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## Genetic variants in BCMO1 and CD36 are associated with plasma lutein concentrations and macular pigment optical density in humans

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1 **Genetic variants in BCMO1 and CD36 are associated with plasma lutein concentrations**  
2 **and macular pigment optical density in humans.**

3

4 **Running title:** Genes involved in plasma lutein and macular pigment

5

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25

26 **Abstract**

27

28 Lutein is recovered at high concentration in the human macula lutea. Recent studies suggest  
29 that this micronutrient might be implicated in prevention of age-related macular degeneration.

30 **Objective:** to identify genes which affect blood and retina lutein concentrations among

31 candidate genes (intestinal sterol transporters and carotenoid oxygenases). **Design:** a

32 comparative plus an observational study. **Participants:** Twenty nine healthy subjects for the

33 comparative study and 622 subjects for the observational study. **Intervention and methods:**

34 All the participants were genotyped for single nucleotide polymorphisms (SNPs) in the

35 candidate genes. Fasting plasma lutein concentrations were measured in all the participants

36 and after six months supplementation, with either a lutein-rich supplement or a placebo, in the

37 29 subjects who participated in the comparative study. Macular pigment optical density

38 (MPOD), which is a measure of macula concentration of lutein, was measured before and

39 after the dietary intervention in the 29 subjects. Associations between SNPs and plasma lutein

40 and MPOD were assessed by Partial Least Square (PLS) regression followed by univariate

41 analysis. Observed associations between SNPs and plasma lutein were verified by haplotype-

42 based associations analysis in the cohort of 622 subjects. **Main outcome measures:** plasma

43 lutein levels and MPOD. **Results:** Six SNPs in four genes (ABCG8, BCMO1, CD36 and

44 NPC1L1) explained 25% and 38% of the plasma and MPOD variance, respectively. Subjects

45 with TT at the BCMO1 rs7501331 locus had lower ( $P < 0.05$ ) plasma lutein than CT subjects.

46 Subjects with CC at the CD36 rs13230419 locus had lower ( $P < 0.05$ ) plasma lutein than

47 subjects who carried a T allele. The association between CD36 and plasma lutein was

48 confirmed in the cohort of 622 subjects. Subjects with TT at the BCMO1 rs7501331 locus had

49 a higher ( $P < 0.05$ ) MPOD, and subjects with GG at rs1761667 CD36 locus had a higher ( $P <$

50 0.05) MPOD than those with an A allele. **Conclusions:** These results suggest that BCMO1

51 and CD36 are implicated in plasma and retina concentrations of lutein and that genetic  
52 variants in these genes can modulate blood and retina concentrations of lutein.

53

54 **Key words:** age-related macular degeneration, bioavailability, carotenoids, eye, genetic  
55 variant, genetic polymorphisms, nutrition, retina, xanthophylls

56

57 **Key messages:** Age-related macular degeneration is an degenerative eye disease that is due, at  
58 least in part, to free radicals, and several studies have suggested that antioxidant  
59 micronutrients, such as vitamin C, E, carotenoids, and selenium, may participate in the  
60 defense of the retina against free radicals. The main carotenoids found in the human retina are  
61 the xanthophylls, lutein, zeaxanthin and meso-zeaxanthin. Our results suggest that genetic  
62 variants in BCMO1 and CD36 modulate plasma and retina lutein concentrations.

63

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64        **Abbreviations:**

65

66        BCMO1:  $\beta$ -carotene monooxygenase 1

67        BCDO2:  $\beta$ -carotene dioxygenase 2

68        BMI: body mass index

69        MPOD: macular pigment optical density

70        HPLC: high pressure liquid chromatography

71        PLS: partial least square regression

72        SNP: single nucleotide polymorphisms

73

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## 74 **Introduction**

75

76 Age-related macular degeneration is an degenerative eye disease that is due, at least in  
77 part, to free radicals (1). Several studies have suggested that antioxidant micronutrients, such  
78 as vitamin C, E, carotenoids, and selenium, may participate in the defense of the retina against  
79 free radicals. The main carotenoids found in the human retina are the xanthophylls, lutein,  
80 zeaxanthin and meso-zeaxanthin (2, 3). Macular meso-zeaxanthin apparently originates from  
81 the metabolism of lutein (4), while lutein and zeaxanthin originate from the diet. Lutein and  
82 zeaxanthin are mainly obtained from fruits and vegetables. It is assumed that carotenoids are  
83 extracted from plant cells in the upper part of the gastrointestinal tract (5, 6), solubilized in fat  
84 lipid droplets (7) and incorporated into mixed micelles (8). Mixed micelles are assumed to  
85 carry carotenoids to the intestinal brush border where they are absorbed by the enterocytes (9).

86 Mechanisms involved in intestinal absorption of carotenoids were initially studied by  
87 the Hollander group (10, 11). This group concluded that the intestinal absorption of beta-  
88 carotene, and, by extension, of all the carotenoids, is passive (12). However, this dogma was  
89 refuted by recent studies that have shown that absorption of several carotenoids (13-17)  
90 involves an enterocyte apical membrane protein, SR-BI (scavenger receptor class B type I),  
91 which has been involved in cholesterol uptake. Interestingly, the result obtained in the study  
92 dedicated to lutein absorption suggested that other transporters are probably involved (13). We  
93 hypothesized that these transporters may be sterol transporters because of the involvement of  
94 SR-BI and because they have low substrate specificity. At present, there are several sterol  
95 transporters that have been identified: 1) NPC1L1, which is apparently the main protein  
96 involved in cholesterol uptake (18) and which has recently been found to be involved in  
97 vitamin E uptake (19); 2) SR-BI, which is involved in the uptake of cholesterol (14),  
98 carotenoids (13, 14, 17) and vitamin E (20); 3) ABCG5 and ABCG8, which are involved in

99 phytosterols and cholesterol efflux back into the intestinal lumen (21) and 4) ABCA1, which  
100 is mainly located at the basolateral side of the enterocyte and is involved in cholesterol (22)  
101 and tocopherol efflux (23, 24).

102         It is assumed that, after absorption, lutein is incorporated into chylomicrons and  
103 transported to the liver. A fraction of lutein is then incorporated into VLDL and distributed to  
104 peripheral tissues by lipoproteins (25-27). A recent study has suggested that SR-BI is involved  
105 in xanthophyll uptake by retina cells (28). Since the sterol transporters mentioned above are  
106 expressed not only in the intestine but also in various other tissues, we hypothesized that they  
107 are good candidates for retina uptake of lutein. Finally, it is assumed that BCMO1 ( $\beta$ -carotene  
108 monooxygenase 1) and/or BCDO2 ( $\beta$ -carotene dioxygenase 2), which are involved in cleavage  
109 of provitamin A carotenoids in retinal and apo-carotenals, respectively (29), might be  
110 involved in the metabolism of lutein and thus in its blood and tissue concentration. This is  
111 supported by a recent study showing that a nonsense mutation in BCDO2 was associated with  
112 the yellow skin phenotype in sheep, suggesting a broad specificity of this enzyme for  
113 carotenoids (30).

114

115         The main objective of the present study was to assess whether some sterol transporters  
116 and carotene oxygenases are involved in blood and macular concentrations of lutein. To attain  
117 our goal, we studied associations between single nucleotide polymorphisms (SNPs) of genes  
118 that encode these transporters and the two carotene oxygenases, and blood and macula  
119 concentrations of lutein in 29 healthy subjects. Observed associations between genetic  
120 variants and plasma lutein were further verified in a cohort of 622 subjects.

121

## 122 **Material and Methods**

123

### 124 *Subject number and characteristics*

125           Since there were no data on the effect of the selected genetic variants on either plasma  
126 lutein levels or macular pigment optical density (MPOD), we were unable to perform a power  
127 analysis to calculate the number of subjects required to observe a significant effect with an  
128 80% power. We decided to work with 30 subjects in order to divide the group into two  
129 subgroups with 15 subjects each: one took a lutein supplement; the other, a placebo (see the  
130 paragraph on lutein supplementation).

