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Associations between serum vitamin D and genetic variants in vitamin D pathways and age-related macular degeneration in the EUREYE study

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This article contains additional online-only material. The following should appear online-only: Table 3, Table 4, Table 5, Table 6.

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Running head: Serum vitamin D, variants in vitamin D genes and age-related macular degeneration

## Abstract

**Objective:** To study associations between early and late age-related macular degeneration (AMD) and the neovascular type of late AMD (nvAMD) with serum 25-hydroxy vitamin D (25(OH)D) and genetic variants in vitamin D pathway genes.

**Design:** Population-based cross-sectional study in a random sample aged 65 and over from seven European countries.

**Participants:** Of 4753 participants, 4496 (2028 men and 2468 women), mean age 73, provided a usable blood sample; 2137 had no signs of AMD, 2209 had early AMD, 150 had late AMD of which 104 nvAMD.

**Methods:** Participants were interviewed for smoking and alcohol use, brief medical history, sunlight exposure and a dietary questionnaire, underwent color fundus photography and provided a blood sample. Fundus images were graded using the modified International Classification System for Age Related Maculopathy. 25(OH)D was measured by liquid chromatography-tandem mass spectrometry and categorized as deficient (< 30 nmol/L), insufficient (30–50 nmol/L), adequate ( $\geq$ 50 nmol/L). Genotyping was performed on a sub-sample of 1284 AMD cases and controls for 93 SNPs from seven genes. Associations were investigated by linear or logistic regression adjusted for potential confounders.

**Main Outcome Measures:** Adjusted odds ratio (AOR) for three outcomes (early AMD, late AMD, nvAMD).

**Results:** No linear association was found with 25(OH)D and early or late AMD or nvAMD.

There was no association between insufficient or deficient status with early or late AMD.

Deficient status was associated with nvAMD (AOR = 1.27, 95% Confidence Interval 1.1-1.45,  $p < 0.0001$ ). Significant ( $p < 0.05$ ) associations with 25(OH)D were found for SNPs in genes *GC*,

*VDR*, *CYP2R1*, *CYP27B1*. Using a initial threshold of  $p < 0.05$ , two SNPs (*VDR*) were associated with early AMD, four SNPs (*RXRA*) and one (*VDR*) with nvAMD and one (*RXRA*), two (*VDR*) and one (*CYP2R1*) with late AMD. After Bonferroni correction, no SNPS were associated with early, late or nvAMD.

Conclusions: 25(OH)D deficient status was associated with nvAMD but the adjusted OR was small and we cannot exclude residual confounding. The hypothesis of a causal association of vitamin D with AMD is not supported by clear evidence for an association of vitamin D pathway SNPs with early, late or nvAMD.

Vitamin D is produced in the skin following exposure to ultraviolet B (UVB) radiation as vitamin D<sub>3</sub> (cholecalciferol), and to a lesser extent, obtained from the diet (principally oily fish).<sup>1</sup> The other natural source of vitamin D, Vitamin D<sub>2</sub> (ergocalciferol) is found in a few foods and provides a much smaller proportion of dietary vitamin D. Other sources of vitamin D include supplements or, in some countries, fortified foods. In the body the biologically active metabolite of vitamin D<sub>3</sub>, 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D<sub>3</sub>) is distributed to the tissues and plays an important role in a number of biological functions, including calcium homeostasis, immune response and insulin metabolism.<sup>2</sup> Serum 25-hydroxyvitamin D (25(OH)D) measures the total circulating vitamin D (D<sub>2</sub> and D<sub>3</sub>) and is used as a measure of vitamin D status. In countries of the European Union where supplement use is relatively low and foods are not fortified, 25(OH)D deficiency has been found in 13% of the overall population.<sup>3</sup> In addition to environmental factors, genetic variants influence 25(OH)D concentrations and single nucleotide polymorphisms (SNPs) in a number of genes related to uptake and metabolism have been identified.<sup>4,5</sup> Recommendations for desirable levels of 25(OH)D of at least 50 nmol/L<sup>6</sup> go beyond avoiding deficiency to maximizing bone health. Vitamin D has also been postulated to influence disease risk across a wide range of other conditions suggesting the potential benefit from increasing vitamin D by diet or supplementation. However, a clear picture has not emerged. A large body of evidence summarized in recent systematic reviews and meta analyses, based on nearly 300 observational studies and 200 randomized controlled trials (RCTs), showed reduced risks of all-cause mortality, cardiovascular disease (CVD) incidence and mortality in the observational studies but not in the RCTs except for all-cause mortality.<sup>7,8</sup>

