mann-Whitney-*U*-test.

The comparison of 25(OH)D₃ and 1,25(OH)₂D₃ levels as continuous variables was performed separately for two centres using the Wilcoxon-Mann-Whitney-*U*-test. A subgroup analysis based on disease phenotype (isolated AAD/APS2) was also performed using the same test. The influence of the confounding factor season (summer, winter) but also sex on 25(OH)D₃ levels was corrected using bi-factorial rank variance analysis.

The Chi square test was used to compare 25(OH)D₃ deficiency (as a dichotomous variable) between AAD patients and healthy controls. Measurements were separated by the threshold of 25(OH)D₃ < 20 ng/mL (deficiency) and > 20 ng/mL (sufficiency) (20). The *P* value for season was adjusted by Cochran-Mantel-Haenszel test.

The correlation analysis between the continuous variable 25(OH)D₃ levels and genotypes was performed primarily using the Kruskal-Wallis test. If a $p_{\text{global}} < 0.05$ was calculated, a subsequent *post hoc* test using an unpaired Wilcoxon-Mann-Whitney-*U*-test with Bonferroni correction was applied and adjusted for season using bi-factorial rank variance analysis.

The Chi square test was used to examine the relationship between 25(OH)D₃ deficiency (as a dichotomous variable) and the investigated SNPs and corrected for season using Cochran-Mantel-Haenszel test. The data are presented as medians, odds ratios (OR) and corresponding 95% confidence intervals (95% CI). *P* values ≤ 0.05 were regarded as being statistically significant.

Results

25(OH)D₃ levels in European cohorts

The 25(OH)D₃ status of most European AAD patients was vitamin D deficient with a plasma level of less than 20 ng/mL. Only 7–14% of the patients had an optimal 25(OH)D₃ level of >30 ng/mL. The proportion of vitamin D insufficient patients (20–30 ng/mL) ranged

A

25(OH)D₃ < 20 ng/ml and autoimmune Addison's disease

B

C

Figure 1

Distribution of autoimmune Addison's disease patients – from five European cohorts – according to 25(OH)D₃ status. Categories (A) 25(OH)D₃ deficiency (<20 ng/mL), (B) severely deficient (■ <10 ng/mL), deficient (□ 10–20 ng/mL), insufficient (□ 20–30 ng/mL), optimal (□ >30 ng/mL) and (C) 25(OH)D₃ levels.

between 28–38%, those deficient (10–20 ng/mL) 34–57% and those severely deficient (<10 ng/mL) 5–22% (Fig. 1A and B).

The median 25(OH)D₃ in the European AAD patients was <20 ng/mL: Italy 16.8 ng/mL; Germany 17.0 ng/mL; Norway 19.4 ng/mL; Poland 17.5 ng/mL), and only the United Kingdom patients' median was 21.3 ng/mL (Fig. 1C). 25(OH)D₃ in AAD patients differed for the season of the blood collection: lower 25(OH)D₃ was found in winter compared to the summer (Supplementary Fig. 1).

Norwegian and German cohorts comparison of vitamin D levels between AAD and healthy controls

A significant difference was found in median 25(OH)D₃ between healthy controls and AAD patients for both

Norwegian (healthy controls 21.2 ng/mL vs AAD 19.4 ng/mL, Hodges–Lehmann estimate for the difference (HLS) was 2 ng/mL (95% CI: 0.9, 3.)), adjusted for season = $P_{\text{adjusted}}=0.03$) and German cohorts (healthy controls 19.9 ng/mL vs AAD 17.0 ng/mL, HLS 2.2 ng/mL (95% CI: 0.8, 3.8), $P_{\text{adjusted}}=0.003$) (Fig. 2A).

Furthermore, the median 1,25(OH)₂D₃ in AAD patients was lower (Norwegian AAD 45.7 pg/mL vs healthy controls 51.9 pg/mL, HLS 6.2 pg/mL (95% CI: 3.7, 8.8), $P_{\text{adjusted}}=1 \times 10^{-5}$ and German AAD 41.7 pg/mL vs healthy controls 51.6 pg/mL, HLS 10.1 pg/mL (95% CI: 7.1, 13.0), $P_{\text{adjusted}} < 1 \times 10^{-7}$) (Fig. 2B).