131           Thirty healthy, non-obese males were recruited. They had no disease history,  
132 hyperlipemia or hyperglycemia. Their characteristics and daily nutrient intakes are reported in  
133 **Table 1**. One subject withdrew for personal reasons during the study. Twenty-nine of the  
134 thirty selected subjects were non-smokers. They were not taking any medication known to  
135 affect lutein or lipid metabolism during the month before the study started or during the study  
136 period. The study was approved by the regional committee on human experimentation (CPP  
137 Sud Est VI, France) and adhered to the tenets of the Declaration of Helsinki. The objectives  
138 and requirements of the study were fully explained to the participants, and informed written  
139 consent was obtained for each subject. The subjects' usual diet was estimated with a three-day  
140 food diary before the study started. Portion sizes were estimated with photographs compiled in  
141 a manual adapted from the Su.Vi.Max picture booklet (31). The dietary diary was analyzed for  
142 nutrient composition with a diet analyzer software (GENI 6.5; Micro6, Villers les Nancy,  
143 France). The software database was extended for carotenoids by mean of the USDA  
144 carotenoid food-composition database (32). The lutein intake was close to that previously  
145 observed in French group of volunteers (33-35), and close to the intake reported in a US  
146 population (36).

147

148 *Choice of candidate SNPs*

149 Candidate SNPs in genes involved in sterol absorption were selected through an  
150 analysis of previous studies describing associations between these SNPs and digestion,  
151 transport or metabolism of sterols. The SNP in BCDO2 was advised by Dr George Lietz  
152 (Newcastle University, UK) from an unpublished study. Characteristics of the SNPs are  
153 presented in **Table 2**. SNPs were validated for the oligo-ligation assay (SNPlex, see below)  
154 by several criteria: 1) genome screening, in which the SNPs may be located in a genome  
155 region that is homologous with at least one other genome region, leading to a lack of assay  
156 specificity and the potential for spurious ligation templates; 2) assay rules, in which an  
157 individual SNP assay cannot be designed due to deleterious sequence contexts or non optimal  
158 interactions among the assay components; for example, aspects of the SNP sequence or assay  
159 components may result in secondary structure and reduce assay performance, including a  
160 series of contiguous Gs or a series of 16 weak contiguous bases (A's or T's) within 25 bases  
161 of the SNP and 3) pooling rules, in which deleterious potential interactions may occur  
162 between specific SNP assays in the assay pools and false signals may be generated due to  
163 components from different assays interacting with genomic DNA.

164 The SNPs that were not validated for SNPlex were replaced by alternate SNPs, in  
165 linkage disequilibrium with the initial SNPs, or analyzed by Taqman (see thereafter).

166

167 *DNA preparation and genotyping methods*

168 Genomic DNA was prepared from 2 ml whole blood and purified with the  
169 NucleoSpin® Blood L kit ref 740 954 (Macherey Nagel, Hoerd, France). A mean of 15 µg of  
170 DNA was isolated from each blood sample. The purity and quantity of DNA was checked by  
171 spectrophotometry at 260 nm and 280 nm. A total of 100 µl of DNA at a concentration of 10

172 ng/μl was added to a plate (Dutscher, Marseille, France) for genotyping. SNPs were  
173 genotyped with an oligo-ligation assay (SNPlex, Applied Biosystems, Foster City)(37, 38) or  
174 a TaqMan method (Applied Biosystems) following the manufacturers' guidelines.

175         The oligo-ligation assay consists of designing 3' specific primers for each SNP, with  
176 two primers carrying the SNP-base specific 3' end and one common primer that starts 5' to  
177 the next base in the target sequence. The two allele specific primers carry unique ZIP codes  
178 that determine each allele. Primers are annealed to the target sequence according to the  
179 manufacturer's recommendations. A ligation reaction will join the allele specific primer with  
180 the common primer if the allele specific 3'-base is present. A short fluorescent dye labeled  
181 probe, homologous to the ZIP code sequence, is then hybridized to the immobilized product.  
182 Up to 48 SNPs can thus be multiplexed into one oligo-ligation reaction. Following the  
183 manufacturer's recommendations, genomic DNA was heat fragmented. The allele specific  
184 fluorescent probes were separated on an automated sequencer (ABI 3730, Applied  
185 Biosystems, Foster City). Alleles were binned and called with the GeneMapper software  
186 (Applied Biosystems, Foster City).

187         The TaqMan assays were performed when selected SNPs could not be analyzed by  
188 SNPlex. This was the case for two SCARB1 SNPs, rs4238001, and the one called "intron 5".  
189 Probes were purchased from the manufacturer and used according to the manufacturer's  
190 guidelines. DNA was PCR-amplified by denaturation at 95°C for 10 min, 40 cycles at 92°C  
191 for 15 sec, 60°C for 1 min, and 72°C for 45 sec, followed by elongation at 72°C for 5 min.  
192 TaqMan assays were then read on a 7900HT Fast Real-Time PCR System (Applied  
193 Biosystems), and alleles were called by the SDS software (Applied Biosystems).

194

195

196

197 *Lutein supplementation*

198           The 29 subjects were first asked to follow a lutein-poor diet for 3 wks. To attain this  
199 objective they were asked to discard lutein-rich foods from their usual diet (a list of lutein-rich  
200 food was given to the volunteers). The subjects then came to a Center for Clinical  
201 Investigation (Centre de Recherche en Nutrition Humaine d’Auvergne) after an overnight fast,  
202 and a blood sample was collected. The same day, the subjects also came to a biophysics  
203 laboratory to have their MPOD measured (the right eye was used for most of the subjects).  
204 Subjects were randomly assigned to one of the two groups. One received a placebo, while the  
205 other received a lutein-rich supplement (Visiobane Protect, Pileje, France) for 6 months. The  
206 supplement was provided as two pills containing 5 mg of lutein esters each. Subjects were  
207 asked to eat the pills during their main meals. The supplement also contains : Porphyra, B  
208 vitamins, vitamin C and E, fish oil, bee wax and gelatine. The placebo contained only refined  
209 sunflower oil. At the end of the supplementation period, all the subjects came back to the  
210 Center for Clinical Investigation for collection of a second fasting blood sample, and to the  
211 biophysics laboratory for another MPOD measurement.

212

213 *Measurement of macular pigment optical density (MPOD)*

214           The MPOD was determined as described by the van Norren team (39, 40). In  
215 summary, the radiant flux of the pattern radiated from photoreceptors at the fovea (sample  
216 field: 2 deg) and the radiant flux of the beam reflected specularly by the ILM in the perifovea  
217 (used as reference) were measured. The measuring light was provided by a 75W Xenon lamp  
218 (Oriol) that was filtered either by an interference filter at 470 nm (FWHM 10 nm, Oriol), or an  
219 interference filter at 532 nm (FWHM 10 nm, Oriol). The retinal illuminances were 4.8 logTd  
220 and 5.5 logTd for the blue and green lights, respectively. The distribution of light in the pupil  
221 was measured with a CCD camera cooled by liquid nitrogen (Princeton Instruments). This

222 camera had a resolution of 512x512 pixels, with an image depth of 16 bits. The pixel size in  
223 the pupil was 0.0255 mm. We combined areas of 2x2 pixels, giving a resolution of 0.051  
224 mm/pixel. Two stepping motors X and Y (Newport) allowed the center J of the entrance pupil  
225 (dia 0.2 mm) to be positioned at the chosen location in the eye's pupil. The head of the subject  
226 was stabilised by a bite bar which was fixed on a three-dimensional positioner. The main  
227 optical components were mounted on a single plate which could be shifted longitudinally,  
228 thus allowing focus adjustment from -12 diopters to +12 diopters of ametropia. The pupil was  
229 dilated by application of 0.5% Mydriacyl to a minimum diameter of 7 mm. The eye was then  
230 aligned to the reflectometer. A fixation target was used to direct the subject's gaze either to  
231 the center of the sampling area or at a site 6 deg temporal to the fovea. At the fovea the center  
232 J of the entrance pupil was aligned to photoreceptor axes. At 6 deg eccentricity, the point J  
233 was positioned at the center of the eye's pupil. For each of these retinal eccentricities, the  
234 measuring green beam bleached the retina for a period of 15 s, then three pupil images were  
235 captured at 532 nm (integration time of 4 s), with an interval of 10 s between each one; they  
236 were followed by three pupil images at 470 nm.

