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A Combination of Single-Nucleotide Polymorphisms Is Associated with the Interindividual Variability in Cholecalciferol Bioavailability in Healthy Men^{1,2,3,4}

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⁴ Abbreviations used: 1,25(OH)₂D (1,25-dihydroxyvitamin D); 25(OH)D (25-hydroxyvitamin D); ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP), member 1); ASBT (apical sodium-dependent bile acid transporter); D₂ (ergocalciferol); D₃ (cholecalciferol); DHCR7 (7-dehydrocholesterol reductase); GC (group-specific component, also known as vitamin D binding protein); PLS (partial least squares); SCARB1 (scavenger receptor class B, member 1); SNPs (single nucleotide polymorphisms); VIP (variable importance in the projection).

⁵ CD and PB contributed equally as joint first authors to this work.

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1 **ABSTRACT**

2 **Background:** Most people require dietary vitamin D to achieve the recommended blood level
3 of 25-hydroxyvitamin D (25(OH)D). However, the blood response to vitamin D supplementation
4 is highly variable among individuals.

5 **Objective:** Our main objective was to assess whether the variability in cholecalciferol (D₃)
6 bioavailability was associated with single nucleotide polymorphisms (SNPs) in candidate
7 genes.

8 **Methods:** In a single-group design, 39 healthy adult men (age= 33 ± 2; BMI=22.9 ± 0.3) were
9 genotyped using whole-genome microarrays. Following an overnight fast, plasma 25(OH)D
10 status was measured and the subjects then consumed a meal providing 5 mg D₃ as a
11 supplement. Plasma chylomicron D₃ concentration was measured over 8 h, and D₃ response
12 was assessed by calculating the postprandial area-under-the-curve. Partial least squares
13 regression was used to test the association of SNPs in or near candidate genes (61 genes
14 representing 3791 SNPs) with the postprandial D₃ response.

15 **Results:** The postprandial chylomicron D₃ concentration peaked at 5.4 h. The D₃ response
16 was very variable among individuals (CV=47%). It correlated with the chylomicron triglyceride
17 response ($r=0.60$, $P<0.001$) but not with the fasting plasma 25(OH)D concentration ($r=0.04$,
18 $P=0.83$). A significant ($P=1.32 \times 10^{-4}$) partial least squares regression model, which included 17
19 SNPs in 13 genes (including 5 that have been associated with chylomicron triglyceride
20 response), was associated with the variance in the D₃ response.

21 **Conclusions:** In healthy men, there is a high interindividual variability in D₃ bioavailability
22 which is associated with a combination of SNPs located in/near genes involved in both vitamin
23 D and lipid metabolism.

24 This trial was registered at <http://www.clinicaltrials.gov> as NCT02100774.

25

Key words: vitamin D, chylomicrons, nutrition, diet and dietary lipids, intestine, kinetics,
nutrigenetics, 25-hydroxycholecalciferol, absorption, postprandial, genetic polymorphisms.

26

27 INTRODUCTION

28

29 Vitamin D is the generic name for compounds exhibiting the biological activity of
30 cholecalciferol (D₃). Vitamin D is essential for bone health and for regulating blood calcium and
31 phosphate concentrations, but it is also involved in other biological functions such as immunity,
32 cell proliferation, or apoptosis. To become hormonally active, D₃ needs two hydroxylations: 25-
33 hydroxyvitamin D (25(OH)D) is produced in the liver upon 25-hydroxylase action (cytochrome
34 P450, family 2, subfamily R, member 1, CYP2R1) and, following its transport via the
35 bloodstream to the kidney bound to vitamin D binding protein (DBP), it undergoes a second
36 hydroxylation by 1- α -hydroxylase (cytochrome P450 family 27, subfamily B, member 1,
37 CYP27B1) to form 1,25-dihydroxyvitamin D (1,25(OH)₂D) (1). In a recent Mendelian
38 randomization analysis, vitamin D status, which is usually estimated by measuring the plasma
39 25(OH)D concentration, has been negatively associated with all-cause and cancer mortality
40 (2). Although a significant fraction of total body vitamin D is produced in the skin after exposure
41 to ultraviolet light, a majority of individuals require at least some dietary vitamin D, either in
42 foods rich in vitamin D (3) or in supplements, to reach the serum level of vitamin D sufficiency.
43 D₃ is the main dietary source of vitamin D and is found in significant amounts in food items
44 such as milk and dairy products, fatty fish or cod liver oil. However, recent data suggests that
45 dietary vitamin D intakes in several Western countries are below recommended levels (current
46 RDA in the U.S. is 15 μ g) (4) and according to NHANES 2005–2006, 37% of the U.S.
47 population uses a dietary supplement containing vitamin D (5).