Median 25(OH)D₃ (effect estimate after Rosenthal $R=0.13$; low effect) and 1,25(OH)₂D₃ ($R=0.3$; medium effect) remained significantly different between AAD and healthy controls when stratifying for sex and disease phenotype (Supplementary Figs 2A, B and 3A, B).

Figure 2

Comparison of (A) 25(OH)D₃ (ng/mL) and (B) 1,25(OH)₂D₃ (pg/mL) between patients with autoimmune Addison's disease (AAD) and healthy controls (HC) in Norway and Germany cohorts. *P* values adjusted for season = *P*_{adjusted}.

Comparing 25(OH)D₃ of controls and AAD patients over seasons, a significant difference was observed only in summers but not in winters: 25(OH)D₃ were higher in healthy controls than in AAD in summers (Norway 21.9 vs 21.2 ng/mL *P* = 0.08 and Germany 21.4 vs 18.0 ng/mL *P* = 0.0002) but winters (Norway 18.2 vs 18.3 ng/mL and Germany 16.2 vs 15.0 ng/mL, both *P* = 0.72) showed no difference (Supplementary Fig. 4). Overall, in the two-cohorts vitamin D deficiency is significantly more frequent in AAD patients than in healthy controls (Germany, OR: 1.92 (95% CI: 1.34–2.76), *P*_{adjusted} = 0.0005 and Norway, OR: 1.24 (95% CI: 1.10–1.95), *P*_{adjusted} = 0.18).

Vitamin D metabolites and SNPs in vitamin D pathway genes

Norwegian AAD patients carrying the genotypes GG of *CYP2R1* rs1553006, had significantly lower 25(OH)D₃ levels compared to healthy controls (*p*_{adjusted} = 0.004) (Table 2).

The allele G of *CYP2R1* (rs1553006) was associated with vitamin D deficiency in the Norwegian patients (OR 1.83, *p*_{adjusted} = 0.0005). Allele C of *CYP24A1* (rs3787555, OR 1.41), allele T of *VDR* (rs2189480, OR 1.37) and allele T of *DBP* (rs4588, OR 1.43) showed no association with vitamin D deficiency after adjustment (*p*_{adjusted} = 0.1, respectively), Table 3. No association was observed between 25(OH)D₃ and other SNPs within *VDR*, *CYP2R1*, *CYP27B1*, *CYP24A1* and *DBP* in the Norwegian cohort (data not shown).

Discussion

We observe a high prevalence of vitamin D deficiency in AAD patients from five European cohorts. Only 7–14% had a sufficient vitamin D status leaving the majority in various degrees of deficiency. AAD patients also suffering from APS were similarly affected by vitamin D deficiency. Vitamin D deficiency is a frequent finding both before and at onset of a range of autoimmune disorders (21). Low vitamin D levels may represent a long-standing cofactor in the pathophysiology of immune perturbations or just be an epiphenomenon. Vitamin D deficiency can be acquired, but has a strong genetic component. It is estimated that heritability accounts for 29% of the vitamin D status. In contrast UV-exposure is the major environmental contributor with nutritional intake having a minor impact (22, 23). Skin pigmentation is the primary threshold for UV light to generate the vitamin D precursor metabolite 7-dehydrocholesterol (7-DHC). Since AAD leads to both

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Table 2 25(OH)D₃ (ng/mL) levels and single nucleotide polymorphisms in vitamin D pathway genes in patients with autoimmune Addison's disease and healthy controls from Norway: correlation between the continuous 25(OH)D₃ levels and genotypes.

	<i>n</i>		25(OH)D ₃ (ng/mL)		<i>*P</i> _{adjusted}
	HC	AAD	HC	AAD	
<i>CYP2R1</i> /rs1553006	290	320			
AA			22.2	21.8	
GA			20.9	19.4	0.7
GG			21.3	16.9	0.004
<i>CYP24A1</i> /rs3787555	360	371			
AA			21.4	17.8	
GA			20.7	20.7	
GG			21.7	18.3	0.02
<i>CYP24A1</i> /rs2181874	358	372			
AA			17.2	16.0	
GA			22.9	20.1	0.04
GG			20.8	18.7	0.1
<i>VDR</i> /rs2189480	356	363			
TT			21.8	17.0	0.12
GT			20.6	18.9	0.06
GG			21.4	20.4	
<i>DBP</i> /rs4588	353	373			
TT			21.3	14.4	0.02
GT			20.9	19.5	0.28
GG			21.7	19.5	0.13