Age related macular degeneration (AMD) is characterized by progressive degenerative changes in the retina. Late stage AMD consists of neovascular AMD (nvAMD) and geographic atrophy (GA). Hypotheses for an association of vitamin D with AMD are based on the anti-inflammatory role of vitamin D, identification of vitamin D receptors in retinal tissues,<sup>9, 10</sup> and the major role of inflammation and of complement-related genes and complement activation in the development of AMD.<sup>11</sup> CVD and risk factors for CVD such as smoking and diabetes are additionally risk factors for nvAMD.<sup>12</sup> There are inconsistent findings from studies investigating the association of 25(OH)D with AMD.<sup>9, 13-17</sup> Most studies lacked power to investigate late AMD<sup>13-16</sup> or reported an association with early<sup>16</sup> or late<sup>17</sup> AMD only in sub-groups (by gender or age). Early AMD was associated with lower concentrations of 25(OH)D in some<sup>13,15</sup> but not all studies.<sup>14</sup> Other studies investigating vitamin D and late AMD were based on diagnoses from medical records<sup>18-20</sup> or from a case series.<sup>21</sup> Two studies reported inconclusive findings for SNPs in genes influencing 25(OH)D concentrations with early<sup>15</sup> or late AMD<sup>9</sup>. In the absence of clear evidence on this important question, we investigated associations between 25(OH)D and SNPs in seven vitamin D related genes with AMD in the European Eye Study (EUREYE).

## **Methods**

Participants were recruited by random sampling of the population aged 65 years and older in seven centers across Europe: Bergen (Norway), Tallinn (Estonia), Belfast (UK), Paris (France), Verona (Italy), Thessaloniki (Greece) and Alicante, (Spain). Participants were interviewed by trained fieldworkers, underwent fundus photography, and provided blood samples. Information collected at interview included education, smoking and alcohol use, a brief medical history, a semi-quantitative food frequency questionnaire, and a detailed questionnaire to estimate

ultraviolet radiation exposure (UVR) (see below). Written informed consent was obtained from all study participants. Institutional Review Board (IRB)/Ethics Committee approval was obtained for each center. The study adhered to the tenets of the Declaration of Helsinki.

#### *Fundus photography and grading*

After pupillary dilation with tropicamide 0.5% and phenylephrine 5%, two 35° non-simultaneous stereoscopic digitized color fundus images centered on the fovea were obtained of each eye. The fundus images were sent to a single reading center (Erasmus University Rotterdam) and graded using the International Classification System for Age-Related Maculopathy<sup>22</sup> and then categorized into five mutually exclusive grades. Grade 0 was defined as a macula free of drusen or pigmentary irregularities or with hard drusen ( $< 63 \mu\text{m}$ ) only. Early AMD was subdivided in grade 1, defined as soft distinct drusen ( $\geq 63 \mu\text{m}$ ) or pigmentary abnormalities, grade 2 as soft indistinct drusen ( $\geq 125 \mu\text{m}$ ) or reticular drusen only or soft distinct drusen ( $\geq 63 \mu\text{m}$ ) with pigmentary abnormalities, and grade 3 as soft indistinct drusen ( $\geq 125 \mu\text{m}$ ) or reticular drusen with pigmentary abnormalities. Grade 4 was defined as presence of either nvAMD (presence of any of the following: serous or hemorrhagic retinal or retinal pigment epithelial detachment, subretinal neovascular membrane, periretinal fibrous scar) or GA (well-demarcated area of retinal pigment atrophy with visible choroidal vessels).