48 It is assumed that vitamin D uptake is governed by the same intra-luminal factors as
49 those that have been described for lipids (6, 7). These include emulsification, solubilization in
50 mixed micelles, and diffusion across the unstirred water layer before uptake by enterocytes.
51 Triglycerides and vitamin D then show marked differences regarding their uptake by intestinal
52 cells (8): although it was thought that vitamin D was absorbed *via* passive diffusion, recent
53 results have shown that, at least at nutritional doses, intestinal cell membrane proteins are
54 involved in the uptake of vitamin D on the apical side of the enterocyte. These proteins are

55 scavenger receptor class B type 1 (SR-BI), cluster determinant 36 (CD36), and Niemann-Pick
56 C1-like 1 (NPC1L1) (9). The list is likely not complete as it has been suggested that other
57 apical membrane transporters, for example the ATP-binding cassette sub-family G member 5
58 and 8 heterodimer (ABCG5/G8), might also be involved (7). Following its uptake, D₃ has to
59 reach the basolateral side of the cell to be secreted in the circulation, yet nothing is known
60 about its transport from the apical to the basolateral side of the enterocyte. It is assumed that
61 most absorbed D₃ is incorporated as such into chylomicrons, which are subsequently secreted
62 into the lymph (apolipoprotein B-dependent route) (10), but it is not known whether a fraction
63 of D₃ is incorporated into HDL secreted by the intestine, as described for cholesterol and
64 vitamin E (11, 12).

65 The heritability of blood vitamin D status is estimated at ~30% (13) and common
66 variants in *CYP2R1* (2, 14-17), vitamin D binding protein (*GC*) (18), *VDR* (vitamin D (1,25-
67 dihydroxyvitamin D) receptor) (19), 7-dehydrocholesterol reductase (*DHCR7*) (14, 15, 20) and
68 cytochrome P450, family 24, subfamily A, member 1 (*CYP24A1*) (14, 15, 19) have been
69 associated with fasting plasma 25(OH)D concentrations. There is also a wide variation in the
70 response of blood 25(OH)D to vitamin D supplementation, and this response has been shown
71 to be partly genetically modulated (2, 16, 17, 19, 21, 22). Moreover, as stated above, at least
72 a portion of vitamin D absorption requires protein transport (6, 7, 9, 23). Several groups,
73 including ours, have previously shown that genetic variants were associated with the variability
74 in the postprandial chylomicron triglyceride response (24-26) and chylomicrons are
75 responsible for the transport of newly absorbed D₃ into the peripheral circulation. Moreover,
76 we have also shown that the variation in the postprandial response to other lipid-soluble
77 micronutrients, i.e. tocopherol, lutein, lycopene and beta-carotene (27-30), which share
78 common absorption processes with D₃, was modulated by SNPs in genes involved in both
79 intestinal transport of these micronutrients and chylomicron metabolism. This set of arguments
80 allows us to suggest that D₃ bioavailability could be, at least partly, genetically modulated.

81 The two main objectives of this study were 1) to describe the postprandial chylomicron
82 D₃ response to a D₃-rich meal and its interindividual variability and 2) to assess whether the

83 interindividual variability in D₃ bioavailability is associated with genetic variants located in or
84 near candidate genes involved in vitamin D or lipid metabolism.
85

86 **SUBJECTS AND METHODS**

87

88 **Subject number and characteristics**

89 Forty healthy, non-obese, non-smoking (31) men were recruited for the study. Subjects
90 reported normal energy consumption (i.e. \approx 2500 kcal/d) with <2% alcohol as total energy
91 intake. Subjects had no history of chronic disease, hyperlipidemia, or hyperglycemia and were
92 not taking any medication known to affect vitamin D or lipid metabolism the month before the
93 study or during the study period. Because of the relatively large volume of blood collected
94 during the study, blood hemoglobin concentration >13 g/dL was an inclusion criteria. The study
95 was approved by the regional committee on human experimentation (N°2008-A01354-51,
96 Comité de Protection des Personnes Sud Méditerranée I, France). Procedures followed were
97 in accordance with the Declaration of Helsinki of 1975 as revised in 1983. Objectives and
98 requirements of the study were fully explained to all participants before beginning the study,
99 and written informed consent was obtained from each subject. One subject left the study for
100 personal reasons before he participated in the postprandial experiment, which left 39 subjects
101 whose baseline characteristics are reported in **Table 1**.

102

103 **DNA preparation and genotyping methods**

104 DNA preparation and genotyping methods were performed as previously described
105 (28). The whole genome was genotyped using HumanOmniExpress BeadChips (Illumina),
106 which allowed for the analysis of $\approx 7.33 \times 10^5$ SNPs/DNA sample. Subjects were also
107 genotyped for eleven additional SNPs as described below (refer to “Choice of candidate
108 genes”) (32).

