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## **CD36 and SR-BI Are Involved in Cellular Uptake of Provitamin A Carotenoids by Caco-2 and HEK Cells, and Some of Their Genetic Variants Are Associated with Plasma Concentrations of These Micronutrients in Humans**

Patrick Borel, G. Lietz, A. Goncalves, F. Szabo de Edelenyi, S. Lecompte, P. Curtis, L. Goumidi, M. Caslake, E. Miles, C. Packard, et al.

### ► **To cite this version:**

Patrick Borel, G. Lietz, A. Goncalves, F. Szabo de Edelenyi, S. Lecompte, et al.. CD36 and SR-BI Are Involved in Cellular Uptake of Provitamin A Carotenoids by Caco-2 and HEK Cells, and Some of Their Genetic Variants Are Associated with Plasma Concentrations of These Micronutrients in Humans. *Journal of Nutrition*, 2013, 143 (4), pp.448-456. 10.3945/jn.112.172734 . inserm-01478566

**HAL Id: inserm-01478566**

**<https://inserm.hal.science/inserm-01478566v1>**

Submitted on 20 Apr 2021

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**Both CD36 and SR-BI are involved in cellular uptake of provitamin A carotenoids by Caco-2 and HEK cells, and genetic variants in their encoding genes are associated with plasma concentrations of these micronutrients in humans<sup>123</sup>**

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<sup>1</sup> (**Supplemental Tables 1,2 and 3**) are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

<sup>2</sup> **Conflict of interest:** No authors have any conflict of interest to declare.

<sup>3</sup> **Grants, supports and funding sources:** The SUVIMAX study was funded by DGS (Ministry of Health) and supported by Médéric, Ipsen, MGEN, SODEXHO and Pierre Fabre. Work on the subset population of SUVIMAX was granted by ANR (n°ANR-05-PNRA-010). The HELENA Study was funded by the European Union's Sixth RTD Framework Programme [grant number Contract FOOD-CT-2005-007034]; Universidad Politécnica de Madrid [grant number CH/018/2008]; Axis-Shield Diagnostics Ltd (Oslo, Norway); Abbot Científica S.A. (Spain); the Spanish Ministry of Education [EX-2007-1124], and Cognis GmbH (Germany). The FINGEN study was supported by contract no. RRD7/N02/A from the UK Food Standards Agency.

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Word count: 6944.

Number of figures: 2

Number of tables: 5

Supplemental online material: 3

Running title: SR-BI and CD36 and blood provitamin A carotenoids

Last name of each author: Borel, Lietz, Goncalves, Szabo de Edelenyi, Lecompte, Curtis, Goumidi, Caslake, Miles, Packard, Calder, Mathers, Minihane, Tourniaire, Kesse-Guyot, Galan, Hercberg, Breidenassel, González Gross, Moussa, Meirhaeghe, Reboul

Abbreviations: ABCA1 (ATP Binding Cassette A1), BLT1 (blocks lipid transport 1), BMI (Body Mass Index), CD36 (cluster determinant 36), DMEM (Dulbecco's modified Eagle's medium), DMSO (Dimethyl sulfoxide), FAT (fatty acid transporter), FBS (Fetal Bovine Serum), HEK (Human Embryonic Kidney), SSO (succinimidyl sulfonyl oleate), SNP (single nucleotide polymorphism), SR-BI (scavenger receptor class B type I).

## 1 **Abstract**

2

3 SR-BI (scavenger receptor class B type I) and CD36 (cluster determinant 36) have been  
4 involved in cellular uptake of some provitamin A carotenoids. However data are incomplete,  
5 e.g. no data on  $\alpha$ -carotene, and it is not known whether genetic variants in their encoding  
6 genes can affect provitamin A carotenoid status. The objectives were i) to assess the  
7 involvement of these scavenger receptors in cellular uptake of the main provitamin A  
8 carotenoids, i.e.  $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin, as well as in that of preformed  
9 vitamin A, i.e. retinol, and ii) to investigate the contribution of genetic variations in genes  
10 encoding these proteins to interindividual variations in plasma concentrations of provitamin A  
11 carotenoids. The involvement of SR-BI and CD36 on carotenoids and retinol cellular uptake  
12 was investigated in Caco-2 and HEK (Human Embryonic Kidney) cell lines. The involvement  
13 of *SCARB1* and *CD36* genetic variants on plasma levels of provitamin A carotenoids was  
14 assessed by association studies in three independent populations. Cell experiments suggested  
15 the involvement of both proteins in cellular uptake of provitamin A carotenoids, but not in that  
16 of retinol. Association studies showed that plasma provitamin A carotenoid concentrations  
17 were significantly different ( $P <$  than alpha values calculated after correction for multiple  
18 comparisons) between subjects who bore different genotypes at single nucleotide  
19 polymorphisms and haplotypes in *CD36* and *SCARB1*. In conclusion, SR-BI and CD36 are  
20 involved in cellular uptake of provitamin A carotenoids, and genetic variations in their  
21 encoding genes may modulate plasma concentrations of provitamin A carotenoids at a  
22 population level.

23

## 24 **Introduction**

25

26 Vitamin A is found in the human diet either as preformed vitamin A in foods from  
27 animal origin, or as provitamin A carotenoids in foods from plant origin. The main dietary  
28 provitamin A carotenoids are, in a decreasing order of provitamin A activity:  $\beta$ -carotene,  $\alpha$ -  
29 carotene and  $\beta$ -cryptoxanthin. Both sources of vitamin A have led to the development of  
30 different strategies to fight vitamin A deficiency in developing countries where it is still a  
31 serious public health problem affecting mostly pregnant or lactating women and preschool  
32 children, with an estimated 250 million at risk of developing vitamin A deficiency disorders  
33 (1). The latest strategies to increase the availability of provitamin A carotenoids are either  
34 through the breeding of new varieties of staple food crops with higher concentrations of  
35 provitamin A carotenoids (biofortification) or through genetic modification of existing crops  
36 (i.e. golden rice) (2-4).

37 The efficiency of vitamin A and carotenoid absorption is determined by the regulation of a  
38 number of proteins involved in the process (5). Free retinol enters into intestinal cells by  
39 either an unknown facilitated process and/or simple diffusion process (6), while its secretion  
40 may require a facilitated transport at physiological doses via ABCA1 (ATP Binding Cassette  
41 A1) (7-9). Conversely, although the intestinal absorption of pro-vitamin A carotenoids was  
42 thought to be a passive diffusion process (5, 10), it is now becoming clear that the absorption  
43 of lutein (11),  $\beta$ -carotene (7, 12, 13),  $\beta$ -cryptoxanthin (13), and lycopene (14) involves  
44 scavenger receptor class B type I (SR-BI), an enterocyte apical membrane transporter. It has  
45 further been suggested that the cluster determinant 36 (CD36), also known as FAT (fatty acid  
46 transporter), is involved in cellular uptake of  $\beta$ -carotene (12). Importantly, the involvement of  
47 CD36 in cellular uptake of carotenoids was confirmed in two recent studies showing that  
48 Cameo2, which is considered homologous to mammalian CD36, is implicated in carotenoid

49 transport in the silk gland in *Bombyx mori* (15) and in uptake of carotenoids by adipose tissue  
50 (16). To date, the involvement of these scavenger receptors in the intestinal uptake of retinol  
51 has been studied only for SR-BI (7). However, it is difficult to draw definite conclusions from  
52 these results as the siRNA strategy used led to only partial (40%) knockdown of SR-BI  
53 expression. Furthermore there is no data on the involvement of SR-BI in the intestinal uptake  
54 of  $\alpha$ -carotene, one of the three main dietary provitamin A carotenoids, as well as on the  
55 involvement of CD36 in the intestinal uptake of retinol,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin.