#### *Measurement of UV exposure*

Full details of UV measurement have been published previously<sup>23</sup>. We used a detailed questionnaire to ascertain lifetime residence and time spent outdoors (from age 14), for work (including homecare), leisure and retirement up to current age. Information from the questionnaire, residence calendar and geographical co-ordinates for residence were sent to the University of East Anglia to estimate years of exposure for different wavelengths of UVR (UVA,



UVB and blue light) using published sources that take into account time of day, month, and latitudinal variations. Personal adult lifetime UV exposure were estimated for each of the three wavelengths of light and summed for a mean annual lifetime dose for all day exposures and for exposures in the middle of the day.

#### *Blood analyses*

Non-fasting venous blood samples were separated within 4 hours of collection and serum was stored at -20°C for up to 4 weeks before frozen samples were transferred to a single laboratory (Queens University Belfast) for storage at -80°C. Antioxidant and cholesterol analyses were carried out within 3 months of storage. Plasma lutein, zeaxanthin, beta cryptoxanthin, alpha and beta-carotene, alpha and gamma tocopherol, lycopene and retinol were measured by reverse phase high pressure liquid chromatography. Plasma vitamin C was measured using an enzyme-based assay in plasma stabilized with metaphosphoric acid. All assays were standardized against appropriate National Institute of Standards and Technology (NIST) standard reference materials. Serum cholesterol was measured using an enzymatic assay (Randox, Crumlin, UK) on a Cobas FARA centrifugal analyzer (Roche Diagnostics, UK). Serum 25-hydroxy vitamin D<sub>2</sub> and D<sub>3</sub> (25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>) were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) 8 to 10 years after initial collection.

#### *SNP selection and Genotyping*

For reason of costs, genotyping was undertaken in a sub-sample of the main study. A random sample of controls (AMD grade 0) was frequency matched on age (within a year), sex and center with all cases of (i) late AMD, and (ii) all cases with early AMD grades 2 and 3. Cases with AMD grade 1 were not investigated. Ninety-three common SNPs located across 7 genes involved in the vitamin D metabolic pathway (*GC* (10), *RXRA* (14), *CYP2R1* (7), *DHCR7* (5),

*VDR* (29), *CYP27B1* (7), *CYP24A1* (21) were selected from Phase III, release 2 HapMap (<http://www.hapmap.org>) CEPH data (Utah residents with ancestry in northern and western Europe; CEU) using Haploview (<http://www.broadinstitute.org/haploview>) to determine linkage disequilibrium. Tag SNPs were selected using multimarker tagging where  $r^2 > 0.8$  for all downloaded SNPs with a minor allele frequency (MAF)  $\geq 5\%$ , genotype call rate  $\geq 95\%$ , and no significant deviation from Hardy Weinberg equilibrium (HWE). Genotyping was performed by KBiosciences (Hoddesdon, United Kingdom). Quality filters for exclusion of SNPs included call rates below 95% and deviation from HWE ( $P < 0.001$ ). DNA samples were excluded if missing genotypes exceeded 10%. Other quality control measures included duplicates on plates, random sample allocation to plates, independent scoring of problematic genotypes by two individuals and re-sequencing of selected DNAs to validate genotypes. KBiosciences quality control also included validation of all SNP assays on a panel of 44 random Caucasian-derived samples plus 4 non-template (negative) controls.

#### *Data processing and statistical analysis*

Statistical analysis was carried out using Stata software version 13 (Stata Corp., College Station, TX) and PLINK (version 1.07) under an additive genotypic model.<sup>24</sup> We investigated total 25(OH)D i.e. 25(OH)D<sub>2</sub> plus 25(OH)D<sub>3</sub> as (i) a continuous variable and (ii) categorized by clinical status based on expert guidelines as: deficient ( $< 30$  nmol/L), insufficient (30–50 nmol/L), and adequate ( $\geq 50$  nmol/L).<sup>6</sup> We excluded extreme values of  $\geq 150$  nmol/L ( $n=17$ ). 25(OH)D was normalized using a square root transformation due to a long right hand tail (110 participants had values  $\geq 100$  and  $< 150$  nmol/L). Dietary vitamin D was estimated using food composition tables<sup>25</sup> and adjusted for total energy intake.<sup>26</sup> All day UVB exposures were square root transformed and lutein concentrations log transformed. We ran preliminary regression