109

110 **Postprandial experiments**

111 In order to assess D₃ bioavailability, we measured the postprandial chylomicron D₃
112 response to a D₃-rich meal. This approach is commonly used for experimental assessment of
113 fat-soluble vitamin and carotenoid bioavailability (33-36). Because it has been shown that

114 season is an important prediction factor for vitamin D response variation (37), the postprandial
115 experiments were performed in a short time period, more precisely between September and
116 November. This postprandial experiment was part of a larger clinical research study where we
117 assessed the bioavailability of other lipid micronutrients and nutrients, *i.e.* lutein (30),
118 tocopherol (27), lycopene (29), beta-carotene (28) and cholesterol (unpublished data), in the
119 same group of subjects. In order to avoid carry-over and/or competition between the studied
120 compounds, a washout period of 2 weeks minimum was respected in between two test-meals.
121 Subjects were asked to refrain from the consumption of vitamin supplements and vitamin D-
122 rich foods 48 h before the postprandial experiment (an exclusion list was provided by a
123 dietitian). In addition, subjects were asked to eat dinner between 19:00 and 20:00 the day
124 before the postprandial experiment and to abstain from any food or beverage consumption
125 afterward with the exception of water. After the overnight fast, subjects arrived at the local
126 Center for Clinical Investigation (Hôpital de la Conception, Marseille, France) and a baseline
127 blood sample was taken. Subjects then consumed the test meal together with 5 mg of
128 supplemental D₃ (2 vials of UVEDOSE 100 kIU, Crinex laboratory, Montrouge, France). The
129 meal also contained semolina (70 g) cooked in 200 mL of hot water, white bread (40 g), cooked
130 egg whites (60 g), peanut oil (50 g), and mineral water (330 mL). Subjects were asked to
131 consume the meal at a steady pace, with one half of the meal consumed in 10 min and the
132 remainder of the meal consumed within 30 min (to diminish the variability due to different rates
133 of intake and, thus, gastric emptying). No other food was permitted over the following 8 h.
134 However, subjects were allowed to drink any remaining water from the meal. Additional blood
135 samples were drawn 2, 3, 4, 5, 6 and 8 h after meal consumption. Blood was collected via
136 evacuated purple-top glass tubes containing K-EDTA. Tubes were immediately placed on ice
137 and covered with aluminum foil to avoid light exposure. Plasma was isolated by centrifugation
138 (10 min at 4 °C and 1620 x g) < 2 h after collection.

139

140 **Chylomicron preparation**

141 Chylomicrons were prepared from plasma samples as previously described (28).
142 Immediately after recovery, chylomicrons were stored at -80°C until analysis.

143

144 **Ability of Caco-2 cells to convert D₃ to 25(OH)D**

145 Caco-2 clone TC-7 cells were cultured as previously described (38). For each
146 experiment, cells were seeded and grown on 6-well plates during 14 days to obtain confluent
147 and highly differentiated cell monolayers. Twelve hours prior to each experiment, the complete
148 medium was replaced with DMEM supplemented with 1% antibiotics and 1% non-essential
149 amino acids. Cells were then incubated with DMEM supplemented with 1% antibiotics, 1%
150 non-essential amino acids, 1% BSA and 50 µM D₃ up to 24h. After incubation, the cell medium
151 was harvested and the cells were scraped in ice-cold PBS. The samples were then stored at -
152 80°C until analysis.

153

154 **Vitamin D extraction and analysis by HPLC**

155 Up to 2 mL of samples were deproteinated by adding 1 volume of ethanol, which also
156 contained ergocalciferol (D₂) as an internal standard, and vortexing for 30 s. After adding
157 2 volumes of hexane, the mixture was vortexed for 10 min and centrifuged at 500 x g for 10 min
158 at 4 °C. The upper phase (containing the different vitamin D forms) was collected and the
159 sample was extracted a second time with hexane following the same procedure. The hexane
160 phases were pooled and completely evaporated under nitrogen gas. The dried residue from
161 chylomicron samples was dissolved in 200 µL of acetonitrile/methanol/water (60/38/2; v/v/v),
162 containing 20 mM sodium perchlorate and 10 mM perchloric acid, whereas the dried residue
163 from cell samples was dissolved in 200 µL of acetonitrile. A volume of 200 µL was used for
164 HPLC analysis.

165 D₃ and D₂ from chylomicron samples were separated using a 250×4.6 mm reverse
166 phase C18, 5-µm Zorbax column (Interchim, Montluçon, France) and a guard column and
167 analyzed as previously described (9).