237

### 238 *Plasma lutein extraction and HPLC analysis of lutein*

239 Plasma lutein was extracted and analyzed as previously described (41). Briefly, 200  
240  $\mu\text{L}$  of plasma was deproteinized by adding one volume of ethanol containing the carotenoid  
241 echinenone (as an internal standard). Lutein was extracted twice by the addition of two  
242 volumes of hexane. The hexane phases obtained after centrifugation (500 g, 10 min, 4°C)  
243 were pooled and evaporated completely under nitrogen. The dried extract was dissolved in  
244 200  $\mu\text{L}$  of dichloromethane/methanol mixture (65/35; V/V). All extractions were performed at  
245 room temperature under yellow light to minimize light-induced damage. A volume of 80  $\mu\text{L}$   
246 was used for HPLC analysis. The HPLC system consisted of a 150 X 4.6 mm, RP C<sub>18</sub>, 3- $\mu\text{m}$

247 Nucleosil column (Interchim, Montluçon, France) coupled with a 250 X 4.6 mm RP C<sub>18</sub>, 5-  
248 µm VydacTP 54 column (Hesperia, CA) and a 10 X 4.6 mm RP C<sub>18</sub>, 5-µm hypersil guard  
249 column. The mobile phase consisted of acetonitrile/methanol containing 50 mmol/L  
250 ammonium acetate/water/dichloromethane (70/15/5/10; V/V/V/V). Solvents were HPLC  
251 grade from Carlo Erba - SDS (Peypin, France). The flow rate was 2 mL/min. The columns  
252 were kept at a constant temperature (30°C). The HPLC system consisted of a Waters system  
253 equipped with a UV-visible photodiode-array detector (Waters 996). Carotenoids were  
254 detected at 450 nm and identified by their retention time compared with pure (> 95%)  
255 standards. Quantifications were performed with Millennium 32 software (version 3.05.01),  
256 comparing peak area with carotenoid standard reference curves. Carotenoid standards were a  
257 generous gift of DSM LTD, Basel, Switzerland.

258

#### 259 *Plasma lipids and apolipoproteins*

260 Triglyceride and total cholesterol concentrations were determined by enzymatic  
261 procedures with commercial kits (Roche, Switzerland). High-density lipoprotein (HDL)  
262 cholesterol was measured after sodium phosphotungstate–magnesium chloride precipitation.  
263 Low-density lipoprotein (LDL) cholesterol was estimated indirectly by use of the Friedewald  
264 formula.

265

#### 266 *Characteristics of the cohort of 622 subjects*

267 In order to verify the associations observed in the study on 29 volunteers we decided  
268 to verify associations observed between genetic variants and plasma lutein on a cohort of 622  
269 french subjects. This cohort was a subset of the french SUVIMAX cohort (42). Characteristics  
270 of this cohort were as follow: 281 males and 341 females, 61.65 ± 0.25 years old, BMI: 25.76  
271 ± 0.17 Kg/m<sup>2</sup>, plasma cholesterol: 2.23 ± 0.01 g/l, plasma lutein: 540 ± 10 nmol/L. Subjects

272 were genotyped for SNPs in CD36 and BCMO1 and haplotype effects of these genes on  
273 plasma lutein were tested.

274

#### 275 *Statistical analysis*

276 As a first approach, we wanted to estimate the relationships between genotypes, and  
277 both plasma lutein and MPOD in the study on 29 volunteers. For this purpose, we used partial  
278 least square (PLS) regression. PLS regression was performed by the SIMCA-P software,  
279 version 11.0 (Umetrics, Umeå, Sweden). The Y matrix was composed of the 2 vectors  
280 containing the initial plasma lutein concentrations and the initial MPOD. The X matrix  
281 contained the genotypes for the 20 SNPs. The values in the Y matrix were scaled with a unit  
282 variance scaling prior to the calculation of the latent vectors. After examination of the  
283 importance of each dependent variable, it appeared that only six SNPs were significant for the  
284 regression model. Therefore, a second regression, which used the genotypes from these six  
285 SNPs in the X matrix, was performed in order to minimize the noise in the model. The PLS  
286 model was calculated with the function “autofit” in the SIMCA-P software in order to find the  
287 optimal number of latent vectors. Note that correction for multiple testing was not compulsory  
288 with PLS regression because independent variables are not treated independently but are used  
289 simultaneously to find a reduced number of latent variables for the model. Furthermore,  
290 model validation, testing for the robustness of the model and its generalization to new data, is  
291 performed in this model with cross-validation.

292 In a second approach, we performed univariate statistical analyses only in associations  
293 that were simultaneously significantly associated with the two independent markers of lutein  
294 metabolism, which were plasma lutein and MPOD. The independence of these two  
295 parameters was checked by assessing the relationship between them (lack of linear Pearson’s  
296 correlation). The fact that SNPs were found associated with two independent markers of

297 lutein metabolism by both PLS and univariate analysis reduces the risk of false positive  
298 associations. Differences between means obtained in the different genotype groups were  
299 analyzed either by ANOVA followed by the *post hoc* Tukey-Kramer test, or by the Student's  
300 t-test when only two genotypes were observed. Values of  $p < 0.05$  were considered  
301 significant. All univariate statistical analyses were performed with Statview software version  
302 5.0 (SAS Institute, Cary, NC, U.S.A.).

303         In a third approach we performed an haplotype-based association analysis of data  
304 which originated from a cohort of 622 french subjects (see above the characteristic of the  
305 subjects). This analysis was performed with the THESIAS software, which is based on the  
306 Stochastic-EM algorithm, and which allows to infer haplotypes from genotypic data and to  
307 test their associations with phenotypes of interest (43). Analysis were performed after  
308 ajustement for the following covariables: gender, age and BMI.

309

## 310 **Results**

311

### 312 *Effect of dietary intervention on dietary lutein intake*

313 Dietary lutein intake of the subjects, fell from 1.3 mg/d to 0.03 mg/d after the lutein  
314 poor diet, showing a good compliance of the subjects to the recommendations, and increased  
315 to 0.4 mg/d during the dietary intervention, which was probably due to the fact that this period  
316 occurred during spring where most subjects increase their intake of fruits and vegetables rich  
317 in lutein. Note that there was no significant difference between the intake of the two groups at  
318 any of these periods..

319

### 320 *Effect of the dietary intervention on plasma lutein concentrations*

321 Initial plasma lutein concentrations ranged between 110 and 470 nmol/L ( $290 \pm 20$   
322 nmol/L). This high interindividual variability (CV=32%) did not change when plasma lutein  
323 was corrected for plasma cholesterol (CV=36%), nor when it was corrected for  
324 lutein+zeaxanthin intake (CV=146%). Surprisingly, the group that took the placebo (placebo  
325 group) exhibited a significant ( $P < 0.05$ ) rise in plasma lutein concentration after the dietary  
326 intervention (**Figure 1A**). Nevertheless, this increase was much higher in the group that took  
327 lutein supplement (+143 %) than in the placebo group (+77 %). Individual plasma lutein  
328 responses to the placebo and the lutein supplement are shown in **Figure 1B and 1C**,  
329 **respectively**. The interindividual response (change from the initial value) strongly varied,  
330 ranging from -100 to 1140 nmol/L. Consequently, the interindividual variability in plasma  
331 lutein increased after lutein supplementation (CV= 29% and 56% before and after  
332 supplementation, respectively).

333 Because lutein is almost exclusively carried by lipoproteins and because it is the reason  
334 why vitamin E ( $\alpha$ -tocopherol) is usually corrected for plasma cholesterol, we corrected

335 plasma lutein for plasma cholesterol. This adjustment led to observe a significant increase ( $P$   
336 = 0.003) in the plasma lutein/cholesterol ratio after lutein supplementation and no significant  
337 increase ( $P = 0.067$ ) of this ratio after supplementation by the placebo (data not shown).

