The Journal of Nutrition
Biochemical, Molecular, and Genetic Mechanisms

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β-Carotene Oxygenase 1 Activity Modulates Circulating Cholesterol Concentrations in Mice and Humans

Jaume Amengual,[1,2] Johana Coronel,[1] Courtney Marques,[2] Celia Aradillas-García,[3] Juan Manuel Vargas Morales,[4] Flavia CD Andrade,[5] John W Erdman, Jr,[1,2] and Margarita Teran-Garcia[2,6,7]

[1]Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL, USA; [2]Division of Nutritional Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA; [3]Facultad de Medicina/Coordination for the Innovation and Application of Science and Technology, CIACYT, Autonomous University of San Luis Potosí (UASLP), San Luis Potosí, Mexico; [4]Facultad de Ciencias Químicas, UASLP, San Luis Potosí, Mexico; [5]School of Social Work, University of Illinois at Urbana-Champaign, Urbana, IL, USA; [6]Department of Human Development and Family Studies, University of Illinois at Urbana-Champaign, Urbana, IL, USA; and [7]Cooperative Extension, University of Illinois at Urbana-Champaign, Urbana, IL, USA

ABSTRACT

Background: Plasma cholesterol is one of the strongest risk factors associated with the development of atherosclerotic cardiovascular disease (ASCVD) and myocardial infarction. Human studies suggest that elevated plasma β-carotene is associated with reductions in circulating cholesterol and the risk of myocardial infarction. The molecular mechanisms underlying these observations are unknown.

Objective: The objective of this study was to determine the impact of dietary β-carotene and the activity of β-carotene oxygenase 1 (BCO1), which is the enzyme responsible for the conversion of β-carotene to vitamin A, on circulating cholesterol concentration.

Methods: In our preclinical study, we compared the effects of a 10-d intervention with a diet containing 50 mg/kg of β-carotene on plasma cholesterol in 5-wk-old male and female C57 Black 6 wild-type and congenic BCO1-deficient mice. In our clinical study, we aimed to determine whether 5 common small nucleotide polymorphisms located in the *BCO1* locus affected serum cholesterol concentrations in a population of young Mexican adults from the Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-Environment (UP AMIGOS) cohort.

Results: Upon β-carotene feeding, $Bco1^{-/-}$ mice accumulated >20-fold greater plasma β-carotene and had ∼30 mg/dL increased circulating total cholesterol ($P < 0.01$) and non–HDL cholesterol ($P < 0.01$) than wild-type congenic mice. Our results in the UP AMIGOS cohort show that the rs6564851 allele of *BCO1*, which has been linked to BCO1 enzymatic activity, was associated with a reduction in 10 mg/dL total cholesterol concentrations ($P = 0.009$) when adjusted for vitamin A and carotenoid intakes. Non–HDL-cholesterol concentration was also reduced by 10 mg/dL when the data were adjusted for vitamin A and total carotenoid intakes ($P = 0.002$), or vitamin A and β-carotene intakes ($P = 0.002$).

Conclusions: Overall, our results in mice and young adults show that BCO1 activity impacts circulating cholesterol concentration, linking vitamin A formation with the risk of developing ASCVD. *J Nutr* 2020;150:2023–2030.

Keywords: atherosclerosis, nutrition, genetic variants, micronutrients, retinoic acid

Introduction

Atherosclerosis is the main contributing factor to the development of myocardial infarction, the most common cause of death worldwide. Several risk factors promote atherosclerotic cardiovascular disease (ASCVD) development, but among those, LDL cholesterol is the most relevant traditional risk factor after sex and age (1). Elevated plasma cholesterol is associated with an enhanced accumulation of apoB-containing lipoproteins, mostly VLDLs and LDL. These lipoproteins are retained within the subendothelial space of the arterial wall, promoting the development and growth of atherosclerotic lesions. These lesions can rupture, causing thrombosis and arterial occlusion, resulting in myocardial infarction or stroke (2, 3).

