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

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

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[123]

Patrick Borel[1-3]*, Georg Lietz[4], Aurélie Goncalves[1-3], Fabien Szabo de Edelenyi[1-3], Sophie Lecompte[5], Peter Curtis[4,6], Louisa Goumidi[5], Muriel J Caslake[7], Elizabeth A Miles[8], Christopher Packard[7], Philip C Calder[8], John C Mathers[4], Anne M Minihane[6], Franck Tourniaire[1-4], Emmanuelle Kesse-Guyot[10], Pilar Galan[10], Serge Hercberg[10], Christina Breidenassel[11], Marcela González Gross[12], Myriam Moussa[1-3], Aline Meirhaeghe[5], Emmanuelle Reboul[1-3]

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*Corresponding author:

Patrick BOREL, Ph.D.

UMR 1260 INRA / 1062 INSERM, Faculté de Médecine, 27 Boulevard Jean-Moulin, 13385 Marseille Cedex 5, FRANCE. Phone number: (+33) 4 91 29 41 11; FAX number: (+33) 4 91 78 21 01. E-mail: Patrick.Borel@univ-amu.fr.

[1] INRA, UMR1260 «Nutrition, Obesity and Risk of Thrombosis», F-13385 Marseille, France (PB, AG, FSdE, FT, MM, ER)

[2] INSERM, UMR U1062, F-13385 Marseille, France (PB, AG, FSdE, FT, MM, ER)

[3] Aix-Marseille Univ, Faculté de Médecine, F-13385 Marseille, France (PB, AG, FSdE, FT, MM, ER)

[4] Human Nutrition Research Centre, Institute for Ageing and Health, Newcastle University, Newcastle NE1 7RU, U. K. (GL, PC, JCM, FT)

[5] INSERM, U744; Institut Pasteur de Lille; Univ Lille Nord de France; UDSL; 59019 Lille France (SL, LG, AM)

[6] Department of Nutrition, Norwich Medical School, University of East Anglia, Norwich NR4 7TJ, U.K. (PC, AMM)

[7] Department of Vascular Biochemistry, University of Glasgow, Glasgow G31 2ER, U.K. (MJC, CP)

[8] Institute of Human Nutrition, School of Medicine, University of Southampton, Southampton SO16 6YD, U.K. (EAM, PCC)

[10] UREN (Unité de Recherche en Epidémiologie Nutritionnelle), U557 Inserm/U1125 Inra/Cnam/Université Paris 13, CRNH IdF, F-93017 Bobigny, France

[11] Institut für Ernährungs- und Lebensmittelwissenschaften- Humanernährung, Rheinische Friedrich-Wilhelms Universität, Bonn, Germany

[12] Faculty of Physical Activity and Sport Sciences, Technical University of Madrid, Madrid, Spain

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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).

Abstract

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

Introduction

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

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

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

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

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

Subjects, materials and methods

Products and reagents

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

Preparation of retinol and carotenoid-rich micelles

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

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

Preparation of retinol and carotenoid-rich complete medium

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

Cell culture

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

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

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

Measurement of carotenoid and retinol uptake by cells

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

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

Retinol and carotenoid apical transport inhibition by BLT1 and SSO

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

All samples were stored at –80°C under nitrogen with 0.5% pyrogallol as a preservative before lipid extraction and HPLC analysis. Aliquots of cell samples without pyrogallol and containing protease inhibitors were used to estimate the protein concentrations using a bicinchoninic acid kit (Pierce, Montluçon, France).

Retinol and carotenoid extraction and HPLC analysis

Retinol, carotenoids and putative retinyl esters were extracted from 500 µL aqueous samples using the following method. After adding ethanol containing two internal standards (retinyl acetate for retinol and putative retinyl esters, and echinenone for carotenoids), the mixture was extracted twice, each time with two volumes of hexane. The hexane phases obtained after centrifugation (500 g, 5 min, 25 ± 3 °C) were evaporated to dryness under nitrogen, and the dried extract was dissolved in 200 µL acetonitrile/dichloromethane (50/50, v/v). A volume of 160 µL was used for HPLC analysis. Retinol, retinyl esters and carotenoids were separated using a 250 × 4.6 nm RP C_{18}, 5 µm Zorbax column (Interchim, Montluçon,

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

Subject samples

The "**Su**pplémentation en **Vi**tamines et **M**inéraux **A**ntio**X**ydants" (SUVIMAX) study was designed to test the benefits of a multivitamin-mineral supplementation on cancer and cardiovascular disease risk in France (28). Out of a total of 6,850 subjects who agreed to participate in a post-supplementation follow-up, 4497 subjects were randomly selected and genotyped for several single nucleotide polymorphisms (SNPs) in 14 candidate genes potentially involved in the metabolism of carotenoids and vitamin E. A subsample of 621 subjects were then selected to obtain groups with contrasting haplotypes in whom plasma provitamin A carotenoids were measured. The characteristics of this subsample are shown in detail in a previous publication (29).