338

### 339 *Relationships between genetic variants and biomarkers of lutein status*

340 The PLS analysis showed that the best model to explain the variance of both plasma  
341 lutein and MPOD was the one with one latent vector only. This model explained 31.4% of the  
342 variance of the Y matrix (38.1% for MPOD and 25.1% for plasma lutein). The predictive  
343 value of the model was estimated after cross-validation. We obtained an estimated variation  
344 of Y explained by the model of 16.1 % (14.6% for MPOD and 18.3% for plasma lutein). The  
345 six SNPs selected by the regression model were found in four genes: BCMO1, CD36,  
346 ABCG8 and NPC1L1. Coefficients of NPC1L1 were, however, not statistically significant.  
347 Furthermore, only the SNP in BCMO1 and two SNPs in CD36 were simultaneously  
348 significantly related to the two independent markers of lutein status. Thus association studies  
349 described thereafter were performed only with these two genes.

350

### 351 *Associations between genetic variants and plasma lutein levels*

352 Univariate analysis of plasma lutein showed that this parameter was higher in subjects  
353 who carried the CT genotype at BCMO1 rs7501331 than in those who carried the TT  
354 genotype (**Figure 2A**), although the difference was not significant. Note that no subject  
355 homozygous for the C allele was found in the study cohort. The difference became significant  
356 ( $P = 0.022$ ) when plasma lutein was corrected for plasma cholesterol: + 33 % in the subjects  
357 with the CT genotype (**Figure 2B**). Finally, no significant difference was observed when  
358 plasma lutein was corrected for lutein intake (data not shown).

359 Univariate analysis also showed that subjects with the CC genotype at rs13230419, a  
360 CD36 locus) had lower plasma lutein ( $P = 0.014$ ) than subjects with a T allele at this locus  
361 (**Figure 3A**). The difference remained significant ( $P = 0.044$ ) when plasma lutein was  
362 corrected for plasma cholesterol (**Figure 3B**). Adjustment for lutein intake led to a  
363 comparable figure (data not shown).

364 **Tables 3 and 4** show results of haplotypes association analysis between BCMO1 and  
365 CD36 haplotypes and plasma lutein/zeaxanthin concentration in a cohort of 622 subjects.  
366 There was no significant effect of the BCMO1 haplotypes on plasma lutein, either adjusted or  
367 not for plasma cholesterol (Table 3). Conversely, a minor haplotype of CD36 (AGA) had a  
368 significant effect on plasma lutein/zeaxanthin concentrations as compared to the most frequent  
369 haplotype (AAG). Note that this effect remained significant ( $P=0.048$ ) when plasma  
370 lutein/zeaxanthin was adjusted for plasma cholesterol (data not shown).

371

#### 372 *Effect of the dietary intervention on MPOD*

373 MPOD measured before supplementation was not significantly different between the  
374 two groups (**Figure 4A**). After the supplementation period, MPOD significantly increased ( $P$   
375  $< 0.05$ ) in both groups. Again, this increase was higher in the lutein group (+22%) than in the  
376 placebo group (+11%) (**Figure 4A**). Interestingly the interindividual variability in initial  
377 MPOD (CV=32%, **Figure 4B**) was similar to that observed for plasma lutein and, as observed  
378 for plasma lutein, increased when MPOD was corrected for lutein intake (CV=142%).  
379 Conversely, in opposite to what was observed for plasma lutein, the interindividual variability  
380 in MPOD decreased after supplementation with lutein (CV decreased from 39% to 25%,  
381 **Figure 4B**).

382 **Figure 5A** shows that the MPOD response was not related to the plasma lutein  
383 response. Furthermore, the initial MPOD was not related to the initial plasma lutein ( $r =$

384 0.009,  $P = 0.96$ ), when either corrected or not for plasma cholesterol ( $r = 0.20$ ,  $P = 0.30$ ) (data  
385 not shown). Conversely, there was a significant inverse relationship between the MPOD  
386 response and the initial MPOD (**Figure 5B**). In other words, the lower the initial MPOD was,  
387 the higher the MPOD response to the dietary intervention.

388

#### 389 *Association between genetic variants and MPOD*

390 **Figure 6** shows results of univariate analysis between the SNPs in BCMO1 and CD36  
391 and the initial MPOD. Subjects with CT at the BCMO1 SNP had significantly lower initial  
392 MPOD values than homozygous TT (**Figure 6A**). Interestingly the CT subjects had a higher  
393 MPOD response (delta from initial values) to the lutein supplement than the TT subjects (data  
394 not shown). Subjects bearing an A allele at rs1761667 (a CD36 locus) had significantly lower  
395 MPOD than homozygous GG (Figure 7B). As observed for BCMO1, the genetic groups with  
396 the lower MPOD (the AA and AG groups) had a higher MPOD response to the lutein  
397 supplement than the group with the higher MPOD (the GG group) (data not shown).

398

**399 Discussion**

400

401           Initially, we verified the effect of lutein supplementation on two markers of lutein  
402 status, which were plasma lutein and MPOD. The dose of lutein provided by the supplement  
403 (10 mg/d) and the duration of the supplementation (6 months) were selected to enable the  
404 detection of significant variations in MPOD (44-46). The fact that, for better compliance,  
405 lutein was provided as pills instead of foods (spinach, for example) is unlikely to have  
406 influenced the results significantly, because it has been shown that lutein bioavailability is not  
407 significantly different between spinach and lutein supplements (47, 48). The significant  
408 increase of both markers in the lutein supplemented group was expected, because it is in  
409 agreement with previous studies (44, 48, 49). The first obvious conclusion is that these  
410 increases were due to the lutein present in the supplement. The significant increase of both  
411 markers in the placebo (control) group did, however, raise some questions about this  
412 conclusion. Nevertheless, it should be remembered that lutein was not only provided by the  
413 supplement, but it was also, of necessity, present in the subjects' diets. We thus hypothesized  
414 that the increase of the lutein markers in the placebo group was due to an increase in dietary  
415 lutein intake. This hypothesis was supported by the fact that a second food diary, kept during  
416 the supplementation period, which occurred in spring where subjects increase their intake of  
417 lutein-rich fruit and vegetables, showed that all subjects increased their dietary intake of lutein  
418 as compared to their initial dietary intake. Given that the food diaries showed that the dietary  
419 intake of lutein during the supplementation period was not significantly different between the  
420 two groups, this demonstrated that the higher increase of both markers in the lutein  
421 supplemented group (+143 % vs. +77 % for plasma lutein and +22 % vs. +11 % for MPOD,  
422 in the lutein and placebo groups, respectively) was due to the lutein supplement.

423 MPOD is assumed to be related to the macula lutea concentration of lutein. Because  
424 some studies showed a significant relationship between blood lutein and MPOD (36, 50),  
425 while other studies did not find this association (49, 51), we aimed to re-assess this  
426 relationship. Our results show that MPOD was not related to plasma lutein, whatever the  
427 parameter used, such as plasma lutein, plasma lutein corrected for plasma cholesterol or  
428 plasma lutein corrected for lutein intake. In fact, this association, when observed, was weak  
429 and found only with a large number of subjects (36, 52). Thus, considering the small number  
430 of subjects in our study, our result is not surprising. Interestingly, and in agreement with a  
431 recent study (53), we found that the MPOD response was inversely and significantly related to  
432 the initial MPOD. This shows that the subjects who respond the most to dietary lutein are  
433 those who have the lowest initial MPOD. This should be taken into account in future studies  
434 of the effect of different parameters on MPOD.