56 The involvement of proteins in cellular absorption of provitamin A carotenoids (5, 6)  
57 led us to hypothesize that genetic variations in the genes encoding these proteins could affect  
58 the uptake efficiency of these compounds (17). In turn, this raises the possibility that genetic  
59 variants in these key genes could affect the pro-vitamin A carotenoid status of affected  
60 individuals and in turn explain some of the large observed inter-individual variations. (17, 18).

61 The objectives of this study were to provide evidences in cell lines to support the  
62 involvement of SR-BI and CD36 in the cellular uptake of  $\beta$ -carotene,  $\alpha$ -carotene,  $\beta$ -  
63 cryptoxanthin and retinol. Furthermore, the study assessed whether genetic variants in genes  
64 that encode these transporters are associated with plasma concentration of provitamin A  
65 carotenoids at a population level using data derived from three independent populations.

66

## 67 **Subjects, materials and methods**

68

### 69 *Products and reagents*

70 All-*trans*-carotenoids ( $\alpha$ -carotene,  $\beta$ -carotene,  $\beta$ -cryptoxanthin, echinenone; all  $\geq$  95%  
71 pure) were a generous gift from DSM LTD, Basel, Switzerland. All-*trans*-retinol ( $\geq$  99%  
72 pure), all-*trans*-retinyl palmitate ( $\geq$  85% pure, retinol-free), retinyl-acetate, 2-oleoyl-1-  
73 palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-  
74 phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium  
75 taurocholate and pyrogallol were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,  
76 France). BLT1 (blocks lipid transport 1), which is a highly specific inhibitor of SR-BI that  
77 does not interfere with receptor-mediated endocytosis or other forms of intracellular vesicular  
78 traffic (19), was purchased from Chembridge (San Diego, CA, U.S.A.). SSO (succinimidyl  
79 sulfonyl oleate), which is a specific chemical inhibitor of CD36 (20), was synthesized as  
80 previously published (21). Its purity was checked by UPLC/ESI(-)MSn and  $^1\text{H-NMR}$  analyses  
81 and was  $> 99\%$ . Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose  
82 and trypsin-EDTA (500 mg/L and 200 mg/L respectively) were purchased from BioWhittaker  
83 (Fontenay-sous-Bois, France), fetal bovine serum was from Biomedica (Issy-les-Moulineaux,  
84 France) and non-essential amino acids and penicillin/streptomycin were purchased from  
85 Gibco BRL (Cergy-Pontoise, France). The protease-inhibitor cocktail was from Roche  
86 (Fontenay-sous bois, France). All solvents used were HPLC grade and purchased from Carlo  
87 Erba - SDS (Peypin, France).

88

### 89 *Preparation of retinol and carotenoid-rich micelles*

90 For the delivery of retinol or carotenoids to human intestinal Caco-2 cell monolayers,  
91 mixed micelles were prepared as published previously (11) to obtain the following final



92 concentrations: 0.04 mmol/L phosphatidylcholine, 0.16 mmol/L lysophosphatidylcholine, 0.3  
93 mmol/L monoolein, 0.1 mmol/L free cholesterol, 0.5 mmol/L oleic acid, 0.5 to 5  $\mu$ mol/L  
94 carotenoid or retinol (22, 23) and 5 mmol/L taurocholate. The concentrations of retinol and  
95 carotenoids in the micellar solutions were checked before each experiment.

96

#### 97 *Preparation of retinol and carotenoid-rich complete medium*

98 For delivery of retinol or carotenoids to HEK cells, an appropriate volume of each  
99 micronutrient solvent stock solutions was carefully evaporated under nitrogen in a glass tube.  
100 The dried residue was solubilized into FBS (Fetal Bovine Serum) overnight and DMEM was  
101 then added to reach a final FBS concentration of 10%. Concentration of retinol or carotenoids  
102 in the medium was checked before each experiment.

103

#### 104 *Cell culture*

105 *Caco-2 cell culture* - Caco-2 clone TC-7 cells (24, 25), which are human intestinal  
106 cells, were a gift from Dr. M. Rousset (UMR S 872, Paris, France). These cells express SR-BI  
107 (11) but not CD36 (11). Cells were cultured in the presence of DMEM supplemented with  
108 20% heat-inactivated fetal bovine serum, 1% non-essential amino acid, and 1% antibiotics  
109 (complete medium) as previously described (11). For each experiment, the cells were seeded  
110 and grown on transwells to obtain confluent differentiated cell monolayers. Twelve hours  
111 before each experiment, the medium used in the apical and basolateral chambers was changed  
112 to a serum-free complete medium, and cell monolayer integrity was checked by measuring the  
113 trans-epithelial electrical resistance as previously described (11).

114

115 *HEK cell culture and transfection* – HEK (Human Embryonic Kidney) 293-T cells,  
116 which came from Human embryonic kidney, were purchased from American Type Culture

117 Collection (Manassas VA, USA). Cells were cultured in 10% FBS complete medium as  
118 previously described (21). For each experiment, cells were seeded at 1:10 dilution in 6-well  
119 plates, and transfected 24 h after with 3  $\mu$ g DNA (i.e. human SR-BI in pCDNA3.1 plasmid,  
120 empty pCDNA3.1 plasmid, human *CD36* in pIRES plasmid and empty pIRES plasmid) and 6  
121  $\mu$ L Jet PeI per well in NaCl 150 mmol/L according to the manufacturer's instructions. The  
122 medium was then changed 10-12 hours after and cells were grown for an additional 24 h.  
123 Transfection efficiency was checked by western blotting as previously published (11). Briefly,  
124 proteins were determined using a bicinchoninic acid kit (Pierce, Montluçon, France), and 50  
125  $\mu$ g were used for Western blot analysis. The blotting membrane was incubated with the  
126 mouse monoclonal IgG against the external domain of human SR-BI (anti-CLA-1, BD  
127 Transduction Laboratories, Lexington, KY) or the mouse monoclonal anti-human CD36 IgM  
128 (Sigma) at 1/1000 dilution. For visualisation, monoclonal anti-mouse IgM or IgG conjugated  
129 to alkaline phosphatase (Sigma) were used as secondary antibodies at 1/5000 dilution.