Carotenoids are a group of plant pigments present in all photosynthetic organisms and some animal products such as

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Manuscript received December 12, 2019. Initial review completed March 16, 2020. Revision accepted April 28, 2020.
First published online May 20, 2020; doi: https://doi.org/10.1093/jn/nxaa143.

salmon and eggs. Approximately 650 carotenoids have been described to date, but only 50 of them are commonly found in our diet, of which 20 are present in significant amounts in our plasma (4). Among these, only β,β'-carotene (β-carotene), α,β-carotene (α-carotene), and β-cryptoxanthin have a provitamin A activity, the most studied role of these micronutrients in mammals. Of these, β-carotene is the most abundant in plasma and the most efficient for vitamin A production. β-Carotene oxygenase 1 (BCO1), a cytosolic enzyme present mostly in the intestine and the liver with high specificity for β-carotene, mediates vitamin A formation (5–7). Clinical studies show that higher β-carotene plasma concentration is associated with a lower incidence of obesity and metabolic syndrome (8, 9). Similarly, results extracted from the NHANES show that serum β-carotene correlates with the reduction in ASCVD risk markers (10). A prospective cohort study also shows an inverse association between plasma β-carotene and mortality caused by myocardial infarction (11).

The promoter and coding region of the *BCO1* gene has several small nucleotide polymorphisms (SNPs) affecting BCO1 activity (12, 13). Among these variants, *BCO1*-rs6564851 was the most significantly associated with circulating β-carotene concentrations in a genomewide association study (14). This variant localizes in the promoter region of *BCO1*, and the G-allele is associated with higher concentrations of β-carotene in plasma and a lower vitamin A conversion rate in comparison to subjects carrying the T-allele, indicating that BCO1 activity is affected upon nucleotide variations in the rs6564851 locus (13).

In this study, we examined whether β-carotene or BCO1 activity affected plasma cholesterol concentrations in both a preclinical and a clinical setting. We found that BCO1 activity, both in mice and in humans, correlates with total cholesterol concentrations and non–HDL cholesterol, the leading indicators of ASCVD risk.

This research was partially funded by the American Heart Association (16SDG27550012 to JA); the NIH (HL147252 to JA), the University of Illinois at Urbana-Champaign Research Board grant no. 09070 (to FCDA); the Center for Health and Aging (to FCDA); ACES Office of Research FIRE grant (to MT-G), and the USDA National Institute of Food and Agriculture, Hatch project nos. ILLU-968-312 and ILLU 971-368 (to MT-G). Funding was also provided by the Autonomous University of San Luis Potosí (UASLP), the Hormones Laboratory at the School of Medicine, the Clinical Biochemistry Laboratory at the Chemical Sciences School, and the UASLP University Health Center under agreement support C09-PIFI-030606 (to CA-G). MT-G is supported by the USDA National Institute of Food (NIFA) and Agriculture Research Initiative competitive grant no. 2015-68001-23248. MT-G also recognizes support from USDA NIFA Hatch project ILLU-793-327. JC is a recipient of the Philip L. and Juanita Fitzer Francis Fellowship.
Author disclosures: The authors report no conflicts of interest.
JWE is an editorial board member of the *Journal of Nutrition* and played no role in the *Journal*'s evaluation of the manuscript
Supplemental Tables 1–4 and Supplemental Figures 1 and 2 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
Address correspondence to JA (e-mail: jaume6@illinois.edu) or MT-G (teranmd@illinois.edu).
Abbreviations used: ASCVD, atherosclerotic cardiovascular disease; BCO1, β-carotene oxygenase 1; FFQ, food-frequency questionnaire; ISX, intestine-specific homeobox; PPARα, peroxisome proliferator-activated receptor α; RAE, retinol activity equivalents; RBP4, retinol binding protein 4; RXRα, rexinoid X receptor α; SNP, single nucleotide polymorphism; SR-B1, scavenger receptor class B type 1; UASLP, Autonomous University of San Luis Potosí; UP AMIGOS, Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-Environment.

Methods

Animal study

Generation of congenic wild-type and $Bco1^{-/-}$ mice, husbandry, and experimental diets.