168 D₃, 25(OH)D and D₂ from cell samples were separated using a 100×3 mm ACE 3 C18-
169 AR column (AIT, Houilles, France). The mobile phase consisted of a gradient of acetonitrile
170 (A) and water (pH10) (B). Flow rate was 0.22 mL/min and column temperature was 20°C. The
171 gradient profile of the mobile phase (A:B) was set at 60:40 from 0 to 1.5 min. It then changed
172 linearly to 100:0 for 6 min. This ratio was maintained for 12 min before the mobile phase was
173 changed back to 60:40 from 18 to 18.5 min. This last ratio was finally maintained for 11.5 min.

174

175 **Plasma and chylomicron 25(OH)D analysis by chemiluminescence**

176 25(OH)D from fasting plasma and chylomicron samples was assessed using a
177 chemiluminescent immunoassay technology (Liaison analyzer, Diasorin, Antony, France),
178 according to the manufacturer's instructions.

179

180 **Choice of candidate genes**

181 Candidate genes included those for which the encoded protein has previously been
182 shown to be involved in D₃ uptake by the enterocyte *in vitro* (9), genes that have been
183 suggested to be involved (directly or indirectly) in the metabolism of fat-soluble micronutrients
184 in the enterocyte (23), and genes that have been associated with circulating blood 25(OH)D
185 concentration in genome-wide (15, 39-41) or candidate gene (18, 20, 21, 42-45) association
186 studies. Consequently, 61 genes were selected (**Supplemental Table 1**), representing 4608
187 SNPs. In addition, we added 27 SNPs in 14 genes associated with the postprandial
188 chylomicron triacylglycerol response in the same subjects (24). Indeed, chylomicrons are the
189 carrier of both newly absorbed triacylglycerols and D₃ (10) in the blood. Thus, we hypothesized
190 that genetic variants that affect the secretion and clearance of chylomicrons also likely affect
191 D₃ response. Finally, we added 11 SNPs that have been previously associated with lipid
192 metabolism and which were not genotyped with the BeadChips (**Supplemental Table 2**). After
193 genotyping of the subjects (see DNA preparation and genotyping methods), SNPs for which
194 the genotype call rate was <95%, or SNPs that presented a significant departure from the
195 Hardy-Weinberg equilibrium ($P < 0.05$; Chi-squared test), were excluded from all subsequent

196 analysis (855 SNPs excluded, leaving 3791 SNPs for the partial least squares (PLS)
197 regression analysis, **Supplemental Figure 1**).

198

199 **Calculation and statistical analysis**

200 The trapezoidal approximation method was used to calculate the AUC of the
201 postprandial plasma chylomicron D₃ concentration over 8 h, henceforth referred to as “D₃
202 response”.

203 All data were expressed as mean ± SEM.

204 Normality was assessed using the Kolmogorov-Smirnov and the Shapiro-Wilk tests.
205 Statistical dependence between two variables was assessed by Pearson’s correlation
206 coefficient. Differences in the postprandial chylomicron 25(OH)D concentrations over time
207 were tested by repeated-measures ANOVA. For all tests, the bilateral alpha risk was $\alpha=0.05$.
208 Statistical analyses were performed using Statview and SAS softwares (SAS Institute, Cary,
209 NC, U.S.A.).

210

211 *Statistics: partial least squares regression*

212 To identify SNPs associated with the variability observed in the D₃ response, we used
213 PLS regression, following previously published rationale and model assumptions (24, 30).
214 Among the 3791 candidate SNPs, PLS identified those which were predictive of the D₃
215 response according to their variable importance in the projection (VIP) value. A general genetic
216 model was assumed. The 3 genotypes of each SNP (i.e. most frequent homozygote allele,
217 heterozygote, less frequent homozygote allele) were treated as separate categories, with no
218 assumption made about the effect conferred by the variant allele on D₃ response. Different
219 PLS regression models were built using increasing VIP threshold values. The selection of the
220 PLS regression model was carried out as previously shown (29). Additional validation criteria
221 and procedures of the PLS regression models are described in **Supplemental Methods**.
222 SIMCA-P13 software (Umetrics, Umeå, Sweden) was used for all multivariate data analyses
223 and modeling.

224 With the knowledge of a subject's genotype at the SNPs associated with the variability
225 observed in the D₃ response, it was possible to calculate the subject's ability to respond to D₃
226 according to the following equation:

$$227 \quad RP = a + \sum_1^b r_i X \text{ genotype. (SNP}_i\text{)} \text{ (Equation 1)}$$

228 with RP as the responder phenotype (i.e. the D₃ response), *a* as a constant (which is equal to
229 the mean D₃ response), *b* the number of SNPs in the selected PLS regression model, *r_i* as the
230 regression coefficient of the *i*th SNP included in the PLS regression model, and
231 "genotype.(SNP_{*i*})" as a Boolean variable indicating the subject's genotype at the *i*th SNP.