analyses to identify factors associated with 25(OH)D as possible confounders of any association with AMD and carried out a similar analysis for vitamin D status. We used univariable and confounder-adjusted logistic regression to investigate associations between 25(OH)D or vitamin D status and early grades of AMD and nvAMD. We also present results for all late AMD (nvAMD and GA combined) as numbers with GA were too small (n=46) to permit meaningful separate analyses. Analyses took account of the study design by including study centre in models and by use of robust errors in Stata.<sup>27</sup> We report associations of individual SNPs with early, late and nvAMD at a nominal significance of  $p < 0.05$ . Since our *a priori* hypothesis of an association between SNPs in vitamin D-related genes and AMD was based on previously demonstrated associations of vitamin D genes with 25(OH)D,<sup>4 5</sup> we applied a Bonferroni correction based on the number of SNPs within each gene in the AMD analyses in preference to a GWAS type correction for all 92 snps. We also investigated whether any association of 25(OH)D status and nvAMD or early AMD was modified by the main AMD genetic risk loci, rs1061170 in *CFH* and rs10490924 in *ARMS2*, as has been reported previously.<sup>15</sup>

## **Results**

### *Serum 25(OH)D*

Of 4753 participants at the clinical examination, 4496 provided a usable blood sample for the vitamin D assays. Of these 2137 had no signs of AMD, 2209 had early AMD; 1635 (grade 1), 460 (grade 2), 114 (grade 3), 46 (GA) and 104 nvAMD. The mean (standard deviation, SD) of 25(OH)D concentrations was 49 (23) nmol/L. The highest mean seasonally-adjusted concentration was observed in the Bergen center (63 (20) nmol/L) compared to all other centers with adjusted differences ranging from 20 nmol/L lower concentrations in Paris, approximately

15 nmol/L lower in Tallinn, Belfast, Verona and Thessaloniki, to 9 nmol/L in Alicante (all comparisons  $p < 0.0001$ ). Vitamin D deficiency was found in 21% ( $n=944$ ) and 32.8% (1475) were classified as insufficient. Only 13.4% (604) had concentrations of 75 nmol/L or greater. A large number of variables were independently associated with 25(OH)D. In multivariable analyses adjusted for center and season, levels were lower with increasing age, in women, in current smokers, participants with diabetes, and higher in those taking fish oil supplements, and drinking alcohol at least weekly. 25(OH)D was associated with higher UVB exposure, dietary vitamin D intake, serum cholesterol, and plasma concentrations of ascorbate, lutein (or zeaxanthin), and retinol (all associations,  $p < 0.05$  or less). There was no association with education. Lutein and zeaxanthin were highly correlated ( $r=0.85$ ) and therefore associations with 25(OH)D were almost identical. The characteristics of people categorized by 25(OH)D status are shown in Table 1 (univariable analyses). Proportionately fewer people classified as insufficient or deficient took fish oil supplements or consumed oily fish at least weekly, were less likely to be in the highest quartile of dietary vitamin D intake, or dietary docosahexaenoic acid intake, and were more likely to be older or obese compared to those with sufficient levels.

#### *Associations with AMD*

Covariate-adjusted models showed no association of 25(OH)D with any grade of early AMD or late or nvAMD (Table 2). 25(OH)D status was not associated with any grade of early or late AMD (Table 2). For nvAMD, deficient status was associated with a covariate adjusted OR of 1.27, 95% confidence interval (1.11-1.45)  $p < 0.0001$ . Although numbers with GA were small there was no indication of increased odds for deficiency status (OR=0.82, 0.36- 1.89). We found no significant interactions with rs1061170 or rs10490924 with 25(OH)D status and nvAMD ( $p=0.32$  and  $0.30$  respectively) or early AMD ( $p=0.28$  and  $0.11$ ).