Animal procedures and experiments were approved by the University of Illinois Urbana Champaign Animal Care Committee. C57/BL6 wild-type mice (Jackson Laboratories) and $Bco1^{-/-}$ mice (15) were cross-bred for 11 generations (16). Wild-type and $Bco1^{-/-}$ mice were maintained at 24°C in a 12:12-h light/dark cycle and had free access to food and water. The experimental design is summarized in **Figure 1**A. Dams and pups were fed a nonpurified breeder diet containing 15 IU vitamin A/g until the pups reached 3 wk of age (Teklad Global 18% protein diet; Envigo). Pups were weaned onto the same diet for another week before being switched to a purified vitamin A–deficient diet based on the AIN-93 formulation for 1 wk (Research Diets, Inc.). To prevent the interference of dietary carotenoids in the nonpurified breeder diet, and to facilitate β-carotene uptake (17), we then fed the 4-wk-old male and female mice a vitamin A–deficient purified diet (Purified-VAD) for 1 wk. After this washout period, age- and sex-matched (n = 7–8/genotype) congenic and $Bco1^{-/-}$ mice received a customized Western-type diet containing 3.08 mg/kg of cholesterol and 50 mg of β-carotene/kg. β-Carotene was incorporated in the diet using a water-soluble formulation of beadlets (DSM Ltd.) and prepared by Research Diets by cold extrusion to protect β-carotene from heat. The detailed diet composition of these diets is shown in **Supplemental Table 1**. After 10 d of dietary intervention, mice were anesthetized by intraperitoneal injection with 80 mg of ketamine per kilogram of body weight. Blood was drawn directly from the heart using EDTA-coated syringes and kept on ice. Mice were immediately killed by cervical dislocation. Plasma was collected by centrifugation at 500 × g for 5 min at 4°C and directly stored at −20°C.

HPLC analysis of murine plasma β-carotene and retinoids.

Nonpolar compounds were extracted from 100 μL of plasma under a dim yellow safety light, as described previously (18). For molar quantification of β-carotene and retinoids, HPLC was scaled with a standard curve using the parent compounds.

Western blot analysis of murine plasma retinol binding protein 4.

Plasma retinol binding protein 4 (RBP4) concentration was quantified as described previously (18). Quantification of scanned immunoblot single bands was performed with ImageJ software (NIH).

Plasma lipid measurements in mice.

Plasma cholesterol concentrations were quantified by colorimetric assays (Wako Chemicals), using an automated microplate reader (Biorad Laboratories). HDL cholesterol was determined after precipitating apoB-containing lipoproteins, and non–HDL cholesterol was calculated by subtracting HDL cholesterol from total plasma cholesterol (19, 20).

Human study

Study subjects and eligibility.

Participants (n = 767) in the cohort of the Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-Environment (UP AMIGOS) were recruited as part of the study. Subjects were healthy young college applicants of Mexican ethnicity. Data collection took place between February and July of 2009. Exclusion criteria are summarized in **Supplemental Figure 1**. Subjects with an incomplete lipid profile, a BMI (in kg/m^2) <16.5, and aged <18 or >25 y old were excluded. Individuals with fasting glucose >126 mg/dL were also excluded from the analysis.