130

### 131 *Measurement of carotenoid and retinol uptake by cells*

132 At the beginning of each experiment, the cell monolayers were washed twice with 0.5  
133 mL PBS. The apical side of the Caco-2 cell monolayers received the micelles, and the other  
134 side received the serum-free complete medium. The HEK cells received the enriched complete  
135 medium. The cells were incubated for 1 h at 37°C. At the end of each experiment, media were  
136 harvested. The cells were washed twice with 0.5 mL ice-cold PBS, then scraped and collected  
137 into 0.5 mL PBS. Uptake was estimated from the concentration of retinol or carotenoids in the  
138 scraped cells (and in the basolateral medium if applicable). Because provitamin A carotenoids  
139 could be cleaved to retinal (26, 27), that is reduced into retinol, which can in turn be esterified  
140 to produce retinyl esters (8), both retinol and retinyl esters were measured to avoid  
141 underestimation of the uptake of vitamin A. However, probably because of the short

142 incubation time, neither retinol no retinyl esters were detected in the scraped cells and in  
143 basolateral medium in any experimental condition.

144

#### 145 *Retinol and carotenoid apical transport inhibition by BLT1 and SSO*

146 The effect of BLT1 and SSO on retinol and carotenoid uptake was assessed as follow:  
147 the cells were pretreated with either DMSO (control) or 10  $\mu\text{mol/L}$  BLT1 or 400  $\mu\text{mol/L}$  SSO,  
148 respectively, for 1 h. The cells then received micelles (in the case of Caco-2) or enriched  
149 complete medium (in the case of HEK cells) supplemented with either DMSO (Dimethyl  
150 sulfoxide alone as control) or scavenger receptors chemical inhibitors (10  $\mu\text{mol/L}$  BLT1 or  
151 400  $\mu\text{mol/L}$  SSO in DMSO). Uptake was then measured as previously described.

152

153 All samples were stored at  $-80^{\circ}\text{C}$  under nitrogen with 0.5% pyrogallol as a  
154 preservative before lipid extraction and HPLC analysis. Aliquots of cell samples without  
155 pyrogallol and containing protease inhibitors were used to estimate the protein concentrations  
156 using a bicinchoninic acid kit (Pierce, Montluçon, France).

157

#### 158 *Retinol and carotenoid extraction and HPLC analysis*

159 Retinol, carotenoids and putative retinyl esters were extracted from 500  $\mu\text{L}$  aqueous  
160 samples using the following method. After adding ethanol containing two internal standards  
161 (retinyl acetate for retinol and putative retinyl esters, and echinenone for carotenoids), the  
162 mixture was extracted twice, each time with two volumes of hexane. The hexane phases  
163 obtained after centrifugation (500 g, 5 min,  $25 \pm 3^{\circ}\text{C}$ ) were evaporated to dryness under  
164 nitrogen, and the dried extract was dissolved in 200  $\mu\text{L}$  acetonitrile/dichloromethane (50/50,  
165 v/v). A volume of 160  $\mu\text{L}$  was used for HPLC analysis. Retinol, retinyl esters and carotenoids  
166 were separated using a  $250 \times 4.6$  nm RP  $\text{C}_{18}$ , 5  $\mu\text{m}$  Zorbax column (Interchim, Montluçon,

167 France) equipped with a guard column. The mobile phase was 70% acetonitrile, 20%  
168 dichloromethane and 10% methanol. The flow rate was 1.5 mL/min and the column was kept  
169 at a constant temperature (35 °C). The HPLC system was composed of a Dionex separation  
170 module (a P680 HPLC Pump and an ASI-100 Automated Sample Injector) and a Dionex  
171 UVD340U photodiode array detector (Dionex SA, Voisins le Bretonneux, France). Retinol,  
172 retinyl acetate and retinyl palmitate were detected at 325 nm, while carotenoids were detected  
173 at 450 nm. All molecules were identified by comparing their retention times and spectra (190  
174 to 500 nm) with pure standards. Quantification was performed using Chromeleon software  
175 (version 6.50 SP4 Build 1000, Dionex), comparing the peak area with standard reference  
176 curves.

177

#### 178 *Subject samples*

179 The “Supplémentation en Vitamines et Minéraux AntioXydants” (SUVIMAX) study  
180 was designed to test the benefits of a multivitamin-mineral supplementation on cancer and  
181 cardiovascular disease risk in France (28). Out of a total of 6,850 subjects who agreed to  
182 participate in a post-supplementation follow-up, 4497 subjects were randomly selected and  
183 genotyped for several single nucleotide polymorphisms (SNPs) in 14 candidate genes  
184 potentially involved in the metabolism of carotenoids and vitamin E. A subsample of 621  
185 subjects were then selected to obtain groups with contrasting haplotypes in whom plasma  
186 provitamin A carotenoids were measured. The characteristics of this subsample are shown in  
187 detail in a previous publication (29).

188 The FINGEN Study was a four-centre trial conducted at the Universities of Glasgow,  
189 Newcastle, Reading and Southampton in the UK between June 2003 and Sept 2005. The  
190 study prospectively recruited 312 volunteers according to apoE genotype, gender and age as  
191 described previously (30, 31). The primary objective of this randomized controlled trial was

192 to examine the impact of *APOE* genotype, age and sex on the responsiveness of an array of  
193 established and putative cardiovascular disease biomarkers to physiological fish oil  
194 supplementation. For the purpose of the current publication, baseline plasma provitamin A  
195 carotenoid concentrations were used.

196 The HELENA cross-sectional study ("Healthy Lifestyle in Europe by Nutrition in  
197 Adolescence") has been described in detail elsewhere (32). Briefly, a total of 3865  
198 adolescents were recruited between 2006 and 2007. Data were collected in a total of ten  
199 centres in nine European countries. Subjects were randomly selected from schools by using a  
200 proportional cluster sampling methodology and taking age into account. One third of the  
201 classes were randomly selected for plasma collection (n=1155). The body mass index (BMI)  
202 and a plasma  $\beta$ -carotene measurement were available for 993 adolescents. Data were collected  
203 on a detailed case report form, in accordance with standardized procedures. Only plasma  $\beta$ -  
204 carotene concentrations are available from this study population as plasma  $\alpha$ -carotene and  $\beta$ -  
205 cryptoxanthin were not analyzed.

206 All the above mentioned human studies have been approved by local research ethic  
207 committees.

208

#### 209 *Choice of candidate SNPs in CD36 and SCARB1 and genotyping methods*

210 In the SUVIMAX and FINGEN samples, SNPs were selected through an analysis of  
211 previous studies describing associations between these SNPs and digestion, transport or lipid  
212 metabolism (33). In the HELENA study, 10 tag SNPs were selected to cover the whole  
213 genetic variability of *CD36* (29) but only the rs5888 SNP was genotyped in *SCARB1*.  
214 Methods of genotyping are described in detail in the previous papers (29, 33). Genotyped  
215 SNPs are presented in **supplemental Table 1**.

216

217 *Statistical analysis*

218

219 • *Cell studies*

220 The results are expressed as the mean  $\pm$  SD. Differences between the uptake efficiency  
221 in the different cell groups (control, HEK cells that overexpressed a scavenger receptor, cells  
222 + chemical inhibitor of either SR-BI or CD36) were analyzed using the Kruskal-Wallis test.  
223 The Mann-Whitney test was used as a *post hoc* test when the Kruskal-Wallis test showed  
224 significant differences ( $P < 0.05$ ) between groups.  $P < 0.05$  was considered significant.