### *Genetic associations*

Genotype data were available for 93 SNPs from 1284 participants selected for genetic analysis. One SNP in *GC* was excluded for failing to meet the quality filters of call rates below 95% or deviation from HWE ( $P < 0.001$ ). The average call rate for the remaining SNPs was 99.3%. No participants were excluded due to insufficient genotype data. No duplicate inconsistencies were observed. Results for all 92 SNPs and their age, sex and center adjusted association with 25(OH)D (additionally adjusted for season), early, late, nvAMD are provided in Tables 3-6 (available at <http://www.aaojournal.org>). Nominal significant results are summarized in Table 7. Our results confirmed that SNPs in *GC* (3), *RXRA* (1), *CYP2R1* (2), *VDR* (1) and *CYP27B1* (1) were associated with 25(OH)D. Two SNPs both in *VDR* (rs11574026, rs4516035) were significantly associated with early AMD. Five SNPs were significantly associated with nvAMD: four in *RXRA* (rs11185644, rs12339187, rs10881582, rs3118536,) and one in *VDR* (rs11574077). Analysis of late AMD identified four significantly associated SNPs following the inclusion of participants with GA. These were rs3118536 in *RXRA*, rs10875693 and rs11574077 in *VDR*, and rs11023371 in *CYP2R1*. After applying Bonferroni corrections of  $p = 0.004$  for 14 *RXRA* SNPs and  $p = 0.002$  for 29 *VDR* SNPS, no SNPs were associated with early AMD, nvAMD or late AMD.

### **Discussion**

We found no linear association between 25(OH)D and early or nvAMD. 25(OH)D deficiency status was associated with nvAMD, adjusted OR of 1.27. Insufficient 25(OH)D status was not associated with nvAMD.

Few studies have investigated associations between 25(OH)D and late AMD with inconsistent results. Several studies have used historical medical records and linked information on vitamin D to a diagnosis of late AMD. No association was found with nvAMD in a Medicare study of 7000 people with a diagnosis of Vitamin D deficiency and matched controls followed for 3 years.<sup>18</sup> Retrospective medical records identification of 146 nvAMD patients tested for vitamin D compared with 100 age, sex and race matched controls with pseudophakia, reported an adjusted OR of 0.37 (CI 0.19- 0.72) between the highest and lowest quintile of 25(OH)D.<sup>20</sup> A health records study in Israel of 9267 participants with a routine 25(OH)D measurement identified 1045 individuals with a diagnosis of AMD (grade and severity not available) and found no differences in 25(OH)D compared to non-AMD participants; results were not adjusted for age although AMD cases were older than non-AMD.<sup>19</sup> Using records of a mobile geriatric clinic of 26 late AMD cases and 34 with no AMD signs, 25(OH)D deficiency was associated with a 3 fold OR, 95% CI, 1-9, results were adjusted only for age, sex and season of blood collection.<sup>28</sup>

An inverse association was found between early AMD and 25(OH)D in the US National Health and Nutrition Examination survey (NHANES III)<sup>13</sup> and in women aged < 75 years in the Carotenoids in Age-Related Eye Disease Study (CAREDS study) but not in those aged 75 and over.<sup>16</sup> Neither study found an association with 25(OH) D and late AMD but the number of cases evaluated was small. In the Korean National Health and Nutrition study there was no association with early or late AMD overall; separate analyses by gender found reduced odds of late AMD with increasing 25(OH)D in men but not in women.<sup>17</sup> No association of 25(OH)D concentrations categorized as deficient (<25 nmol/L) or insufficient (25–49 nmol/L) compared to

sufficient ( $\geq 50$  nmol/L) with either early or late AMD, were observed in a small population based study of elderly residents in France.<sup>14</sup>