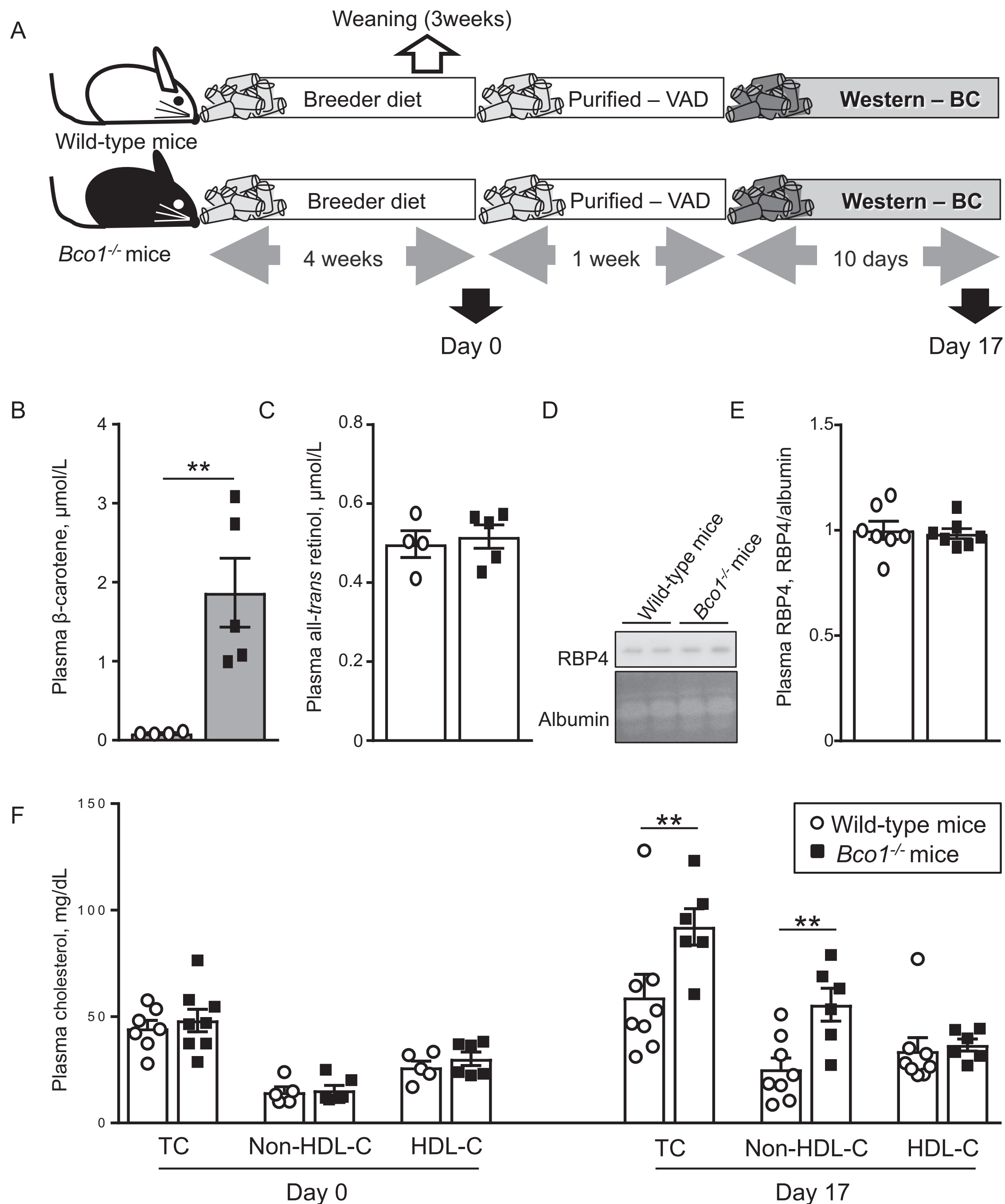

FIGURE 1 Effects of β-carotene dietary supplementation on congenic wild-type and *Bco1*$^{-/-}$ mice. (A) Experimental design. Four-week-old wild-type and *Bco1*$^{-/-}$ mice were fed a vitamin A–deficient purified diet for 1 wk, then switched to a vitamin A–deficient Western-type diet containing 50 mg/kg of β-carotene for 10 d. After this period, mice were killed and plasma collected. (B) Plasma β-carotene and (C) all-*trans* retinol concentrations were measured by HPLC. (D) Representative Western blot for plasma RBP4 and albumin (loading control). (E) Relative quantification of plasma RBP4. (F) Plasma cholesterol concentrations before (day 0) and after the dietary intervention with β-carotene (day 17). Values are means ± SEMs, $n = 4$–8 mice/group. Statistical differences were evaluated using the 2-tailed Student's t test. **Different from wild-type mice, $P < 0.01$. *Bco1*, β-carotene oxygenase 1; HDL-C, HDL cholesterol; Non-HDL-C, non–HDL cholesterol; RBP4, retinol binding protein 4; TC, total cholesterol; VAD, vitamin A deficient; Western-BC, Western-type diet supplemented with 50 mg/kg of β-carotene.