232

233 In a second approach, we performed univariate analyses to compare the D₃ response
234 between subgroups of subjects who bore different genotypes for the SNPs present in the
235 selected PLS model. Differences obtained between the various genotype subgroups were
236 analyzed by a Student's *t*-test with the Benjamini-Hochberg correction with QVALUE software
237 (version 1.0, designed by researcher) (46) and R software (version 3.0.2, R foundation of
238 statistical computing). For all tests, an adjusted *P*-value <0.05 was considered significant.

239

240 RESULTS

241

242 Vitamin D status of the subjects

243 As shown in **Table 1**, most subjects displayed a fasting plasma 25(OH)D concentration
244 lower than the recommended concentration of 75 nmol/L (47), with 16 out of the 39 subjects
245 exhibiting a concentration <50 nmol/L. The fasting plasma 25(OH)D concentration did not
246 correlate with any of the reported characteristics of the subjects (all r were lower than 0.11 and
247 all P -values were higher than 0.49, data not shown).

248

249 Chylomicron vitamin D responses to the D₃-rich meal

250 The mean D₃ response after consumption of the test meal is shown in **Figure 1**. Thirty-
251 seven out of the 39 subjects had D₃ concentrations below our limit of detection in their fasting
252 chylomicron fraction, and the remaining 2 subjects had extremely low D₃ concentrations (0.27
253 \pm 0.01 nmol/L). The maximal chylomicron D₃ concentration was obtained 5.4 \pm 0.3 h after meal
254 intake. Note that the subject with the lowest response exhibited an increase in his chylomicron
255 D₃ concentration only 8 h after meal intake. The individual responses ranked by increasing
256 AUC are shown in **Figure 2**. They followed a normal distribution ($P=0.20$ and $P=0.92$ following
257 the Kolmogorov-Smirnov and the Shapiro-Wilk test respectively; skewness=0.165 and
258 kurtosis=-0.394). The CV of the D₃ response was 47% and the ratio between the highest and
259 the lowest responder was \approx 34. The D₃ response correlated relatively well with the chylomicron
260 triglyceride response ($r=0.60$, $P < 0.001$) as well as with fasting TG ($r=0.478$; $P=0.002$). Note
261 that the D₃ response was not related to the vitamin D status of the subjects (as estimated by
262 their fasting plasma 25(OH)D concentrations) ($r=0.04$, $P=0.83$).

263 The postprandial chylomicron 25(OH)D concentrations are shown in **Figure 3**. These
264 concentrations did not vary significantly over the postprandial period ($P=0.20$).

265

266

267

268 **Genetic variants associated with the D₃ response to the D₃-rich meal**

269 The PLS regression model that included all 3791 candidate SNPs (used as qualitative
270 X variables) in 61 candidate genes (**Supplementary Table 1**) described the D₃ response with
271 good accuracy ($R^2 = 0.879$), but was not predictive of this response ($Q^2 = -0.1$; R^2 after cross-
272 validation). Therefore, to improve the model and find an association of SNPs more predictive
273 of the D₃ response, we filtered out those that displayed the lowest VIP value (i.e. those that
274 made no important contribution to the PLS regression model). After the application of several
275 increasing VIP value thresholds, we selected a model with a VIP threshold value of 2.1 that
276 included 18 SNPs, of which 17 were not in linkage disequilibrium. The 17 SNPs were located
277 in or near 13 genes (**Table 2**) and described 63.5% of the variance (R^2), with a prediction index
278 Q^2 of 47.6%. The measured and the predicted D₃ responses were positively correlated ($r=0.80$,
279 $P < 0.001$). The robustness and the stability of the model was validated by 3 additional methods
280 (see **Supplemental methods**). Of note, when the quantitative X variables BMI, age, fasting
281 plasma triglycerides, cholesterol, glucose and hemoglobin concentrations were included, only
282 triglyceride concentration, age and BMI made a significant contribution to the PLS regression
283 model (with VIP values of 7.4, 4.0 and 2.1 and regression coefficients of 1.32, -0.02, -0.07
284 respectively).

285 The association of the 17 selected SNPs with the D₃ response was further evaluated
286 using univariate statistics by comparing the D₃ response of subjects who bore different
287 genotypes for each SNP (**Table 2**). For 11 of these 17 SNPs, subjects who bore different
288 genotypes exhibited a significantly different D₃ response (adjusted P -value < 0.05).

289

290 **Genetic score to calculate the D₃ response of a genotyped subject**

291 With the knowledge of a subject's genotype at the 17 aforementioned loci, it was
292 possible to calculate the subject's ability to respond to D₃ according to equation 1 with
293 $a=114.18$ and $b=17$. A list of regression coefficients calculated by the SIMCA-P13 software
294 can be found in **Supplemental Table 4**.

295

296 **Conversion of D₃ to 25(OH)D by Caco-2 cells**

297 Following a 24 h-incubation with 50 µM D₃, only about 2.3 % of vitamin D recovered in
298 the apical medium was found as 25(OH)D (Fig. 4A) and only about 0.2% of the vitamin D taken
299 up by the cells was recovered as 25(OH)D (Fig. 4B) (secondary outcomes).