In our study, 25(OH)D was measured in stored blood samples collected at the same time as the ascertainment of AMD. We cannot therefore exclude the possibility of reverse causation i.e. that nvAMD, directly or indirectly, influenced the 25(OH)D levels, for example as a result of complement-related or other inflammatory effects.<sup>11</sup> We had no information on co-morbidities which may be either consequences of AMD such as depression and anxiety,<sup>29</sup> low physical activity<sup>30</sup> or other age-related conditions associated with AMD such as cognitive impairment<sup>31, 32</sup> or complement-related conditions such as diabetic nephropathy,<sup>33, 34</sup> some of which have been associated with 25(OH)D.<sup>7, 8</sup> The conclusion of one substantial review of vitamin D and a wide range of health outcomes highlighted a lack of highly convincing evidence for a clear role of vitamin D for many health related outcomes in both randomized and observational studies.<sup>8</sup> Furthermore, Autier and colleagues suggest that low 25(OH)D is a marker of ill health and that inflammatory processes involved in disease manifestation reduce 25(OH)D, explaining why low vitamin D status has been reported for a wide range of disorders<sup>7</sup>. Restoration of vitamin D deficits due to ageing and lifestyle changes induced by ill health could explain why low-dose supplementation may lead to slight gains in survival in the elderly.

Genetic variants in vitamin D pathway members are not subject to concerns of temporality and confounding and therefore provide stronger evidence on the possible association of vitamin D with AMD. SNPs in the *CYP24A1* and *VDR* gene were identified in the discovery phase of a family nvAMD study but in the replication component, only variants in *CYP24A1* were found to be associated.<sup>9</sup> In the CAREDS study, SNPs in *CYP2R1* and *VDR* but not *CYP24A1* were associated with early AMD.<sup>15</sup> In our study, we found no significant associations between

variants in *CYP2R1* and early AMD but a nominal association with two *VDR* SNPs. A *VDR* SNP (rs11574077) was significantly associated with nvAMD in our study but this SNP was not associated with nvAMD in a family study.<sup>9</sup> We found the strongest evidence of nominal association for SNPs located within the *RXRA* gene region (rs12339187, rs3118536, rs11185644, rs10881582), although these did not withstand correction for multiple testing. A limitation of our study was the small number of cases of early and nvAMD for investigation of genetic associations.

Genetic determinants of vitamin D bioactivity have been identified previously in the genes encoding *VDR* and the retinoic acid-X-receptor (*RXRA*), which forms a complex with 1,25 OH<sub>2</sub> D bound to *VDR* and is recognized by vitamin D response elements on target genes.<sup>35</sup> We directly genotyped rs1570669 and rs2274130 in *CYP24A1*, together with rs927651 as a proxy for rs1570670, rs2296239 and rs4809957, all SNPs in high linkage disequilibrium. We found no evidence to support their previously reported association with AMD.<sup>13</sup> In addition, previous reports of *VDR* SNPs associated with AMD offer little support for our study findings and vice versa,<sup>19</sup> although lack of support may result from discrepancies in recruitment and composition, study size and characterization of the AMD cases within each study. In contrast to a previous finding we found no evidence that major AMD genetic risk variants (rs1061170 and rs10490924) modified the association between 25(OH)D deficiency and early AMD and nvAMD.<sup>19</sup> We did not find a relationship with *RXRA* SNPs and 25(OH)D; however, a previous association with one *RXRA* SNP (rs9409929) has been reported only with the metabolite 1,25 OH<sub>2</sub> D but not the circulating serum level.<sup>36</sup> It is possible that the nominal association we report between *RXRA* variants and nvAMD may not relate to vitamin D. *RXRA* receptors are involved in a large number of pathways other than those related to vitamin D including, of relevance to AMD,



glucose and lipid metabolism<sup>37</sup> and the omega 3 fatty acid, docosahexaenoic acid.<sup>38</sup> Previously reported genome wide association data evaluated several SNPs across the *RXRA* gene in 2157 mixed early and late AMD cases and 1150 controls (<http://www.ncbi.nlm.nih.gov/gap>) including rs10881582,  $p=0.15$ , but no evidence of association was found.<sup>39</sup>

We conclude therefore that the hypothesis of a causal association of vitamin D with AMD is not supported by clear evidence for an association of vitamin D pathway SNPs with early, late or nvAMD. Moreover concerns about residual confounding and reverse causation make it unlikely that there is an association between vitamin D deficiency and AMD.