435 The main objective of this study was to check the hypothesis that genetic variants can  
436 explain, at least in part, the interindividual variability in blood lutein concentrations and  
437 MPOD observed in this study (figures 1 and 5), as well as in previous studies. To that aim, we  
438 compared plasma lutein concentrations and MPOD in volunteers bearing different SNPs in  
439 genes potentially involved in lutein metabolism. Because we wanted to predict blood lutein  
440 concentrations and MPOD (dependent variables) from a large set of SNPs (independent  
441 variable), we used PLS regression. As stated in the material and method section this analysis  
442 has the advantage of avoiding false positive results that could be obtained in multiple testing  
443 and small sample size. This analysis showed that a significant part of the variability (38% for  
444 MPOD and 25% for plasma lutein) can be assigned to genetic variants in CD36, BCMO1 and  
445 ABCG8. Furthermore, three SNPs were simultaneously and significantly related to plasma  
446 lutein and MPOD: one in BCMO1, and two in CD36. Since plasma lutein and MPOD were  
447 not related, this double association further supports the involvement of these genes in lutein

448 metabolism. However, in order to reinforce the validity of these associations we decided to  
449 perform an haplotype-based association analysis of data which originated from a cohort of  
450 622 subjects. This analysis confirmed the association of plasma lutein with CD36 but failed to  
451 find a significant association between plasma lutein and BCMO1. This is likely due to an  
452 insufficient statistical power because a recent genome wide association study supported a role  
453 of BCMO1 in plasma lutein levels (54).

454         The association of plasma lutein concentration with BCMO1 suggests that this gene,  
455 which encodes an enzyme responsible for the cleavage of provitamin A carotenoids into  
456 retinal (29), is also involved in the metabolism of lutein, which is not a provitamin A  
457 carotenoid. This association is probably indirect because zeaxanthin (a xanthophyll which  
458 exhibits a chemical structure very close to lutein) was not cleaved *in vitro* by BCMO1 (55),  
459 supporting the assumption that BCMO1 is not involved in lutein cleavage. One hypothesis  
460 might be that vitamin A status, related to BCMO1 activity, can modulate lutein metabolism  
461 by modulating expression of transporters of lutein. This is supported by a publication showing  
462 that *all-trans* retinoic acid strongly up-regulate CD36 (56). The association between BCMO1  
463 and MPOD was observed for the first time and suggests that BCMO1 activity modulate the  
464 concentration of lutein in the retina. The effect of this SNP in BCMO1 on plasma and retina  
465 concentration of lutein is probably related to an effect on BCMO1 activity. Indeed this SNP  
466 leads to a change in an amino acid (A379V), and this change is functional, as a recombinant  
467 267S + 379V double mutant showed a reduced BCMO1 catalytic activity and carriers of the  
468 379V genotype had a reduced ability to convert  $\beta$ -carotene (57).

469         The second gene that is apparently involved in lutein metabolism is the scavenger  
470 receptor CD36, also known as FAT. This is not very surprising as, SR-BI, a scavenger  
471 receptor like CD36, has been involved in the uptake of lutein by both intestinal (13) and  
472 retinal cells (28). Because CD36 is expressed in the intestine and in several other tissues, we

473 hypothesize that functional variants in its gene may affect lutein absorption efficiency, or  
474 tissue uptake of lutein, or both, leading to lower concentrations of plasma and retina lutein.  
475 Unfortunately, there are no functional data available about the effect of the studied variants in  
476 CD36 on the activity, or expression, of CD36.

477         The lack of association between the two lutein biomarkers and the SNPs in SCARB1,  
478 the gene that encodes SR-BI, was rather unexpected, since this transporter has been involved  
479 in lutein absorption (13) and retinal cell uptake (28). Furthermore, two out of the three  
480 genotyped SNPs (rs5888 and rs4238001) have been associated with lower SR-BI protein  
481 expression and function (58, 59). Thus, the lack of association between these SNPs and  
482 plasma lutein and MPOD, together with the fact that CD36 (another scavenger receptor which  
483 share many structural characteristics with SR-BI) was associated with plasma and retina  
484 lutein, suggests that CD36 is probably more important for lutein uptake by cells than SR-BI  
485 and that it can compensate for lower expression/function of SR-BI.

486         The fact that two genes were associated with the studied biomarkers of lutein status  
487 raises questions about interactions between these genes. Unfortunately, the number of subjects  
488 enrolled in this study was too low to detect a significant interaction between BCMO1 and  
489 CD36 (two factors ANOVA).

490

491         In summary, our results suggest that genetic variants in BCMO1 and CD36 modulate  
492 plasma and retina lutein concentrations. This observation may have consequences with regard  
493 to the recommended dietary intake of lutein in groups of subjects bearing unfavourable  
494 genetic variants in these genes.

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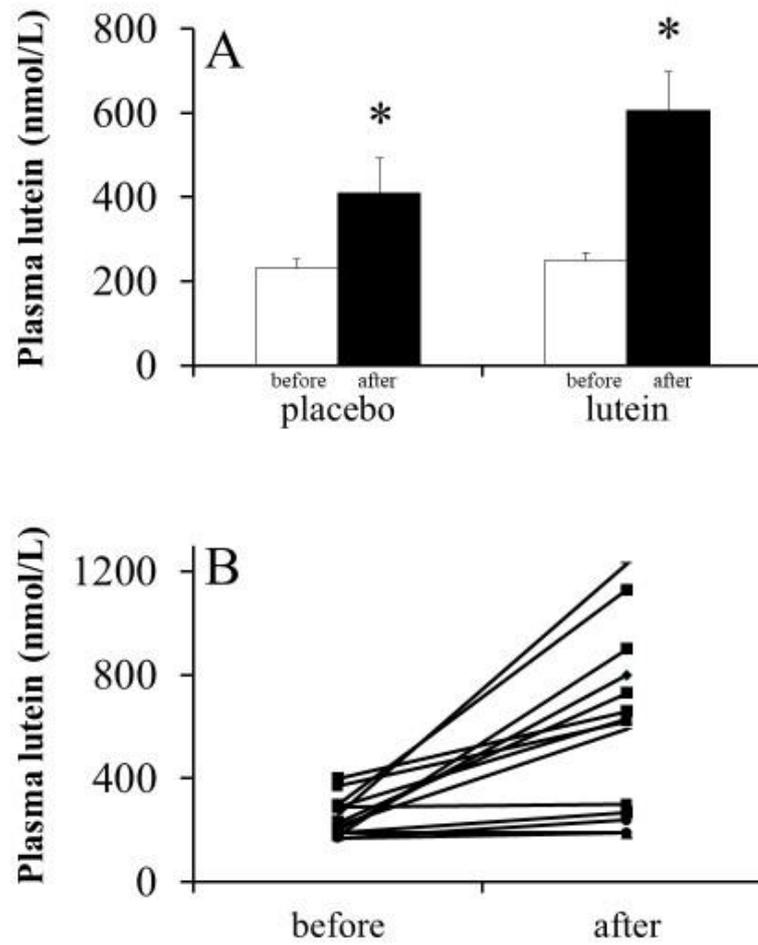
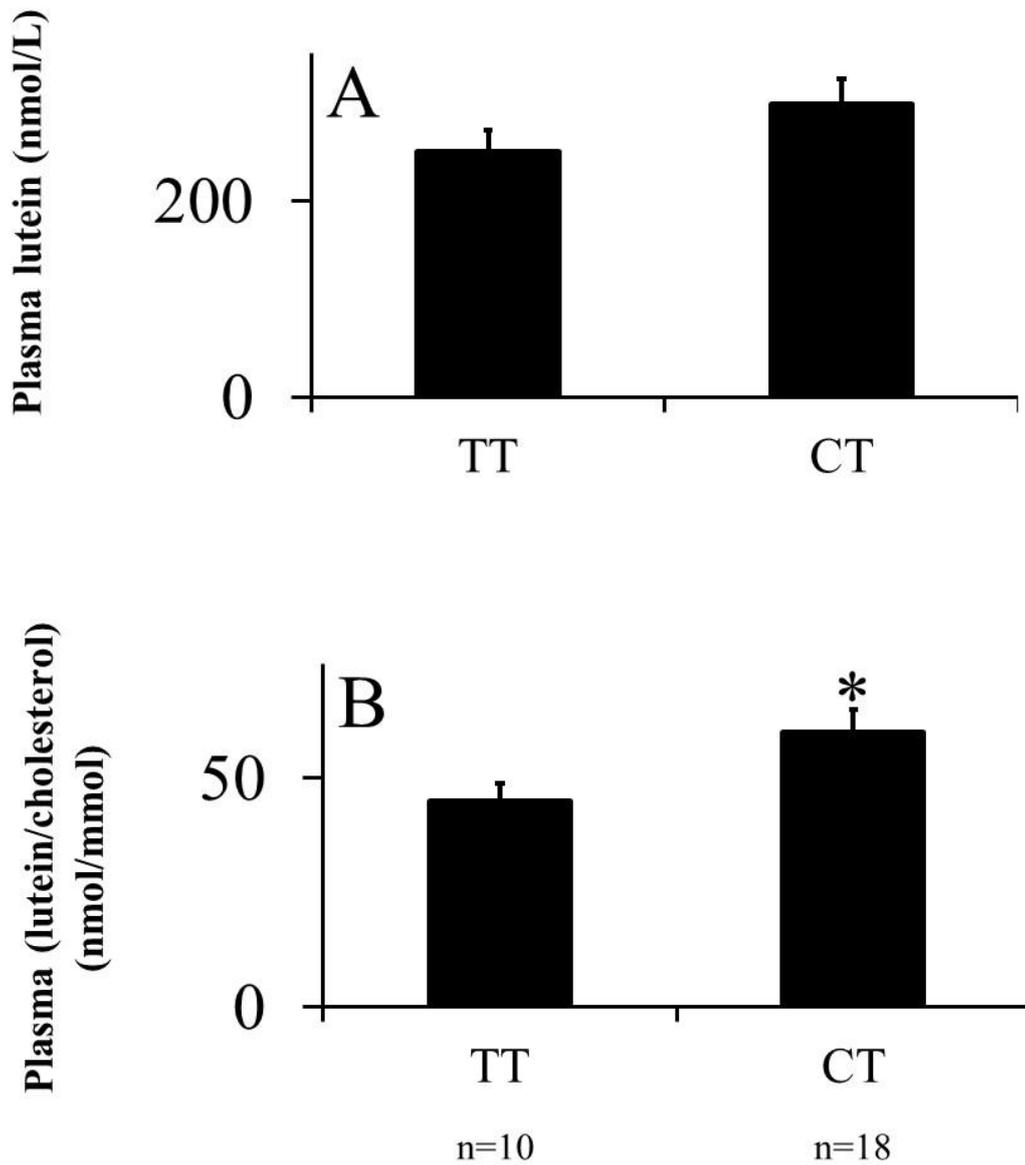