225

226 • *Candidate gene association studies in the three studies*

227 Associations between SNPs and plasma concentrations of provitamin A carotenoids in  
228 the three independent samples were first assessed by using the general linear model on log  
229 transformed data. In a first step, interfering covariables (adjustment factors) were identified.  
230 Any covariable that was significant at  $P < 0.05$  (namely age, gender and BMI) was included as  
231 an adjustment factor for testing the genotype effect. The effects of the genotypes on the  
232 dependent variables (i.e. plasma provitamin A carotenoids) were tested systematically in the 3  
233 populations and additionally in the SUVIMAX and FINGEN studies, for all subjects and for  
234 men and women separately. The dominant model specifically tests the association of having  
235 at least one minor allele versus not having it at all. The recessive model specifically tests the  
236 association of having the minor allele as both alleles versus having at least one major allele.  
237 Under the additive model, testing is designed specifically to reveal associations depend  
238 additively upon the minor allele that is, where having two minor alleles rather than having no  
239 minor alleles is twice as likely to affect the outcome in a certain direction as is having just one  
240 minor allele rather than no minor alleles.

241 In a second approach we performed a haplotype-based association analysis of data with the  
242 THESIAS software, which is based on the Stochastic-EM algorithm. This permits haplotypes  
243 to be inferred from genotypic data and to test their associations with phenotypes of interest  
244 (34). Analyses were performed after adjustment for gender, age and BMI.

245 In order to correct for multiple comparisons, Bonferroni corrections to account for the  
246 number of comparisons that were performed in each data set were performed. The alpha  
247 values to reach significance were lowered as follows:  $P < 0.0083$  for the SUVIMAX study (6  
248 tested SNPs),  $P < 0.0063$  for the FINGEN study (8 tested SNPs), and  $P < 0.0045$  for the  
249 HELENA study (11 tested SNPs). Similar corrections were applied to the haplotype-based  
250 association analysis.

251

## 252 **Results**

253

### 254 *Retinol and provitamin A carotenoid absorption efficiency in Caco-2 TC-7 cells*

255 After 1h incubation, retinol was more efficiently absorbed than provitamin A  
256 carotenoids (up to  $30.3 \pm 0.2\%$  absorbed by the cells for retinol *versus*  $2.2 \pm 0.3\%$  for  $\beta$ -  
257 cryptoxanthin,  $2.4 \pm 0.3\%$  for  $\alpha$ -carotene and  $4.2 \pm 0.3\%$  for  $\beta$ -carotene (data not shown).  
258 Figure 1 shows that the addition of BLT1 (a specific chemical inhibitor of SR-BI (19))  
259 significantly ( $p < 0.05$ ) decreased uptake of all provitamin A carotenoids (~by 50% to 70%)  
260 but had no effect on retinol uptake.

261

### 262 *Effect of SR-BI and CD36 transfection on provitamin A carotenoid and retinol uptake in HEK* 263 *cells*

264 Transfection with a human SR-BI expression vector led to a significant 1- to 2-fold  
265 increase in provitamin A carotenoid uptake when compared with control (cells transfected  
266 with an empty plasmid). Furthermore this increase was completely suppressed by addition of  
267 BLT1 (**Figure 2A**). Conversely, HEK cell transfection with human SR-BI did not modify  
268 retinol uptake, and BLT1 had no effect on retinol uptake by SR-BI transfected cells. **Figure**  
269 **2B** shows that HEK cell transfection with a human *CD36* expression vector also significantly  
270 increased provitamin A carotenoid uptake from about 0.4- to 1-fold. This increase was  
271 suppressed by the addition of SSO (the chemical inhibitor of CD36). As for SR-BI, the  
272 transfection of HEK cells with *CD36* and the addition of SSO had no effect on retinol uptake.

273

### 274 *Associations between SNPs and haplotypes in SCARB1 and CD36 and plasma concentrations* 275 *of provitamin A carotenoids in the three studies*

276



277 • *SCARB1 gene variants and plasma provitamin A carotenoid concentrations*

278 In the SUVIMAX sample, significant Bonferroni corrected associations between the  
279 rs61932577 SNP in *SCARB1* and plasma provitamin A carotenoids were observed (**Table 1**).  
280 More precisely, subjects bearing the TT genotype had higher provitamin A carotenoid  
281 concentrations than subjects with the C allele (recessive and additive models). Note that  
282 similar findings were observed in men and in women suggesting no gender effect on this  
283 association. There were no significant associations between the two other *SCARB1* SNPs  
284 genotyped in the SUVIMAX study, i.e. rs5888 and rs4238001, and plasma provitamin A  
285 carotenoid concentrations (data not shown). This was confirmed in the HELENA and  
286 FINGEN studies where no significant association between the genotyped *SCARB1* SNPs and  
287 plasma provitamin A carotenoid concentrations was observed (data not shown). Finally,  
288 haplotype analysis suggested that SUVIMAX subjects bearing the CCT haplotype (rs5888;  
289 rs4238001; rs61932577) had lower (about 18% data not shown) plasma  $\beta$ -cryptoxanthin than  
290 subjects bearing the TCC haplotype (**Table 2**).

291

292 • *CD36 genetic variants and plasma provitamin A carotenoid concentrations*

293 **Table 3** shows that plasma  $\beta$ -cryptoxanthin concentration in women from the  
294 SUVIMAX study were significantly associated (even after Bonferroni correction) with  
295 rs1984112 and with rs1761667 (recessive effects). Women who were homozygous for the G  
296 minor allele at either rs1984112 or rs1761667 had about 25% less plasma  $\beta$ -cryptoxanthin  
297 than women who bore an A allele at either of these loci. Conversely there was no association  
298 between these SNP and plasma  $\beta$ -cryptoxanthin concentration in men. There was also a  
299 significant Bonferroni corrected associations between plasma  $\beta$ -cryptoxanthin and rs7755 that  
300 differed between genders. Women homozygous for the A minor allele at this locus had about

301 23% less  $\beta$ -cryptoxanthin than women carrying the G allele. Conversely, men carrying the AA  
302 genotype in rs7755 had about 21% more  $\beta$ -cryptoxanthin than men who bore a G allele.

303 **Table 4** shows that, in the FINGEN study, plasma concentrations of the provitamin A  
304 carotenoid,  $\alpha$ -carotene, were associated with three *CD36* SNPs (two of these associations  
305 remained significant after Bonferroni correction). Haplotype analysis showed that, in the  
306 FINGEN study (**Table 5**), one haplotype constituted by 5 SNPs, GGACC (rs1984112;  
307 rs1761667; rs1527479; rs1527483; rs13230419), with a frequency of ~ 29% in the sample,  
308 achieved nominal association with higher (+12% data not shown) plasma  $\alpha$ -carotene  
309 ( $p=0.035$ ) and higher (+16% data not shown)  $\beta$ -cryptoxanthin concentrations in comparison  
310 with the most frequent haplotype (AAGCT, frequency of 47%). A nominal association  
311 between the rs1984112 SNP and plasma  $\beta$ -cryptoxanthin concentration was also observed in  
312 the SUVIMAX sample when the haplotypic background was G at rs1761667 and A at rs7755  
313 (**supplemental Table 2**). Finally, no significant association was detected (in individual SNP  
314 analysis or haplotype analysis) between the *CD36* SNPs and plasma  $\beta$ -carotene concentrations  
315 in any of the three populations.