**P* values adjusted for season.

CYP2R1, 25-hydroxylase; *CYP24A1*, 24-hydroxylase; *DBP*, VD binding protein; *VDR*, vitamin D receptor.

acute and chronic elevations of ACTH levels, (24) equimolar elevations of alpha-MSH enhance skin pigmentation. Many patients remain hyperpigmented despite adequate glucocorticoid and mineralocorticoid supplementation (25). Reduced UVB exposure is an established cause of vitamin D deficiency (26). This may contribute to vitamin D deficiency in AAD but needs to be studied further. Another reason is the regular glucocorticoid supplementation that may reduce the absorption of vitamin D in the gut. Taken together AAD patients will benefit from vitamin D supplementation throughout the year.

Our second finding is the genetic association of vitamin D deficiency with vitamin D pathway genes in patients. Hereby we can not distinguish whether the genetic determinants for vitamin D deficiency confer disease susceptibility via the low 25(OH)D₃ levels or by a linked genetic mechanism, since most SNPs reside in introns and will affect regulation. Therefore only part of the explanation for vitamin D deficiency in AAD is genetic. Out of three key genes involved in vitamin D metabolism, two have been previously reported to be associated with AAD; namely variants within the *DBP*/GC and *CYP27B1* loci (6, 27, 28) alongside variants in the *VDR* gene (29).

The gene encoding *DBP*/GC has the greatest effect on vitamin D status with genotypes in the top quartile displaying a two-fold risk for vitamin D insufficiency (30).

Another major gene is *CYP2R1* that controls the hepatic hydroxylation to 25(OH)₂D₃. Allelic variation at this locus is consistently associated with vitamin D deficiency across several populations (30, 31). We find this gene to associate with vitamin D deficiency in Norwegian AAD patients. Another gene with major impact on vitamin D metabolism is *NADSYN1/DHCR7* encoding the enzyme required for the generation of 7-DHC. Mutations in *DHCR7* lead to the autosomal recessive Smith-Lemli-Opitz-syndrome, a rare polyendocrine disease manifesting with adrenal insufficiency, hypoparathyroidism, hypothyroidism and immune deficiency in the neonatal period accompanied by reduced cholesterol biosynthesis (32). Adequate supplementation of vitamin D in these cases may improve developmental outcomes in children.

Rare gene mutations with extreme phenotypes can illustrate a mechanism by which an endocrine metabolite such as vitamin D perturbs adrenal function. The more common genetic variants impacting vitamin D metabolism have more subtle effects which can be studied by Mendelian randomization where variants of the vitamin D system show effects on cytokine signalling (33). Furthermore, vitamin D regulates the gene expression of innate and acquired immunity genes, in particular the major histocompatibility complex class I and class II genes. For example, 1,25(OH)₂D₃ has the capacity to

Table 3 Association between 25(OH)D₃ deficiency (<20 ng/mL; as dichotomous variable) and alleles in patients with autoimmune Addison's disease and healthy controls from Norway. Data is presented as *n* (%).

Alleles	Healthy controls		P	Autoimmune Addison's disease patients			*P adjusted
	<20 ng/mL	>20 ng/mL		<20 ng/mL	>20 ng/mL	OR (95% CI)	
CYP2R1/rs1553006							
A	98 (39.8%)	145 (43.4%)	0.44	131 (38.1%)	153 (51.7%)	0.54 (0.40–0.75)	0.0005
G	148 (60.2%)	189 (56.6%)		213 (61.9%)	143 (48.3%)	1.83 (1.33–2.53)	
CYP24A1/rs3787555							
A	90 (29.0%)	110 (26.8%)	0.57	83 (21.1%)	95 (27.3%)	0.71 (0.50–1.00)	0.1
C	220 (71.00)	300 (73.2%)		311 (78.9%)	253 (72.7%)	1.41 (1.00–1.99)	
CYP24A1/rs2181874							
A	52 (17.0%)	87 (21.2%)	0.19	85 (21.6%)	75 (21.4%)	1.00 (0.70–1.42)	1
G	254 (83.0%)	323 (78.8%)		309 (78.4%)	275 (78.6%)	1.00 (0.70–1.43)	
VDR/rs2189480							
T	109 (35.4%)	152 (37.6%)	0.59	154 (39.3%)	103 (30.8%)	1.37 (1.00–1.88)	0.1
G	199 (64.6%)	252 (62.4%)		238 (60.7%)	231 (69.2%)	0.72 (0.53–0.99)	
DBP/rs4588							
T	85 (28.3%)	102 (25.1%)	0.38	109 (27.4%)	71 (20.4%)	1.43 (1.01–2.02)	0.1
G	215 (71.7%)	304 (74.9%)		289 (72.6%)	277 (79.6%)	0.70 (0.49–0.98)	