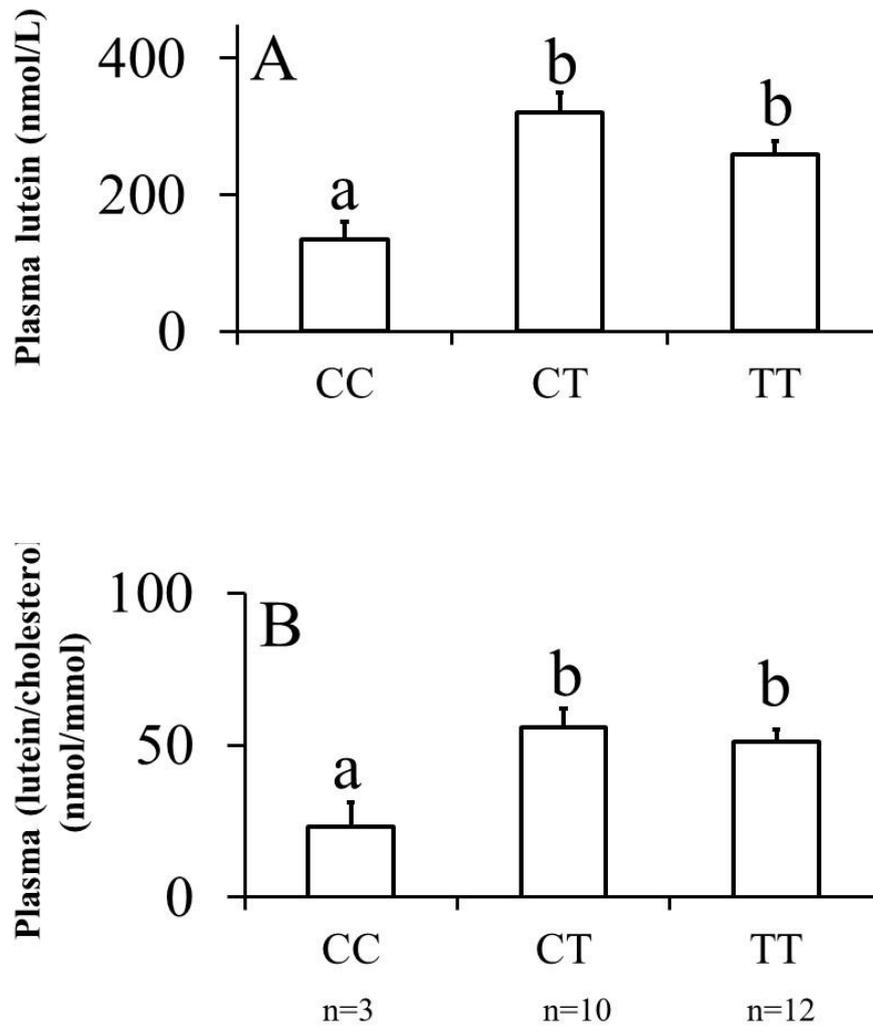
Figure 1

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**Figure 1:** (A) Plasma lutein concentrations, before and after supplementation, in both the group that took the placebo and the group that took the lutein supplement. White bars: values measured before supplementation. Black bars: values measured after supplementation. Means  $\pm$  SEM of 15 subjects in placebo group and 14 subjects in lutein group. An asterisk indicates a significant difference ( $P < 0.05$ ) between values measured before and after the supplementation period in each group (paired t test). (B) Individual plasma lutein concentrations, before and after supplementation, in the placebo group. (C) Individual plasma lutein concentrations, before and after supplementation, in the lutein supplemented group.