316

**317 Discussion**

318

319 The scavenger receptor family is a multiligand, multifunction receptor system  
320 including class A, class B, mucin-like and endothelial receptors. SR-BI is a class B receptor  
321 found in numerous tissues including the intestine. It is involved in the cellular uptake of a  
322 wide range of lipid molecules including cholesterol (12, 35), vitamin E (36), vitamin D (21),  
323 and the non-provitamin A carotenoids lutein (11) and lycopene (14). Its involvement in the  
324 cellular uptake of these carotenoids has raised questions as to its role in the cellular uptake of  
325 vitamin A, either as retinol or as provitamin A carotenoids as well as to the involvement of  
326 other scavenger class B receptors, such as CD36/FAT. It has been shown that CD36 is  
327 involved both in  $\beta$ -carotene uptake by mouse brush border membrane vesicles (12), and in  
328 cellular uptake of lutein and lycopene by adipocytes (16).

329 Our first objective was to investigate for the first time the involvement of SR-BI and  
330 CD36 in cellular uptake of  $\alpha$ -carotene, as well as on the involvement of CD36 in  $\beta$ -  
331 cryptoxanthin and retinol uptake. Our second objective was to assess whether genetic variants  
332 in the genes that encode these scavenger receptors may affect plasma concentrations of  
333 provitamin A carotenoids. To achieve these objectives we used two different approaches: 1)  
334 uptake studies in a human intestinal cell line (Caco-2) and in HEK cells that can be easily  
335 transfected, and 2) association studies between genetic variants in the genes that encode these  
336 transporters and plasma concentrations of provitamin A carotenoids in three independent  
337 populations.

338 Our initial objective was to work with a human intestinal cell line and either to  
339 modulate the expression of the scavenger receptors (siRNA or overexpression by transfection)  
340 or to inhibit the scavenger receptors by using specific chemical inhibitors: BLT1 for SR-BI  
341 and SSO for CD36. Nevertheless, it was not possible to fully inhibit SR-BI expression in

342 Caco-2 cells with the siRNA methodology (13), and the fact that these cells do not express  
343 CD36 (35) did not allow to study the inhibitory effect of the specific chemical inhibitor of  
344 CD36, i.e. SSO, on vitamin A uptake. We thus used HEK cells transfected with either human  
345 *SCARB1* or *CD36* to confirm and extend the data obtained in Caco-2 cells.

346 Our first result shows, in both human intestinal cells and in *SCARB1* transfected HEK  
347 cells, that the chemical inhibitor of SR-BI, i.e. BLT1, impairs provitamin A carotenoid uptake.  
348 This result is in agreement with the two studies that have suggested the involvement of SR-BI  
349 in cellular uptake of  $\beta$ -carotene (12, 13) and  $\beta$ -cryptoxanthin (13). Furthermore, it shows for  
350 the first time that SR-BI is also involved in the uptake of the third main provitamin A  
351 carotenoid of the human diet:  $\alpha$ -carotene. The lack of effect of BLT1 on retinol uptake, both in  
352 Caco-2 and in *SCARB1* transfected HEK cells, confirms the result obtained by During and  
353 Harrison using siRNA (7) and shown for the first time that this scavenger receptor is also  
354 involved in the cellular uptake of  $\beta$ -cryptoxanthin and  $\alpha$ -carotene, but apparently not in that of  
355 retinol.

356 The second objective of this paper was to evaluate whether genetic variants in *SCARB1*  
357 and *CD36* are related to plasma vitamin A concentrations in humans. To diminish the risk of  
358 associations which occur only in a particular genetic background we assessed these  
359 associations in three independent populations. We focused on provitamin A carotenoids  
360 because cell experiments showed that cellular uptake of retinol is not mediated by these  
361 scavenger receptors and because our sample size was underpowered to detect SNP effects on  
362 circulating retinol concentrations. Indeed, a previous GWAS study indicated that a sample  
363 size of  $n = 1242$  only gives a power of 13% to detect a SNP explaining 1.5% of the variation  
364 in serum retinol concentrations (37).

365 The first important findings of these association studies are the significant associations  
366 between plasma  $\alpha$ -carotene and  $\beta$ -cryptoxanthin concentrations and SNPs, or haplotypes, in

367 *CD36*. Indeed, this suggests that *CD36* is involved in affecting the plasma concentration of  
368 provitamin A carotenoids. Nevertheless, the fact that no significant association was observed  
369 between plasma  $\beta$ -carotene and *CD36* genetic variants in any of the three populations was  
370 surprising. We hypothesized that this was due to a confounding effect of *BCMO1* genetic  
371 variants on  $\beta$ -carotene cleavage (18, 38). To verify this hypothesis we evaluated associations  
372 between two SNPs in *BCMO1* (rs7501331 and rs12934922 (33)) and plasma provitamin A  
373 carotenoid concentrations in both the SUVIMAX and FINGEN samples (SNPs in *BCMO1*  
374 were not genotyped in the HELENA study). Results of these analyses (**supplemental table 3**)  
375 clearly showed that these two SNPs, as well as one of their haplotypes (data not shown), were  
376 significantly associated with blood concentrations of  $\beta$  and  $\alpha$ -carotene, but not with that of  $\beta$ -  
377 cryptoxanthin. This result supports the hypothesis that the effects of the genetic variants in  
378 *BCMO1* masked the effect of the *CD36* SNPs with regard to blood  $\beta$ -carotene in the three  
379 studies.

380         The mechanism by which *CD36* affects plasma provitamin A carotenoid  
381 concentrations is not known. *CD36* recognizes a broad variety of lipid ligands, including fatty  
382 acids, oxidized LDL, apoptotic cells and  $\beta$ -carotene (12). Thus, recognition of the fat-soluble  
383 provitamin A carotenoids by *CD36* is plausible. Because *CD36* is expressed in the human  
384 enterocyte, although not in the Caco-2 cell line (11), our result in *CD36* transfected HEK cells  
385 suggests that *CD36* may facilitate the transfer of provitamin A carotenoids from mixed  
386 micelles (39) across the apical membrane of the enterocyte. However, since *CD36* has been  
387 shown to be involved in active transport of pro-vitamin A carotenoids into the adipose tissue  
388 (16), it is unclear at this point in time whether the observed effect of genetic variants in *CD36*  
389 on fasting plasma  $\alpha$ -carotene and  $\beta$ -cryptoxanthin concentrations is caused at the site of the  
390 intestine or other tissues involved in provitamin A uptake. The potential functional  
391 importance of SNPs in *CD36* has been shown by a recent study of monocyte and/or platelet

392 CD36 protein expression in African Americans (40). In this work, the A allele of rs1761667  
393 was associated with decreased CD36 expression in monocytes. This functional data allows us  
394 to hypothesize that the higher plasma  $\beta$ -cryptoxanthin concentration observed in subjects  
395 carriers of the A allele at rs1761667 (**Table 4**) might be related to a lower CD36 expression in  
396 some tissues, and therefore to a lower uptake of this carotenoid by these tissues, which may  
397 lead to an accumulation of this carotenoid in the plasma compartment. If confirmed, this  
398 mechanism might help explain why high plasma concentrations of carotenoids could result  
399 not only from high intakes and/or high bioavailability of carotenoids, but also from an  
400 impaired uptake of these compounds by tissues. In other words a high plasma concentration in  
401 provitamin A carotenoids would not always mean that tissue status for these carotenoids is  
402 also high.