Institutional review boards of both the Autonomous University of San Luis Potosí (UASLP) and the University of Illinois at Urbana Champaign approved the study protocol. Each participant filled out semiquantitative food frequency questionnaires (FFQs) under the supervision of a facilitator. Participants completed a 116-item FFQ based and adapted from the FFQ developed by Hernandez-Avila et al. (22), and validated in the Mexican population (21–24). Surveys missing >20% of information were not included in the data set. Survey data were entered into the "SNUT" software developed by the National Institute of Public Health in Mexico (22). A total of 475 participants met the study's criteria and provided complete questionnaire data and, therefore, were included in the statistical analyses (Supplemental Figure 1).

Blood sample collection and plasma lipid measurements. Blood samples were collected from participants following an overnight fast. All samples were processed on the same day of collection using automatized methods. Fasting blood glucose was measured using the glucose oxidase-peroxidase (GOD-PAP) method utilizing the Alcyon 300 autoanalyzer (Abbott Laboratories). Reagents were purchased from BioSystems. Triglyceride concentrations were measured via the glycerol phosphate oxidase peroxidase method based on a colorimetric enzymatic reaction (Wako Chemicals). Total cholesterol was measured using a colorimetric assay based on the cholesterol oxidase method (Wako Chemicals). Cholesterol carried by HDL (HDL cholesterol) was quantified from the supernatant after precipitating apoB-containing lipoproteins with a detergent (Wako Chemicals). Cholesterol present in the non-HDL fraction (non–HDL cholesterol) was calculated by subtracting HDL cholesterol from total cholesterol. For human samples, all blood biomarker measurements were conducted at the UASLP clinical chemistry laboratory, which is a state-certified reference laboratory for blood analysis in San Luis Potosí, Mexico (25).

DNA extraction and genotyping

Blood samples were collected in the fasted state by trained medical professionals using Vacutainer (Becton Dickinson, Franklin Lakes, NJ) tubes containing EDTA. Blood was kept at −80°C until extraction. Genomic DNA was extracted from whole blood using commercial DNA extraction kits (Gentra Puregene Blood Kits; Qiagen). DNA was subsequently quantified using NanoDrop 2000 (Thermo Fisher). Genomic DNA was stored at −80°C until analysis quality was confirmed using PCR, in line with past research (25, 26). *BCO1* SNPs rs10048138 and rs6564851 were genotyped using predesigned allelic discrimination Taqman assays (Applied Biosystems). Genotypes were assigned using Sequence Detection Software 2.4 (Applied Biosystems). *BCO1* SNPs rs6420424, rs12934922, and rs7501331 were genotyped using the Fluidigm® (Fluidigm Corporation, San Francisco, CA) SNP genotyping platform. Briefly, 300 ng of genomic DNA was sent to the Functional Genomic Unit of the WM Keck Center at the University of Illinois for preamplification and genotyping. The assay design was constructed on the Fluidigm® D3™ website. Genotypes were analyzed using Fluidigm® Genotyping Analysis software version 4.1.2 at a minimum of 85–90% confidence.

Statistical analysis

For the preclinical study, values represented are expressed as means ± SEMs (Figure 1). Normal distribution of the sample groups was confirmed using the D'Agostino-Pearson omnibus and the Shapiro-Wilk normality tests using GraphPad Prism Software (GraphPad Software, Inc.). Statistical significance was then determined by the unpaired 2-tailed Student's t test with a threshold of significance set at $P < 0.05$.