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

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

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

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

Choice of candidate SNPs in CD36 and SCARB1 and genotyping methods

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

Statistical analysis

- *Cell studies*

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

- *Candidate gene association studies in the three studies*

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

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

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

Results

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

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

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

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

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

- *SCARB1 gene variants and plasma provitamin A carotenoid concentrations*

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

- *CD36 genetic variants and plasma provitamin A carotenoid concentrations*

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

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

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

Discussion

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

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

Our initial objective was to work with a human intestinal cell line and either to modulate the expression of the scavenger receptors (siRNA or overexpression by transfection) or to inhibit the scavenger receptors by using specific chemical inhibitors: BLT1 for SR-BI 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 β-carotene (12, 13) and β-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: α-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 β-cryptoxanthin and α-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 α-carotene and β-cryptoxanthin concentrations and SNPs, or haplotypes, in

CD36. Indeed, this suggests that *CD36* is involved in affecting the plasma concentration of provitamin A carotenoids. Nevertheless, the fact that no significant association was observed between plasma β-carotene and *CD36* genetic variants in any of the three populations was surprising. We hypothesized that this was due to a confounding effect of *BCMO1* genetic variants on β-carotene cleavage (18, 38). To verify this hypothesis we evaluated associations between two SNPs in *BCMO1* (rs7501331 and rs12934922 (33)) and plasma provitamin A carotenoid concentrations in both the SUVIMAX and FINGEN samples (SNPs in *BCMO1* were not genotyped in the HELENA study). Results of these analyses (**supplemental table 3**) clearly showed that these two SNPs, as well as one of their haplotypes (data not shown), were significantly associated with blood concentrations of β and α-carotene, but not with that of β-cryptoxanthin. This result supports the hypothesis that the effects of the genetic variants in *BCMO*1 masked the effect of the *CD36* SNPs with regard to blood β-carotene in the three studies.

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

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

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

In summary, our results show that CD36 and SR-BI are involved in cellular uptake of the three main dietary provitamin A carotenoids, but not in that of retinol. Genetic variants in both genes are associated with plasma concentrations of provitamin A carotenoids. All these data corroborate the importance of these receptors in regulating plasma concentrations of 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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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 (*µmol/L*) according to the rs61932577 SNP in *SCARB1* in the SUVIMAX clinical study.

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

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

[1]Number of subjects in each genotype group. Data are means ± 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 β-cryptoxanthin concentration in the SUVIMAX clinical study.

rs5888	rs4238001	rs61932577	Haplotype Frequencies[1] *(%)*	*P* value for the haplotype effect[2] on plasma β-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 β-cryptoxanthin concentrations were assessed in 622 subjects. [1]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. [2]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 β-cryptoxanthin concentrations (*µmol/L*) according to genotypes of *CD36* in the SUVIMAX clinical study.

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

[1]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 α-carotene concentrations (*μmol/L*) according to genotype of *CD36* SNPs in the FINGEN clinical study.

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

[1]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 α-carotene and β-cryptoxanthin concentrations in the FINGEN clinical study.

rs1984112	rs1761667	rs1527479	rs1527483	rs13230419	Haplotype Frequencies[1] *(%)*	*P* value for the haplotype effect[2] on plasma α-carotene	*P* value for the haplotype effect[2] on plasma β-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). [1]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. [2]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[1] on plasma β-cryptoxanthin concentrations in the SUVIMAX clinical study.

rs1984112	rs1761667	rs7755	Statistical effect[2]
-	A	G	-[3]
-	G	A	0.02*
-	G	G	0.45
A	-	A	0.11
A	-	G	0.44
G	-	G	-[2]
A	A	-	0.63
A	G	-	0.87
G	G	-	0.44

Plasma β-cryptoxanthin and *CD36* SNPs were assessed in 622 subjects. [1]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 [2]Comparisons were made using the Thesias software (34). The most frequent haplotype was AAG. [3]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/ *BCMO1* **SNP/carotenoid**		***P*** **(dominant model)**	***P*** **(recessive model)**	***P*** **(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