300

301

302 DISCUSSION

303

304 To the best of our knowledge, this study is only the second report of the chylomicron D₃
305 response to a D₃-rich meal in humans (48). The increased subject number and longer duration
306 of time points taken post-prandially provide a more complete picture of D₃ bioavailability in
307 humans, as compared to the previous work.

308 The subjects enrolled in our study were apparently healthy. However, most of them
309 presented sub-deficient vitamin D levels, as classified by a fasting plasma 25(OH)D
310 concentration <75 nmol/L (47). This observation was not surprising, since the prevalence of
311 vitamin D insufficiency is high in France, as elsewhere (49, 50).

312 The first major observation of this study was that the interindividual variability in D₃
313 bioavailability was quite large, with a 34-fold difference in AUC observed between the highest
314 and lowest D₃ responders. The lack of a significant correlation between the chylomicron D₃
315 response and the fasting plasma 25(OH)D concentration in our subjects could suggest that the
316 variability in D₃ bioavailability does not significantly affect long-term vitamin D status in these
317 individuals. This is perhaps due to the fact that the fasting plasma 25(OH)D concentration is
318 the result of interactions between several factors, including vitamin D synthesis by the skin,
319 dietary vitamin D intake, vitamin D absorption efficiency, hepatic vitamin D metabolism to
320 25(OH)D, and rates of renal conversion to 1,25(OH)₂D.

321 In order to identify genetic variants that are associated with the interindividual variability
322 in the chylomicron D₃ response, we employed a statistical approach that previously allowed us
323 to identify combinations of candidate SNPs involved in the variability of the postprandial
324 chylomicron response to other fat-soluble vitamins, i.e. vitamin E and A (27, 28). This approach
325 revealed that a significant 63.5% of the interindividual variability in the postprandial D₃
326 response was explained by 17 SNPs in or near 13 genes. Five of these genes [ATP-binding
327 cassette, sub-family A, member 1 (*ABCA1*), apolipoprotein B (*APOB*), blocked early in
328 transport 1 homolog (*BET1*), lipoprotein lipase (*LPL*) and N-acetyltransferase 2 (*NAT2*)] have
329 been associated with the postprandial chylomicron triacylglycerol response in the same group

330 of subjects (24). Since it is assumed that most newly absorbed D₃ is carried from the intestine
331 to peripheral organs and the liver via chylomicrons (48), it is plausible that SNPs in these genes
332 have an indirect effect on the D₃ response by modulating chylomicron metabolism. Indeed,
333 three of these SNPs (*BET1*-rs10464587, *LPL*-rs10096561 and *NAT2*-rs4921920) have been
334 previously associated with the variability in the postprandial chylomicron triglyceride response
335 in the same group of subjects (24). This is further illustrated by the significant correlation
336 between the D₃ and the chylomicron triglyceride responses.

337 Yet, this correlation was only partial, highlighting the fact that a significant part of the
338 variance of the D₃ response can be attributed to factors other than the variance in the
339 chylomicron TG response, such as processes specific to D₃ metabolism. Indeed, several SNPs
340 associated with the interindividual variability in the D₃ response were located in genes involved
341 more directly in vitamin D metabolism and transport. Intestine specific homeobox (*ISX*) is an
342 intestinal transcription factor that modulates *SCARB1* (scavenger receptor class B, member 1)
343 expression (51). *SCARB1* encodes SR-BI, an apical membrane protein which is involved in D₃
344 uptake by enterocytes (9). Additionally, a SNP in *SCARB1* was significantly associated with
345 the D₃ response in our PLS regression model. *SLC10A2* (solute carrier family 10 (sodium/bile
346 acid cotransporter), member 2) encodes for the apical sodium-dependent bile acid transporter
347 (ASBT), the main transporter involved in bile acid uptake in the distal ileum (52), and a
348 candidate transporter for D₃ uptake. Indeed, data from our laboratory suggests that ASBT is
349 involved in cellular uptake of D₃ by Caco-2 TC7 cells and by ASBT-transfected cells (data not
350 shown). *GC* encodes for vitamin D binding protein, which is responsible for the plasma
351 transport of 25(OH)D and several SNPs in this gene have been associated with plasma
352 25(OH)D concentration (20, 53). The association of a SNP in *GC* with the variability in D₃
353 bioavailability could suggest the existence of a feedback loop that regulates the circulating
354 25(OH)D concentration. *ABCB1* (ATP-binding cassette, sub-family B (MDR/TAP), member 1)
355 encodes for P-glycoprotein (also known as multidrug resistance protein 1, MDR1) which is
356 involved in the efflux of xenobiotic compounds with a broad substrate specificity. Unpublished
357 data from our lab, which have shown that *ABCB1*-transfected cells effluxed more D₃ than