For the human study, each variable was examined for normality (Shapiro-Wilk test, skewness, and kurtosis test). Nonnormal variables identified were all variables of the lipid profile, except for HDL, total, and individual carotenoid intake per day, which included β-carotene, α-carotene, β-cryptoxanthin, lycopene, and lutein. Each nonnormal variable was log-transformed (log10), and means reported here were back-transformed for interpretation. BMI was used to categorize individuals as normal weight (BMI <25), or affected by overweight or obesity, according to WHO guidelines (27). Values in **Table 1** are expressed as means ± SEMs. Results are presented for the entire sample and separated by sex. Next, allele frequencies were assessed using the allele counting method, while the divergence from Hardy-Weinberg equilibrium was identified using chi-square tests with 1 df. Associations between variables were tested for additive, dominant, and recessive models with general linear models using SAS version 9.3 (SAS Institute, Inc.). Models adjusted for age, sex, BMI, and retinol intake. Results presented in **Table 2** further adjust for total carotenoid intake, and those in **Table 3** for β-carotene intake. Statistical significance was set to $P < 0.05$. Bonferroni correction was used to adjust for multiple testing. The Bonferroni critical value was set at $P < 0.017$.

TABLE 1 Characteristics of the young adults from the UP AMIGOS study[1]

Variable	All ($n = 475$)	Males ($n = 216$)	Females ($n = 259$)	P
Age, y	18.6 ± 0.1	18.5 ± 0.1	18.6 ± 0.1	0.01
BMI, kg/m^2	23.7 ± 0.2	24.3 ± 0.3	23.2 ± 0.3	0.24
Serum TC, mg/dL	171 ± 2	169 ± 2	173 ± 3	0.14
Serum non–HDL-C, mg/dL	121 ± 2	121 ± 2	120 ± 2	0.76
Serum HDL-C, mg/dL	49.7 ± 0.6	47.6 ± 0.8	51.4 ± 0.8	0.0007
Serum TGs, mg/dL	107 ± 2	109 ± 3	106 ± 3	0.47
Serum glucose, mg/dL	90 ± 0.4	92 ± 1	89 ± 1	<0.0001
Retinol intake, RAE	1035 ± 51	1202 ± 91	901 ± 55	0.005
Provitamin A[2] intake, RAE	202 ± 7	191 ± 11	210 ± 10	0.19

[1]Values are means ± SEMs. Significance was calculated using the Student's t test. HDL-C, HDL cholesterol; non–HDL-C, non–HDL cholesterol; RAE, retinol activity equivalents; TC, total cholesterol; TG, total triglyceride; UP AMIGOS, Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-Environment.
[2]Provitamin A carotenoids in the diet (β-carotene + α-carotene + β-cryptoxanthin).

Results

Animal study: β-carotene supplementation reduces plasma cholesterol in wild-type mice but not *Bco1*$^{-/-}$ congenic mice

The experimental design is briefly described in the Methods section and represented in Figure 1A. At the moment of killing (day 17), *Bco1*$^{-/-}$ mice accumulated >20-fold greater β-carotene in plasma in comparison to congenic wild-type mice (Figure 1B). These changes occurred without affecting systemic vitamin A concentration (all-*trans* retinol) or its carrier, RBP4 (Figure 1C–E).

Baseline total cholesterol, non–HDL-cholesterol, and HDL-cholesterol concentrations were not different between genotypes before the dietary intervention (day 0; Figure 1F). However, upon feeding with a Western-type diet supplemented with β-carotene, total cholesterol and non–HDL cholesterol were significantly lower in wild-type mice in comparison to *Bco1*$^{-/-}$ mice, without affecting HDL-cholesterol concentration (day 17; Figure 1F).

Human study: association between BCO1 activity in humans with plasma cholesterol

The demographic characteristics of the final sample ($n = 475$) are summarized in Table 1. There were no significant differences between sexes in total plasma cholesterol ($P = 0.14$) and non–HDL cholesterol ($P = 0.76$), although male subjects showed significantly lower HDL cholesterol than females (males: 47.6 ± 0.8 mg/dL; females: 51.4 ± 0.8 mg/dL; $P = 0.0007$). Fasting glucose concentrations (males: 92 ± 1 mg/dL; females: 89 ± 1 mg/dL; $P < 0.0001$) and preformed vitamin A (retinol) consumption were higher in male subjects [males: 1202 ± 91 retinol activity equivalents (RAE); females: 901 ± 55 RAE; $P = 0.005$]. No significant differences were observed in provitamin A carotenoid intake ($P = 0.19$) (Table 1).