671  
672 **Figure 2:** Baseline plasma lutein concentrations for each BCMO1 rs7501331 genotype. (A) uncorrected values.  
673 (B) values corrected for plasma total cholesterol. Data are means  $\pm$  SEM of 28 subjects. n is the number of  
674 subject in each genotype group. An asterisk indicates a significant difference ( $P < 0.05$ ) between genotype  
675 groups (Student's t test).  
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692 **Figure 3:** Baseline plasma lutein concentrations for each CD36 rs13230419 genotype. (A) uncorrected values.  
 693 (B) Values corrected for plasma total cholesterol. Data are means  $\pm$  SEM of 25 subjects. n is the number of  
 694 subject in each genotype group. In each figure, different letters indicate significant differences ( $P < 0.05$ )  
 695 between genotype groups (ANOVA).  
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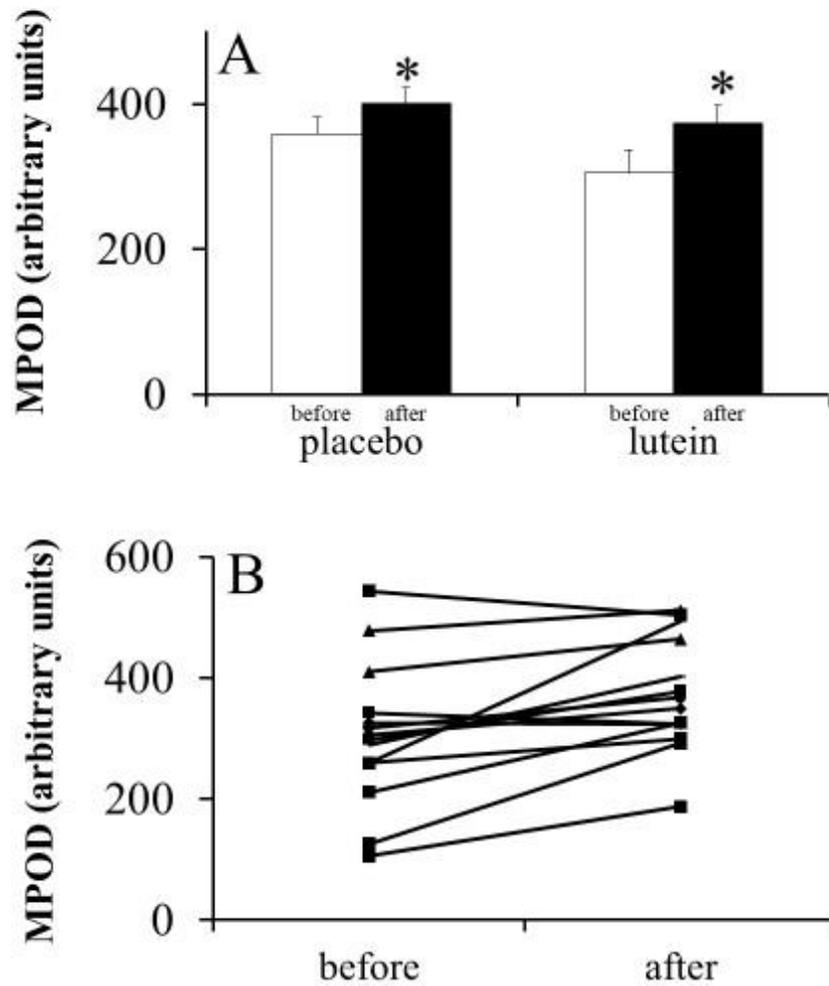
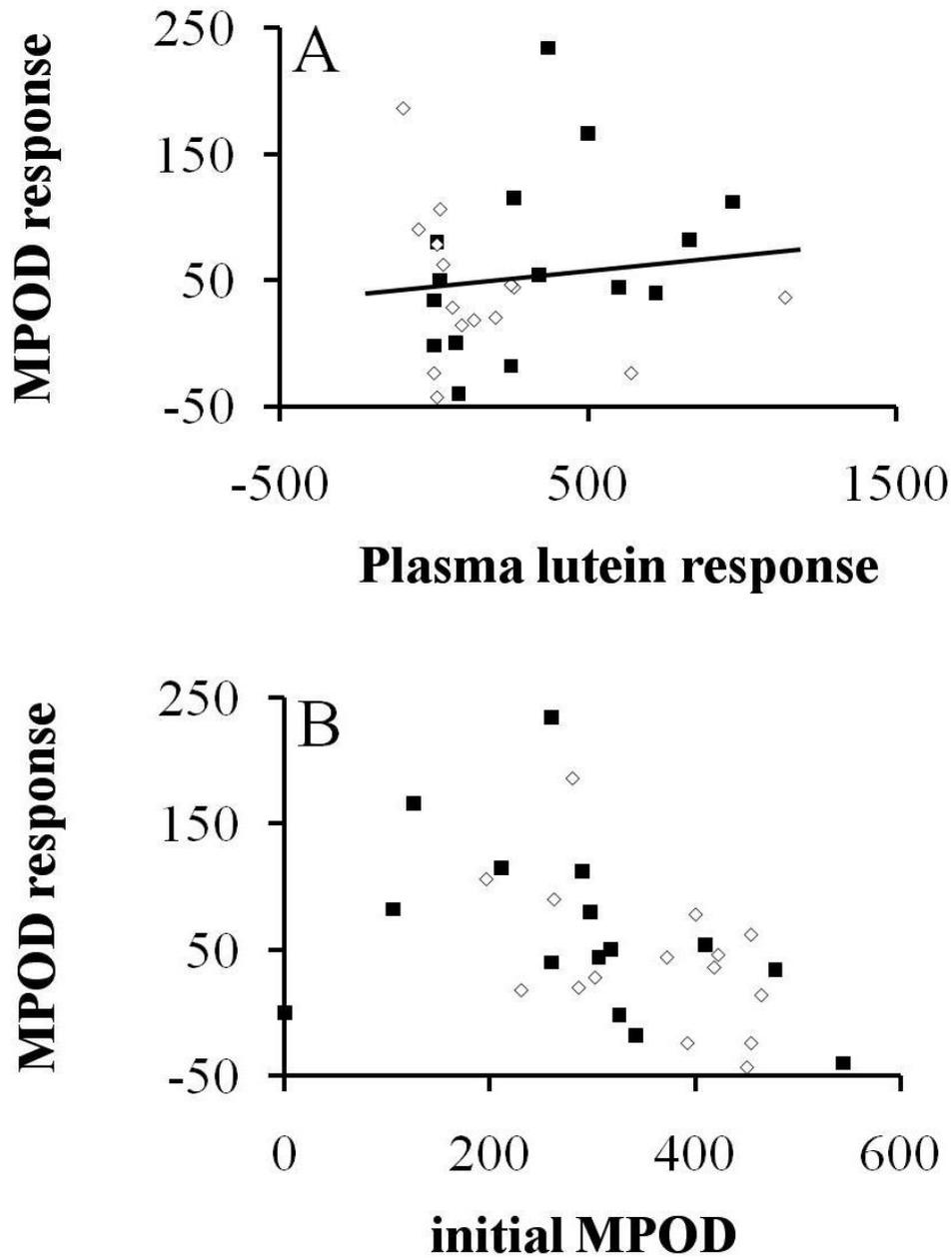


Figure 4

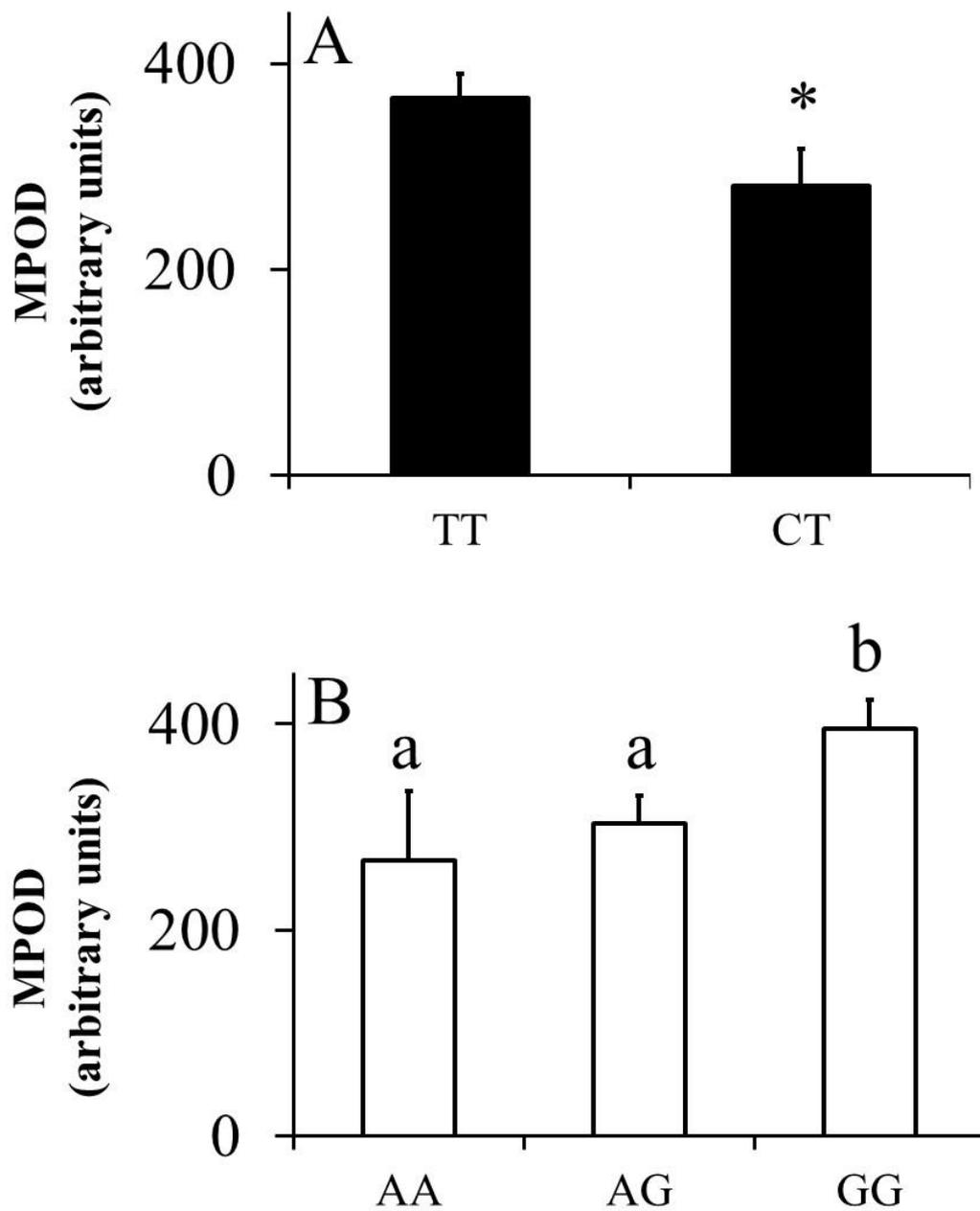
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**Figure 4:** (A) MPOD (macular pigment optical density), before and after supplementation, in the group that took the placebo and the group that took the lutein supplement. White bars: values measured before supplementation (before). Black bars: values measured after supplementation (after). Means  $\pm$  SEM of 15 subjects in placebo group and 14 subjects in lutein group. An asterisk indicates a significant difference ( $P < 0.05$ ) between values measured before and after the supplementation period in each group (paired t test). (B) Individual MPOD before and after supplementation in the lutein supplemented group (n=14).