358 control cells, further support that this apical membrane protein could also be involved in D₃
359 efflux. The association of 2 SNPs in pancreatic lipase (*PNLIP*) with the D₃ response suggests
360 that the hydrolysis efficiency of dietary triglycerides, in which D₃ is assumed to be solubilized,
361 is an important factor regarding D₃ absorption efficiency. This can be due either to the fact that
362 pancreatic lipase allows the release of D₃ from triglycerides and its incorporation in micelles or
363 that the lipolysis products of triglycerides by pancreatic lipase, i.e. fatty acids, facilitate D₃
364 absorption (38). In support of this hypothesis it has been shown that patients with cystic fibrosis
365 with pancreatic insufficiency display lower D₃ absorption (54). Finally, we observed the
366 association of a SNP in *DHCR7* with D₃ bioavailability. *DHCR7* encodes for 7-
367 dehydrocholesterol reductase, an enzyme that catalyzes the conversion of 7-
368 dehydrocholesterol to cholesterol. 7-dehydrocholesterol also serves as a precursor of D₃ in
369 keratinocytes upon UVB exposure (55). It could be hypothesized that increased activity of
370 *DHCR7* would reduce the pool of 7-dehydrocholesterol available for D₃ synthesis following sun
371 exposure, inducing in turn an upregulation in D₃ bioavailability to maintain homeostatic levels.
372 This hypothesis is further supported by the fact that *DHCR7* variants have been previously
373 associated with plasma 25(OH)D concentrations (2, 14, 15, 20), although future studies are
374 required to test this hypothesis.

375 25(OH)D was observed in the chylomicron-containing fraction of blood sampled during
376 the postprandial period. This measurement was carried out to assess whether a fraction of
377 ingested D₃ was immediately metabolized to 25(OH)D in the enterocyte, and then packaged
378 into the chylomicrons. Indeed, although it is well-known that the liver is the main organ that
379 converts D₃ to 25(OH)D, there is no clinical data on the possibility that the intestine can convert
380 a significant fraction of newly absorbed D₃ to 25(OH)D and incorporate it into chylomicrons.
381 Yet, it has been shown that the human intestinal Caco-2 cells possess 25-hydroxylase activity
382 (56) suggesting that the intestinal cell can convert a fraction of newly absorbed D₃ to 25(OH)D.
383 Furthermore, it is possible that, if newly absorbed D₃ is converted to 25(OH)D in the intestine,
384 it is directly transported to the liver via the portal vein, as was reported in rats (57). In our study,
385 the fact that 25(OH)D concentration did not significantly vary in the chylomicron fraction during

386 the postprandial period, together with the fact that our complementary experiment in Caco-2
387 showed that the fraction of D₃ converted to 25(OH)D in the intestinal cell was very low (i.e.
388 2.3%), and even non detectable when D₃ was provided to the Caco-2 monolayers in micelles
389 containing cholesterol (data not shown), suggests that 25(OH)D recovered in the chylomicron
390 fraction did not come from the D₃ ingested by the subjects. 25(OH)D in the chylomicron fraction
391 was most likely due to the presence of plasma 25(OH)D, i.e. 25(OH)D associated to DBP, in
392 the triglyceride-rich lipoprotein fraction isolated from blood.

393 To conclude, results of this study allow us to describe the interindividual variability in D₃
394 bioavailability. They also suggest for the first time that this variability is, at least partly,
395 explained by a combination of SNPs in genes involved in D₃ and chylomicron metabolism, and
396 that ASBT and ABCB1 are involved in D₃ bioavailability, likely in its intestinal uptake. We
397 acknowledge some limitations of the study: it is likely that SNPs that have a significant effect
398 on D₃ bioavailability were not entered in the PLS regression analysis because they were not
399 present on the BeadChips, they were excluded from the analysis (for not following the Hardy-
400 Weinberg equilibrium) or simply because they were not located in the candidate genes.
401 Moreover, these findings need to be tested in other population groups (based on *e.g.* sex, age,
402 or genetic backgrounds). Thus future studies are required to confirm these associations and
403 to identify other genes/SNPs involved in D₃ bioavailability.

AUTHORS' CONTRIBUTIONS TO MANUSCRIPT:

All authors have read and approved the final manuscript.

PB designed the research project. PB and ER designed the protocol; MN, SM and NL conducted the clinical study; AG analyzed chylomicron D₃ by HPLC with assistance from ER; HP analyzed plasma and chylomicron 25(OH)D; ER conducted the cellular study from culture to result interpretation; CD and PB analyzed the results; CD performed statistical analyses; PB and CD wrote the paper with consultation from ER and RK; PB and CD had primary responsibility for the final content of the manuscript.