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**Table 1. Characteristics of participants by vitamin D status**

Serum 25(OH)D status nmol/L)	Sufficient >50 (nmol/L)	Insufficient ≥30<50 (nmol/L)	Deficient <30 (nmol/L)	
N	2077	1475	944	p trend
Serum 25(OH)D (nmol/L) <sup>1</sup>	70.2 (16.0)	39.9 (5.7)	21.2 (5.9)	
Age <sup>1</sup>	72.6 (5.4)	73.3 (5.7)	74.5 (6.0)	<0.0001
Women <sup>2</sup>	47.9 (1055)	53.7 (823)	61.7 (590)	0.11
Education (lowest tertile of years) <sup>2</sup>	37.4 (769)	36.5 (561)	39.5 (380)	0.42
Ever smoker <sup>2</sup>	49.2 (1020)	44.8 (681)	43.3 (414)	0.13
Alcohol at least weekly <sup>2</sup>	52.2(903)	44.4 (555)	44.0 (374)	0.18
Annual UVB exposure (MED) <sup>3,5</sup>	237 (123-393)	229 (112-412)	202 (69-380)	0.17
Obese <sup>2</sup> (BMI ≥30)	32.1 (645)	40.4 (567)	40.7 (352)	0.05
Diabetes <sup>2</sup>	12.9 (218)	17.9 (219)	16.7 (147)	0.02
CVD <sup>2</sup>	12.5 (268)	13.6 (215)	14.3 (142)	0.29
Fish oil supplement use <sup>2</sup>	8.2 (381)	3.9 (109)	1.5 (22)	0.22
<i>Blood measures μmol/L</i>				
Cholesterol <sup>1</sup>	5.8 (1.1)	5.6 (1.1)	5.6 (1.0)	0.12
Vitamin C <sup>1</sup>	48.4 (24.8)	42.2 (25.4)	40.6 (25.6)	0.02
Lutein <sup>3,5</sup>	1.26 (0.47-2.79)	0.99 (0.46-2.42)	0.90 (0.46-2.42)	0.05
Alpha- tocopherol <sup>1</sup>	30.6 (7.0)	30.5 (7.0)	29.9 (6.7)	0.43
Retinol <sup>1</sup>	2.23 (0.77)	2.19 (0.79)	2.18 (0.74)	0.36
<i>Dietary variables</i>				
≥ 1 Oily fish serving per week <sup>2</sup>	45.8 (892)	36.7 (522)	27.5 (254)	0.02
Vitamin D <sup>4</sup> (highest quartile)	30.9 (593)	24.7 (355)	18.2 (153)	0.01
DHA <sup>4</sup> (highest quartile)	33.7 (617)	25.9 (350)	17.9 (150)	0.01

<sup>1</sup> Mean (SD)<sup>2</sup> % (n)<sup>3</sup> Median, IQR (interquartile range)<sup>4</sup> Energy adjusted<sup>5</sup> p trend based on log lutein

**Table 2. Association of early, late and neovascular AMD with (i) 25(OH)D (nmol/L) distribution (ii) Vitamin D status**

	Serum 25(OH)D (nmol/L) <sup>a</sup>		Sufficient >50 nmol/L	Insufficient ≥30 -<50 nmol/L		Deficient <30 nmol/L	
	OR (95% CI) <sup>b,c,d</sup>	p	Reference	OR (95% CI) <sup>b,c</sup>	p	OR (95% CI) <sup>b,c</sup>	p
Early AMD							
Grade 1	0.98 (0.94-1.01)	0.24	1	0.98 (0.88-1.10)	0.78	0.97 (0.79-1.18)	0.73
Grade 2	1.04 (0.93-1.17)	0.36	1	0.94 (0.71-1.23)	0.65	0.88 (0.54-1.45)	0.62
Grade 3	1.03 (0.89-1.20)	0.65	1	0.65 (0.38-1.10)	0.11	1.17 (0.84-1.64)	0.36
Late AMD	1.02 (0.94-1.11)	0.59	1	0.76 (0.48-1.20)	0.19	1.21 (0.88-1.68)	0.19
Neovascular AMD	1.02 (0.95 -1.08)	0.64	1	0.92 (0.57-1.48)	0.73	1.27 (1.11-1.45)	<0.0001