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**Figure 5:** (A) MPOD response as a function of plasma lutein response. (B) MPOD response as a function of initial MPOD. “Response” indicates the differences between the values measured after the dietary intervention and the values measured before the intervention ( $n = 29$ ). White dots: subjects in the placebo group, black dots: subjects in the lutein supplemented group.



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**Figure 6:** MPOD for each BCMO1 and CD36 genotype. (A) BCMO1 rs7501331 genotypes. An asterisk indicates a significant difference ( $P < 0.05$ ) between genotype groups (Student's t test). (B) CD36 rs1761667 genotypes. Different letters indicate significant differences ( $P < 0.05$ ) between genotype groups (ANOVA). n is the number of subject in each genotype group.

756 **Tables**

757

758 **Table 1** Characteristics and nutrient intake of the 29 male subjects enrolled in the clinical study<sup>1</sup>

<b>Item</b>	<b>Mean ± SD</b>
Age, <i>y</i>	49.7 ± 0.8
Height, <i>cm</i>	174.2 ± 1.2
Weight, <i>kg</i>	71.1 ± 1.3
Plasma analytes	
Glucose, <i>mmol/L</i>	4.88 ± 0.09
Triglycerides, <i>mmol/L</i>	0.93 ± 0.07
Total cholesterol, <i>mmol/L</i>	4.91 ± 0.14
HDL cholesterol, <i>mmol/L</i>	1.63 ± 0.08
LDL cholesterol, <i>mmol/L</i>	2.99 ± 0.13
Nutrient intake <sup>1</sup>	
Total Energy, <i>kJ/d</i>	2406 ± 103
Alcohol, <i>g/d</i>	9.0 ± 2.2
Carbohydrate, <i>g/d</i>	279.1 ± 14.2
Protein, <i>g/d</i>	94.2 ± 4.4
Fat, <i>g/d</i>	98.0 ± 4.9
Lutein+zeaxanthin <i>mg/d</i>	1.3 ± 0.4

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760 <sup>1</sup>Nutrient intake of the subjects as evaluated, at the beginning of the study (i.e. before the lutein-poor diet), by a

761 3-day food diary.

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763 **Table 2** Characteristics of the studied SNPs  
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Gene	dbSNP ID	SNP ID	Main allele	Minor allele	MAF (%) <sup>1</sup>	Intron (I)/Exon (E)/N(Near the gene)	Possible effect on protein activity/expression
ABCA1	rs2230808	hCV2741104	G	A	24.3	E	NS <sup>2</sup>
ABCA1	rs2066718	hCV11720789	G	A	3.5	E	NS
ABCA1	rs2230806	hCV2741051	G	A	28.8	E	NS
ABCA1	rs2230805	hCV2741050	G	A	27.4	E	silent
ABCG5	rs6720173	hCV29001998	G	C	15.2	E	NS
ABCG8	rs4148211	hCV29535502	A	G	35.0	E	NS
ABCG8	rs4148217	hCV375061	C	A	20.2	E	NS
ABCG8	rs6544718	hCV25642779	C	T	20.5	E	NS
BCDO2	rs7123686	hCV22274763	A	C	33.6	I	-
BCMO1	rs12934922	hCV25745282	A	T	45.7	E	NS
BCMO1	rs7501331	hCV <sup>3</sup>	T	C	23.6	E	NS
CD36	rs1527483	hCV8315330	C	T	8.0	I	-
CD36	rs1761667	hCV8314499	A	G	45.1	N	?
CD36	rs13230419	hCV1803768	T	C	44.0	N	?
CD36	rs7755	hCV8315318	G	A	43.9	N	?
CD36	rs1984112	hCV12093946	A	G	35.8	N	?
NPC1L1	rs217434	hCV2292875	A	G	18.2	E	silent
NPC1L1	rs217428	hCV29736499	T	G	24.1	I	-
NPC1L1	rs17655652	hCV2625150	T	C	31.8	N	?
SCARB1	rs5888	hCV7497008	C	T	47.2	E	silent
SCARB1	rs4238001	hCV26062113	C	T	13.0	E	NS
SCARB1	intron 5 <sup>3</sup>	hCV25608758	C	T	10.0	I	-

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766 <sup>1</sup>MAF: minor allele frequency as observed in a cohort of 2994 french subjects which was a subset of the french767 SUVIMAX cohort (42). <sup>2</sup>NS: non synonymous SNP (missense), indicating a change of amino acid. <sup>3</sup>not

768 referenced in SNP ID or dbSNP. ?: effect not known, although it may have a possible effect on gene expression.

769 SNPs were genotyped with the SNPlex assay except hCV26062113 and hCV25608758, which were genotyped

770 by Taqman (see Materials and methods).

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778 **Table 3** BCMO1 haplotypes effects on plasma lutein/zeaxanthin

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780 Haplotypes

rs12934922	rs7501331	Frequencies <sup>1</sup> (%)	Haplotype effect <sup>2</sup>
A	C	34.1	0.571
A	T	21.1	0.887
T	C	42.7	-
T	T	2.1	0.368

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782 Plasma lutein/zeaxanthin and BCMO1 SNPs were assessed in 622 french subjects (281 males, 341 females).

783 <sup>1</sup>Haplotype frequencies were estimated using Thesias software (43). The most frequent haplotype (TC) is the

784 reference haplotype. <sup>2</sup>Haplotype effect on plasma lutein/zeaxanthin by comparison to the most frequent

785 haplotype (p value calculated by Student t-test).

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788 **Table 4** CD36 haplotypes effects on plasma lutein/zeaxanthin  
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rs1984112	rs1761667	rs7755	Haplotype Frequencies <sup>1</sup> (%)	Haplotype effect <sup>2</sup>
A	A	A	10.2	0.992
A	A	G	45.2	-
A	G	A	8.4	0.018
A	G	G	1.9	0.567
G	A	G	0.1	- <sup>3</sup>
G	G	A	26.2	0.908
G	G	G	7.9	0.074

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791 Plasma lutein/zeaxanthin and CD36 SNPs were assessed in 622 french subjects (281 males, 341 females).

792 <sup>1</sup>Haplotype frequencies were estimated using Thesias software (43). The most frequent haplotype (AAG) is the

793 reference haplotype. <sup>2</sup>Haplotype effect on plasma lutein/zeaxanthin by comparison to the most frequent

794 haplotype (p value calculated by Student t-test). <sup>3</sup>could not be calculated because of the too low frequency of this

795 haplotype.

796

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798

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806 Severine Bieuvelet.

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