403 The second important finding of these association studies was the significant  
404 associations observed between the rs61932577 SNP in *SCARB1* and plasma provitamin A  
405 carotenoid concentrations. This confirms, in a new population, findings from a previous study  
406 showing an association between this SNP and plasma  $\alpha$ - and  $\beta$ -carotene concentration (41).  
407 This association can be explained by an effect of the genetic variants on the ability of SR-BI  
408 to either absorb provitamin A carotenoids at the intestinal level and/or to bind HDL, which  
409 transports a fraction of carotenoids in the plasma (42).

410

411 In summary, our results show that CD36 and SR-BI are involved in cellular uptake of  
412 the three main dietary provitamin A carotenoids, but not in that of retinol. Genetic variants in  
413 both genes are associated with plasma concentrations of provitamin A carotenoids. All these  
414 data corroborate the importance of these receptors in regulating plasma concentrations of  
415 provitamin A carotenoids. The presented data therefore add to our understanding of the

416 molecular regulation of pro-vitamin A uptake and adds information to explore the observed  
417 inter-individual variations in plasma carotenoid concentrations.

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**Acknowledgements:**

The authors would like to thank Dr D. Lairon, Dr B. Vialettes and Dr Y. Barra for their helpful criticisms of the manuscript. We also thank M. Maillot for his statistical advices.

**Authors' contribution to manuscript**

PB designed research and has primary responsibility for final content. ER designed and performed the cell experiments with AG and MM. AMM, principle investigator for FINGEN. GL, PC, MJC, EAM, CP, PCC, JCM and AMM were responsible for data collection and FT for additional DNA extraction from the FINGEN population, whereas EKG, PG and SH constituted the SUVIMAX 2 study management team. CB and MGG were responsible for data collection from the HELENA population. MM carried out the plasma carotenoid analysis for both the FINGEN and the SUVIMAX samples. FSE carried out general linear model and haplotype analysis in the SUVIMAX and FINGEN samples. PB performed the ecological statistics with the help of LG. AM and SL carried out general linear model and haplotype analysis in the HELENA sample. PB drafted the manuscript. All authors contributed to the final version of the manuscript. All the authors have read and approved the final version of the paper.

**Table 1.** Plasma provitamin A carotenoid concentrations ( $\mu\text{mol/L}$ ) according to the rs61932577 SNP in *SCARB1* in the SUVIMAX clinical study.

carotenoid/subject groups	<i>n</i>	Genotypes			<i>P</i>	<i>P</i>	<i>P</i>
		CC	CT	TT	(dominant model)	(recessive model)	(additive model)
$\alpha$ -carotene							
all subjects	494-113-15 <sup>1</sup>	0.29 $\pm$ 0.24	0.26 $\pm$ 0.21	0.38 $\pm$ 0.20	NS	NS	NS
		0.36 $\pm$ 0.26	0.30 $\pm$ 0.24	0.43 $\pm$ 0.20			
women	266-65-10	0.21 $\pm$ 0.18	0.22 $\pm$ 0.16	0.28 $\pm$ 0.20	NS	0.008	NS
men	228-48-5				NS	NS	NS
$\beta$ -carotene							
all subjects	494-113-15	1.10 $\pm$ 0.81	0.99 $\pm$ 0.69	1.41 $\pm$ 0.59	NS	NS	NS
		1.38 $\pm$ 0.89	1.10 $\pm$ 0.76	1.62 $\pm$ 0.51			
women	266-65-10	0.77 $\pm$ 0.54	0.85 $\pm$ 0.57	1.00 $\pm$ 0.55	NS	0.007	NS

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men	228-48-5				NS	NS	0.04*
$\beta$ -cryptoxanthin							
all subjects	494-113-15	0.33 $\pm$ 0.28	0.30 $\pm$ 0.26	0.35 $\pm$ 0.25	NS	NS	NS
		0.38 $\pm$ 0.28	0.33 $\pm$ 0.23	0.35 $\pm$ 0.20			
women	266-65-10	0.28 $\pm$ 0.27	0.26 $\pm$ 0.29	0.36 $\pm$ 0.37	NS	NS	NS
men	228-48-5				NS	NS	NS

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<sup>1</sup>Number of subjects in each genotype group. Data are means  $\pm$  SD. NS: non significant ( $P > 0.05$ ). \*: nominal association prior to Bonferroni correction (i.e.  $0.0083 < P < 0.05$ ).  $P$  values were calculated using a general linear model and were adjusted for age and BMI



**Table 2.** *SCARB1* haplotype effects on plasma  $\beta$ -cryptoxanthin concentration in the SUVIMAX clinical study.

rs5888	rs4238001	rs61932577	Haplotype Frequencies <sup>1</sup> (%)	<i>P</i> value for the haplotype effect <sup>2</sup> on plasma $\beta$ - cryptoxanthin concentration
C	C	C	0.378	0.75
C	C	T	0.076	0.05*
C	T	C	0.052	0.90
T	C	C	0.400	-
T	T	C	0.054	0.38

The associations between *SCARB1* SNPs and plasma  $\beta$ -cryptoxanthin concentrations were assessed in 622 subjects. <sup>1</sup>Haplotype frequencies were estimated using Thesias software (34). The most frequent haplotype (TCC) is the reference haplotype. Haplotypes with a frequency lower than 2.5% are not shown. <sup>2</sup>Haplotype effect on plasma provitamin A carotenoids by comparison to the most frequent haplotype (*P* value calculated by Student t-test). \*: nominal association prior to Bonferroni correction (i.e.  $0.0125 < P < 0.05$ ).

**Table 3.** Plasma  $\beta$ -cryptoxanthin concentrations ( $\mu\text{mol/L}$ ) according to genotypes of *CD36* in the SUVIMAX clinical study.