<sup>a</sup> Square root transformed serum 25(OH)D nmol/L

<sup>b</sup>Odds ratio (OR) and 95% Confidence Interval (95% CI)

<sup>c</sup>Adjusted for age, sex, center, season, smoking, CVD, diabetes, alcohol consumption, BMI, plasma cholesterol, alpha-tocopherol, retinol ,lutein, ascorbate.

<sup>d</sup> Odds ratio (OR) per unit increase of square root transformed serum 25(OH)D

**Table 7. Genetic association of SNPs with P<0.05 for (a) serum 25(OH)D (b) early AMD, (c) late AMD and (d) neovascular AMD**

<b>(a) Vitamin D</b>	<b>CHR</b>	<b>SNP</b>	<b>Gene</b>	<b>BP</b>	<b>BETA (SE)<sup>a</sup></b>	<b>P</b>
	4	rs2298850	<i>GC</i>	72833131	-0.414 (0.116)	<0.001
	4	rs1155563	<i>GC</i>	72862352	-0.276 (0.116)	0.018
	4	rs11939173	<i>GC</i>	72891022	-0.242 (0.106)	0.023
	9	rs11185644	<i>RXRA</i>	136350684	-0.277 (0.133)	0.038
	11	rs10832306	<i>CYP2R1</i>	14813670	-0.342 (0.169)	0.043
	11	rs7935792	<i>CYP2R1</i>	14866037	-0.556 (0.206)	0.007
	12	rs2239186	<i>VDR</i>	46555677	-0.390 (0.125)	0.002
	12	rs4760169	<i>CYP27B1</i>	56405114	-0.483 (0.167)	0.004
	<b>CHR</b>	<b>SNP</b>	<b>Gene</b>	<b>BP</b>	<b>OR (95% CI)<sup>b</sup></b>	<b>P</b>
<b>(b) Early AMD</b>	12	rs11574026	<i>VDR</i>	46574513	0.74 (0.56-0.98)	0.03
	12	rs4516035	<i>VDR</i>	46586093	1.21 (1.02-1.44)	0.03
<b>(c) Late AMD</b>	9	rs3118536	<i>RXRA</i>	136448283	0.60 (0.41-0.90)	0.01
	12	rs10875693	<i>VDR</i>	46555917	1.44 (1.08-1.93)	0.01
	12	rs11574077	<i>VDR</i>	46539194	0.32 (0.12-0.84)	0.02
	11	rs11023371	<i>CYP2R1</i>	14852847	1.67 (1.01-2.74)	0.04
<b>(d) nvAMD</b>	9	rs11185644	<i>RXRA</i>	136350684	0.53 (0.32-0.87)	0.01
	9	rs12339187	<i>RXRA</i>	136369148	0.54 (0.33-0.88)	0.01
	9	rs10881582	<i>RXRA</i>	136395899	0.64 (0.43-0.97)	0.04
	9	rs3118536	<i>RXRA</i>	136448283	0.54 (0.33-0.87)	0.01
	12	rs11574077	<i>VDR</i>	46539194	0.18 (0.04-0.76)	0.02

<sup>a</sup> Regression coefficient (Beta) of square root transformed 25(OH)D and Standard error (SE) adjusted for age, sex, center and season.

<sup>b</sup> Odds Ratio (OR) and 95% Confidence Interval (CI) for early, late and nvAMD adjusted for age, sex, center

Abbreviations: AMD, age related macular degeneration; nvAMD, neovascular age related macular degeneration; CHR, chromosome; SNP, single nucleotide polymorphism; BP, base pair;