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FIGURE LEGENDS

Figure 1: Baseline-adjusted chylomicron D₃ concentration over 8 h after consumption of a meal providing 5 mg of supplemental D₃ in 39 healthy men.

For each subject, postprandial chylomicron D₃ concentrations were baseline-adjusted by subtracting the fasting chylomicron D₃ concentration at time 0.

Figure 2: Individual AUCs of the postprandial chylomicron D₃ concentration after consumption of 5 mg supplemental D₃ in 39 healthy men.

Subjects were sorted by increasing postprandial chylomicron D₃ response. Each bar represents the baseline-adjusted AUC over 8 h.

Figure 3: Chylomicron 25(OH)D concentration over 8 h after consumption of 5 mg supplemental D₃ in 39 healthy men.

Data points represent the mean \pm SEM at each time point in the 39 subjects.

Figure 4: Conversion of D₃ to 25(OH)D by Caco-2 TC7 cells.

The differentiated cell monolayers received serum-free complete medium supplemented with 1% BSA and 50 μ M D₃. Incubation time ranged from 4 to 24 h. **A:** 25(OH)D appearance in the apical medium was expressed as a percentage of total vitamin D (D₃ + 25(OH)D) recovered in this medium. **B:** 25(OH)D cellular content was expressed as a percentage of total vitamin D recovered in the cells. Data are means \pm SEM of 3 assays.

TABLES

Table 1. Baseline characteristics of healthy men¹

Parameter	Value
Age, y	33 ± 2
Weight, kg	73.5 ± 1.3
BMI, kg/m ²	22.9 ± 0.3
Glucose ² , mmol/L	4.8 ± 0.1
Triacylglycerols ² , g/L	0.78 ± 0.06
Total cholesterol ² , g/L	1.65 ± 0.06
Hemoglobin ³ , g/dL ²	15.1 ± 0.2
25-hydroxyvitamin D ^{2, 4} , nmol/L	51.4 ± 2.8

¹ Values are means ± SEM, n = 39.

² Fasting plasma values.

³ Fasting total blood value.

³ Min-max (11.2-86.7), 3 subjects > 75 nmol/L.

Table 2. Genes and single nucleotide polymorphisms associated with the postprandial chylomicron cholecalciferol response in healthy men¹

Gene and SNP rs no. ²	Biological role of the encoded protein ³	VIP value	SNP minor allele frequency	Adjusted <i>P</i> -value
<i>LPL</i> -rs6586874	Chylomicron clearance	3.85	0.173	0.013
<i>LPL</i> -rs10096561	Chylomicron clearance	3.71	0.128	0.013
<i>ISX</i> -rs5754862	Transcriptional regulation	2.95	0.253	0.025
<i>SLC10A2</i> -rs9558203	Apical transport	2.78	0.198	0.016
<i>GC</i> -rs6845026	Plasma transport	2.67	0.133	0.032
<i>SCARB1</i> -rs12580803	Apical transport	2.64	0.150	0.032
<i>PNLIP</i> -rs2915775	Dietary fat hydrolysis	2.50	0.257	0.046
<i>DHCR7</i> -rs11604724	Conversion of 7-dehydrocholesterol to cholesterol	2.44	0.197	0.036
<i>ABCA1</i> -rs7043894	Basolateral efflux transport	2.41	0.240	0.040
<i>ABCB1</i> -rs2235023	Apical efflux transport	2.31	0.106	0.040
<i>ABCB1</i> -rs10260862	Apical efflux transport	2.29	0.215	0.062
<i>PNLIP</i> -rs3010494	Dietary fat hydrolysis	2.26	0.293	0.062
<i>APOB</i> -rs2854725	Chylomicron formation	2.26	0.087	0.042

<i>MAPRE2</i> -rs1125425	Unknown	2.23	0.279	0.055
<i>BET1</i> -rs10464587	Transit of chylomicrons through the enterocyte	2.21	0.297	0.062
<i>NAT2</i> -rs4921920	Unknown	2.19	0.101	0.062
<i>SLC10A2</i> -rs9555166	Apical transport	2.17	0.197	0.055

¹ Abbreviations: rs, reference single nucleotide polymorphism; SNP, single nucleotide polymorphism; VIP, variable importance in the projection.

See **Supplemental Table 1** for a complete list of gene names and symbols.

² Single nucleotide polymorphisms (SNPs) present in the selected partial least squares regression model. SNPs are ranked by decreasing variable importance in the projection (VIP) value. Note that *ABCB1*-rs2235015 and *ABCB1*-rs10260862 were in linkage disequilibrium. We kept one of them, chosen at random (*ABCB1*-rs10260862), in the final selected partial least squares regression model and removed the other as it provided redundant information.

³ The given biological role of the protein is that in relation to vitamin D bioavailability.