SNP/subject groups	<i>n</i>	genotypes			<i>P</i>	<i>P</i>	<i>P</i>
		AA	AG	GG	(dominant model)	(recessive model)	(additive model)
rs1984112							
all subjects	312-189-119 <sup>1</sup>	0.35 $\pm$ 0.31	0.38 $\pm$ 0.25	0.28 $\pm$ 0.21	NS	NS	NS
		0.40 $\pm$ 0.31	0.39 $\pm$ 0.25	0.27 $\pm$ 0.17			
women	176-102-63	0.28 $\pm$ 0.31	0.26 $\pm$ 0.24	0.29 $\pm$ 0.24	NS	4.10 <sup>-5</sup>	0.0009
men	138-87-56				NS	NS	NS
rs1761667							
all subjects	249-192-179	0.33 $\pm$ 0.31	0.34 $\pm$ 0.26	0.31 $\pm$ 0.23	NS	NS	NS
		0.39 $\pm$ 0.30	0.40 $\pm$ 0.29	0.31 $\pm$ 0.19			
women	136-105-99	0.27 $\pm$ 0.31	0.26 $\pm$ 0.21	0.31 $\pm$ 0.28	NS	0.0052	0.02*

men	113-87-80				NS	NS	NS
rs7755		GG	AG	AA			
all subjects	216-250-151	0.35 ± 0.30	0.32 ± 0.29	0.31 ± 0.23	NS	NS	NS
		0.39 ± 0.28	0.39 ± 0.30	0.30 ± 0.20			
women	125-132-82	0.29 ± 0.32	0.24 ± 0.25	0.32 ± 0.25	0.04*	0.0013	0.0022
men	91-118-69				NS	0.0024	0.03*

<sup>1</sup>Number of subjects in each genotype group. Data are means ± SD. NS: non significant ( $p > 0.05$ ).  $P$  values were calculated using a general linear model and were adjusted for age and BMI. \*: nominal association prior to Bonferroni correction (i.e.  $0.0083 < P < 0.05$ ). Models explained in Table 2 legend.

**Table 4.** Plasma  $\alpha$ -carotene concentrations ( $\mu\text{mol/L}$ ) according to genotype of *CD36* SNPs in the FINGEN clinical study.

SNP/subject	<i>n</i>	genotypes			<i>P</i>	<i>P</i>	<i>P</i>
groups					(dominant model)	(recessive model)	(additive model)
rs1984112		AA	AG	GG			
all subjects	92-106-33 <sup>1</sup>	0.22 $\pm$ 0.13	0.26 $\pm$ 0.17	0.25 $\pm$ 0.13	0.03*	NS	0.03*
women	45-64-12	0.22 $\pm$ 0.13	0.29 $\pm$ 0.19	0.23 $\pm$ 0.13	0.05*	NS	NS
men	47-42-21	0.21 $\pm$ 0.13	0.20 $\pm$ 0.09	0.25 $\pm$ 0.13	NS	NS	NS
rs1761667		AA	AG	GG			
all subjects	63-123-48	0.21 $\pm$ 0.13	0.26 $\pm$ 0.17	0.25 $\pm$ 0.12	0.005	NS	0.02*
women	28-68-26	0.22 $\pm$ 0.13	0.29 $\pm$ 0.19	0.24 $\pm$ 0.12	NS	NS	NS

men	35-55-22	0.20 ± 0.13	0.22 ± 0.11	0.25 ± 0.13	0.02*	NS	0.01*
rs1527479		GG	GA	AA			
all subjects	68-121-47	0.21 ± 0.13	0.26 ± 0.17	0.26 ± 0.12	0.005	NS	0.01*
women	31-67-24	0.22 ± 0.12	0.29 ± 0.20	0.25 ± 0.12	NS	NS	NS
men	37-54-23	0.20 ± 0.13	0.22 ± 0.11	0.26 ± 0.13	0.04*	0.04*	0.01*

<sup>1</sup>Number of subjects in each genotype group. Data are means ± SD. NS: non significant ( $P > 0.05$ ).  $P$  values were calculated using a general linear model and were adjusted for age, BMI and smoking habits, and. \*: nominal association prior to Bonferroni correction (i.e.  $0.00625 < P < 0.05$ ). Models explained in Table 2 legend.

**Table 5.** *CD36* haplotype effects on plasma  $\alpha$ -carotene and  $\beta$ -cryptoxanthin concentrations in the FINGEN clinical study.

rs1984112	rs1761667	rs1527479	rs1527483	rs13230419	Haplotype Frequencies <sup>1</sup> (%)	<i>P</i> value for the haplotype effect <sup>2</sup> on plasma $\alpha$ -carotene	<i>P</i> value for the haplotype effect <sup>2</sup> on plasma $\beta$ -cryptoxanthin
A	A	G	C	T	0.465	-	-
A	A	G	C	C	0.041	0.39	0.98
A	G	A	C	C	0.034	0.10	0.23
A	G	A	T	C	0.045	0.93	0.54
G	G	A	C	T	0.061	0.34	0.43
G	G	A	C	C	0.294	0.04*	0.05*

The associations between *CD36* SNPs and plasma provitamin A carotenoids were assessed in 207 subjects (59% men). <sup>1</sup>Haplotype frequencies were estimated using Thesias software (34). The most frequent haplotype (AAGCT) is the reference haplotype. Haplotypes with a frequency lower than 2.5% are not shown. <sup>2</sup>Haplotype effect on plasma provitamin A carotenoids by comparison to the most frequent haplotype (*P* value calculated by Student t-test). \* nominal association prior to Bonferroni correction (i.e.  $0.01 < P < 0.05$ ).

## Online Supporting Material

**Supplemental Table 1** Genotyped SNPs in the different studies.

Gene	dbSNP ID	Major allele	Minor allele	studies		
				HELENA	SUVIMAX	FINGEN
CD36	rs1984112	A	G	X	X	X
CD36	rs1761667	A	G	X	X	X
CD36	rs1527479	G	A	X	-	X
CD36	rs3211816	G	A	X	-	-
CD36	rs3211867	C	A	X	-	-
CD36	rs3211883	T	A	X	-	-
CD36	rs3211908	C	T	X	-	-
CD36	rs3211931	C	T	X	-	-
CD36	rs1527483	C	T	X	-	X
CD36	rs1049673	G	C	X	-	-
CD36	rs13230419	T	C	-	-	X
CD36	rs7755	G	A	-	X	-
SCARB1	rs5888	C	T	X	X	X
SCARB1	rs4238001	C	T	-	X	X
SCARB1	rs61932577	C	T	-	X	X

## Online Supporting Material

**Supplemental Table 2.** *CD36* polymorphism effect under haplotypic background<sup>1</sup> on plasma  $\beta$ -cryptoxanthin concentrations in the SUVIMAX clinical study.

rs1984112	rs1761667	rs7755	Statistical effect <sup>2</sup>
-	A	G	- <sup>3</sup>
-	G	A	0.02*
-	G	G	0.45
A	-	A	0.11
A	-	G	0.44
G	-	G	- <sup>2</sup>
A	A	-	0.63
A	G	-	0.87
G	G	-	0.44

Plasma  $\beta$ -cryptoxanthin and *CD36* SNPs were assessed in 622 subjects. <sup>1</sup>Effect under haplotypic background means that, for each polymorphism, the allelic effect according to the three haplotypic backgrounds of the two other SNPs was tested <sup>2</sup>Comparisons were made using the Thesias software (34). The most frequent haplotype was AAG. <sup>3</sup>could not be calculated because of the too low frequency of one or both of the haplotypes. \*: nominal association prior to Bonferroni correction (i.e.  $0.00625 < P < 0.05$ ).