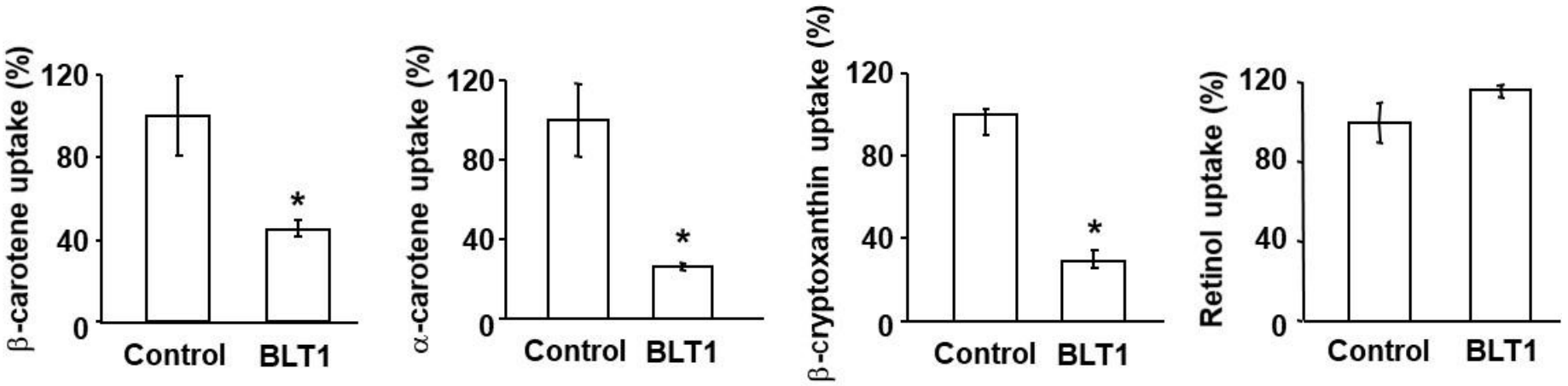

Fig. 1: **Effect of BLT1 on provitamin A carotenoids and retinol uptake by differentiated Caco-2 TC-7 cell monolayers.**

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

Figure 2

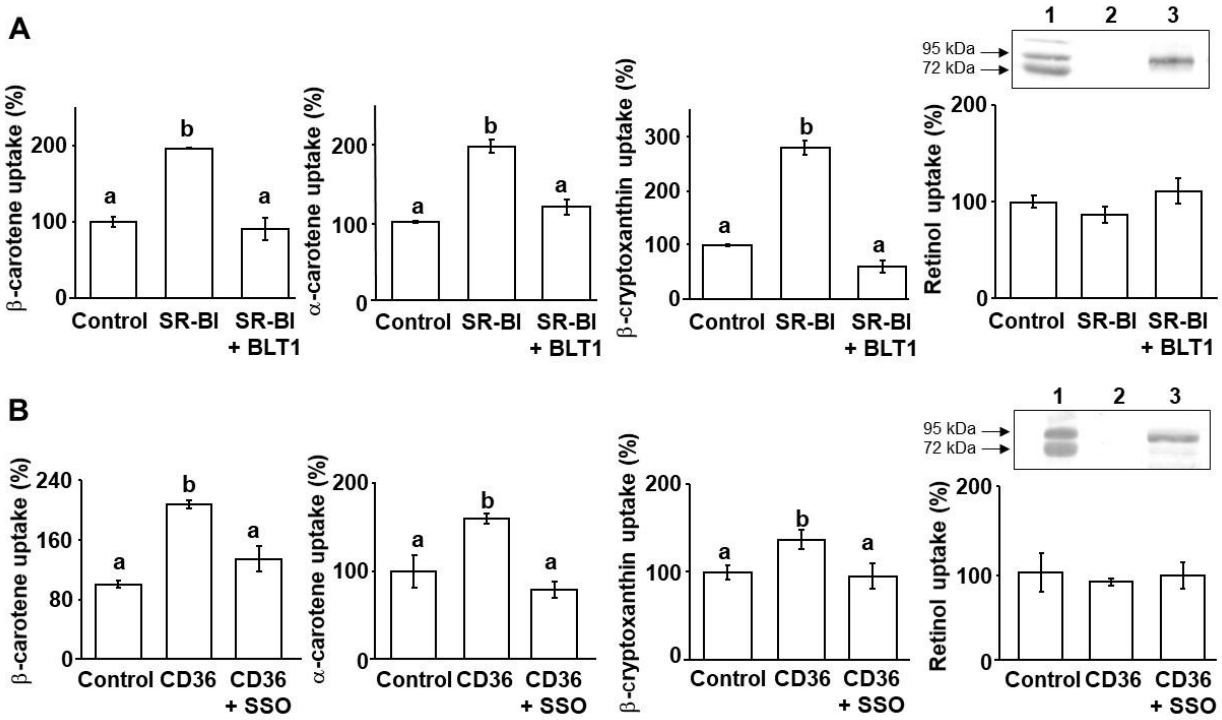

Fig. 2: **Effect of transfection with human SR-BI and *CD36*, and chemical inhibitors of SR-BI and CD36, on uptake of retinol and provitamin A carotenoids by 293-T HEK cells.**

Data are means ± 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).