*P values adjusted for season

CYP2R1, 25-hydroxylase; CYP24A1, 24-hydroxylase; DBP, VD binding protein; VDR, vitamin D receptor.

downregulate HLA-DR antigens on the surface of human monocytes and can dampen the immune response (34).

Active vitamin D acts on several genomic regions that are critical for the immune response, in particular the *HLA* gene cluster on chromosome 6p (35). Elevated 25(OH)D₃ levels after vitamin D treatment enhance chromatin accessibility allowing an epigenetic regulation of target genes. The epigenetic vitamin D response appears to be stronger in immune cells that are often capable of paracrine 1,25(OH)₂D₃ activation (36).

Vitamin D sufficiency in AAD should be achieved for three reasons: adequate intestinal calcium resorption, preventing secondary hyperparathyroidism and reducing fracture risk. Furthermore, a sufficient vitamin D status is required for a robust immune response. Patients with AAD depend on stable immunity because they are more vulnerable to infective agents that might trigger an adrenal crisis. Vitamin D supplementation may reduce the risk for acute respiratory infections particularly if patients were vitamin D deficient before supplementation (37). Patients with AAD are also at increased risk for additional autoimmune diseases, malignancies and cardiovascular disease (38). Vitamin D supplementation has a potential benefit in all three disease entities as systematic reviews suggest: by improving T regulatory function (39), reducing the risk of cancer death (40) or colorectal adenoma occurrence (41), and by lowering several risk factors for cardiovascular disease (42). Benefits of vitamin D supplementation were shown to vary depending on *VDR* genotypes (41). Another *VDR* SNP and one of *CYP2R1* were associated with vitamin D deficiency in the Norwegian AAD cohort, illustrating that effects of vitamin D supplementation vary in different populations and dosing may need to be individualized to reach sufficiency.

Strengths of this study are the large size of the AAD patient cohorts from five European countries, and their vitamin D targeted clinical, biochemical and genetic analyses representing the biggest vitamin D investigation of this rare disease. A shortcoming is that healthy controls from only Norway and Germany were studied.

In summary, patients with AAD are at increased risk to develop vitamin D deficiency for clinical and genetic reasons. Vitamin D should be supplemented in order to prevent deficiency. Due to genetic variability in the vitamin D response, the treatment should be to target by sufficiency testing. At present, vitamin D supplementation is not part of routine endocrine care in AAD but should be considered as such, in particular due to steroid comedication. Whether AAD patients will benefit from prophylactic effects on other comorbidities requires further study.

Limitations

The Euradrenal consortium recruited the largest number of AAD patients possible. Healthy controls were only studied in Norway and Germany for this project part but not from Poland, Great Britain and Italy. Therefore AAD patients from these countries were studied for their vitamin D status without local controls.

25(OH)D₃ serum concentrations are largely confounded by nutrition, ethnicity and season (reflecting UVB exposure) which we reported for a German cohort (18). Here we corrected for the major confounding factor season (summer, winter). Since patients and controls were of Caucasian origin, ethnic confounders are negligible. The potential influence of diet was not determined.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-20-0956>.

Declaration of interest

Simon H S Pearce has received speaker fees from Sanofi, Quidel and Berlin Chemie, and has consulted for Apitope BV. The other authors have nothing to disclose.

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Author contribution statement

M P M and K B wrote the manuscript and the other authors contributed to the final manuscript. G M, A S B W, B K, C B, A F, W O, D U, E S H, S H S P, A L M and K B recruited the study participants and collected clinical data. M P M, A L M and A S B W planned the study and were responsible for the genotyping and vitamin D metabolite analysis. M P M performed statistical analyses. All authors have approved the manuscript and agreed with its submission. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

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