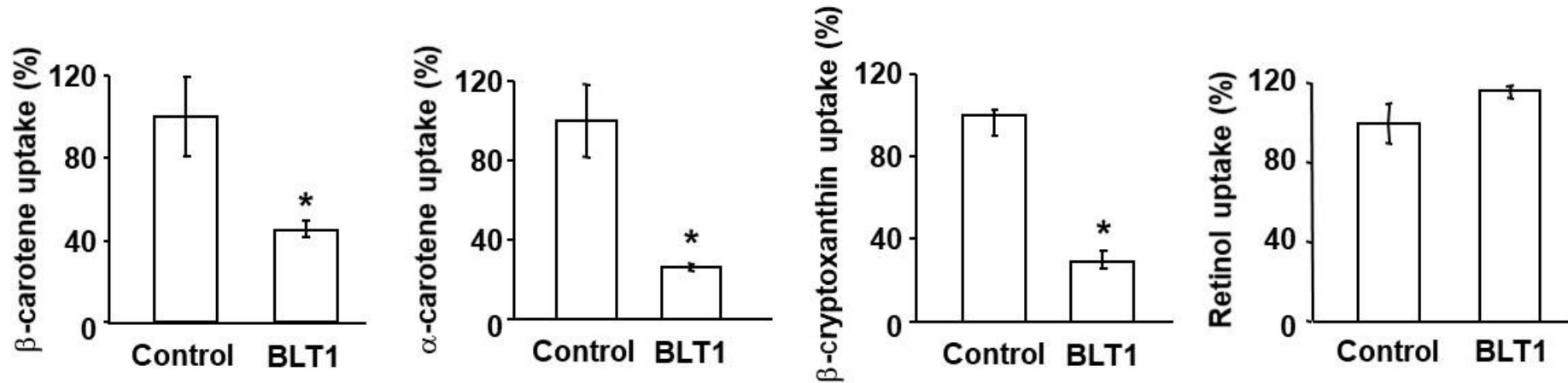


### Online Supporting Material

**Supplemental Table 3.** Associations between plasma provitamin A carotenoids and SNPs in *BCMO1* in the SUVIMAX and FINGEN studies.

Study/ <i>BCMO1</i> SNP/carotenoid	<i>P</i> (dominant model)	<i>P</i> (recessive model)	<i>P</i> (additive model)
SUVIMAX			
rs750331			
β-carotene	0.004	NS	0.004
α-carotene	0.014	NS	0.015
β-cryptoxanthin	NS	NS	NS
rs12934922			
β-carotene	NS	NS	NS
α-carotene	NS	NS	NS
β-cryptoxanthin	NS	NS	NS
FINGEN			
rs750331			
β-carotene	NS	NS	0.031
α-carotene	NS	0.004	0.011
β-cryptoxanthin	NS	NS	NS
rs12934922			
β-carotene	0.008	0.04*	0.003
α-carotene	NS	0.0004	0.001
β-cryptoxanthin	NS	NS	NS

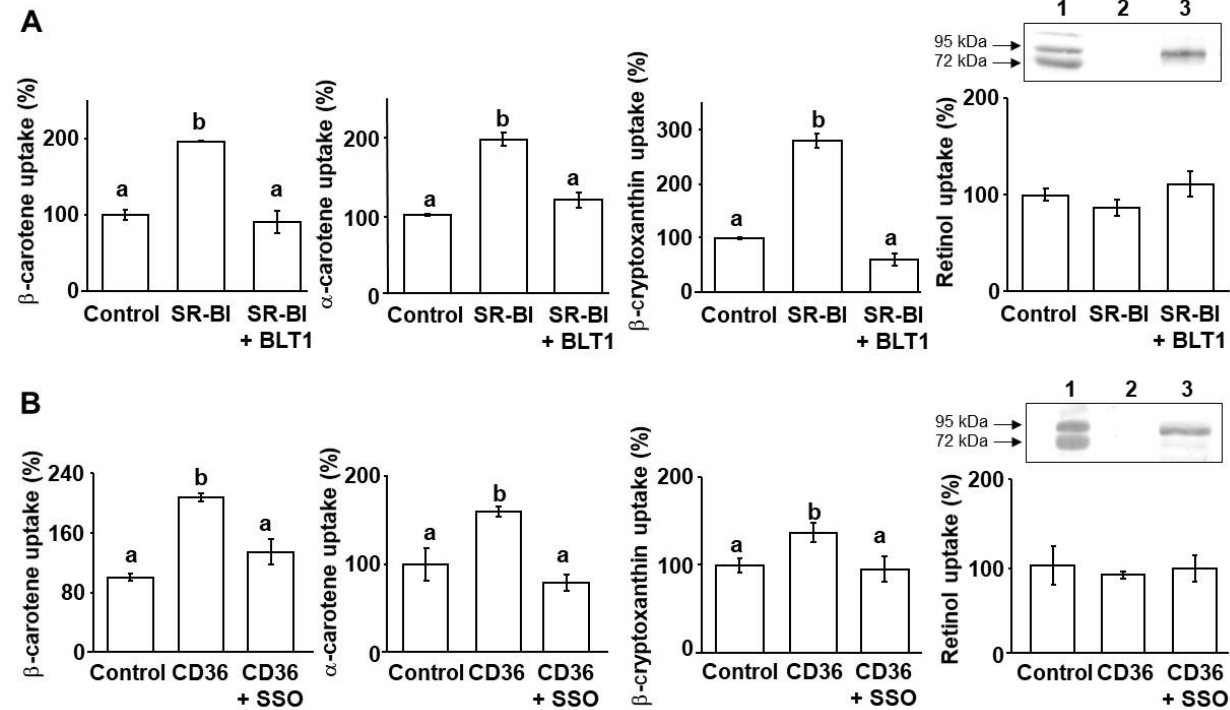
*P* values were calculated using a general linear model and were adjusted for age and BMI. NS: non significant ( $P > 0.05$ ). \*: nominal association prior to Bonferroni correction (i.e.  $0.025 < P < 0.05$ ).

**Figure 1**

**Fig. 1: Effect of BLT1 on provitamin A carotenoids and retinol uptake by differentiated Caco-2 TC-7 cell monolayers.**

Cells were incubated with either  $\beta$ -carotene,  $\beta$ -cryptoxanthin,  $\alpha$ -carotene-enriched mixed micelles or retinol-enriched mixed micelles  $\pm$  BLT1 at 10  $\mu$ M. Data are means  $\pm$  SEM of 3 assays. Data are from one experiment representative of at least 2 independent experiments. An asterisk indicates a significant difference ( $P < 0.05$ , Mann-Whitney  $U$  test) from the control (an assay performed with DMSO).

Figure 2



**Fig. 2: Effect of transfection with human SR-BI and CD36, and chemical inhibitors of SR-BI and CD36, on uptake of retinol and provitamin A carotenoids by 293-T HEK cells.**

Data are means  $\pm$  SEM of 3 assays. Data are from one experiment representative of at least 2 independent experiments. Labeled means without a common letter indicate significant difference ( $P < 0.05$ ) between groups. Insets: Western blots of protein expression in HEK cells (1: Size Marker, 2: Control cells, 3: HEK cells overexpressing SR-BI (A) or CD36 (B)). *A: Effect of SR-BI overexpression and BLT1:* Cells were transfected with either an empty pCDNA3.1 plasmid (control) or with a pCDNA3.1 plasmid containing human SR-BI (SR-BI). *B: Effect of CD36 overexpression and succinimidyl sulfonyl oleate:* Cells were transfected with either an empty pIRES plasmid (control) or with a pIRES plasmid containing human CD36 (CD36).