Minor allele frequency was calculated for each of the 5 candidate SNPs (**Figure 2**A) and compared with the reported values from the 1000 Genomes Project database (28, 29). To determine genetic associations between the different loci, we

TABLE 2 Serum lipid concentrations by *BCO1*-rs6564851 genotype variants calculated using general linear models and adjusted for age, sex, BMI, retinol intake, and total carotenoid intake[1]

Variables	Genotype			*P* value		
	GG	GT	TT	Additive model: GG vs GT vs TT	Dominant model: GG vs GT/TT	Recessive model: TT vs GG/GT
n	130	227	118		130 vs 345	118 vs 357
Serum TC, mg/dL	175 ± 3	166 ± 2	166 ± 3	0.03	0.009	0.43
Serum non–HDL-C, mg/dL	125 ± 3	114 ± 2	115 ± 3	0.007	0.002	0.41
Serum HDL-C, mg/dL	49.3 ± 1.0	49.8 ± 0.8	49.6 ± 1.1	0.94	0.74	0.98
Serum TGs, mg/dL	102 ± 4	97 ± 2	99 ± 4	0.39	0.21	0.89

[1]Values are means ± SEMs. Statistical significance was set to $P < 0.05$. After applying the Bonferroni correction to consider the measurement of 3 independent tests (TC, HDL-C, and TG), we established the Bonferroni critical value to $P < 0.017$. *BCO1*, β-carotene oxygenase 1; HDL-C, HDL cholesterol; non–HDL-C, non–HDL cholesterol; TC, total cholesterol; TG, total triglyceride.

represented the linkage disequilibrium map for the 5 *BCO1* loci. We observed that the *BCO1*-rs6564851 locus showed the strongest association with 2 neighboring loci, both of which are located in the promoter region (Figure 2B). All SNPs, except for *BCO1*-rs12934922, were in Hardy-Weinberg equilibrium; therefore, this SNP was excluded from further analysis (**Supplemental Table 2**). All the other markers were evaluated for associations with plasma lipid profiles using general linear models adjusted for age, sex, and BMI. Based on the data provided by FFQs, we also adjusted the associations for total vitamin A (retinol) intake, as well as for total carotenoid (Table 2) or β-carotene (Table 3) intake (21–24).

Only the noncoding *BCO1*-rs6564851 variant was associated with individual differences in total cholesterol (Tables 2 and 3; **Supplemental Tables 3** and **4**). The allele frequency in our cohort was similar to the frequency reported in the 1000 Genomes Project database as well as by the Lietz et al. (13) (Figure 2). Using an additive model, where the 3 independent allelic combinations are considered individually, total cholesterol ($P = 0.03$) did not reach statistical significance when we considered the Bonferroni critical value ($P < 0.017$). Non–HDL cholesterol, however, was significantly different between groups when we adjusted for age, sex, BMI, retinol intake, and total carotenoid intake using the additive model ($P = 0.008$) (Table 2).

We evaluated the association between the *BCO1*-rs6564851 variants and lipid profile under the dominant model of inheritance, where individuals with ≥1 copy of the rs6564851 T allele were compared with those with 2 copies of the rs6564851 G allele. We observed significantly lower total cholesterol ($P = 0.009$) and non–HDL-cholesterol ($P = 0.002$) concentrations in individuals with the rs6564851 T allele. We did not observe differences in HDL-cholesterol or triglyceride concentrations in the additive or dominant models. There were no significant associations between the SNP variants and the serum lipids in the recessive model (Table 2).

Next, we adjusted the data for age, sex, BMI, retinol intake, and β-carotene intake. We chose β-carotene because this carotenoid is the main substrate of BCO1 (4), and the most consumed provitamin A carotenoid in our cohort, as shown by the FFQ records (data not shown). With these adjustments, total cholesterol concentrations were not significant for the additive model ($P = 0.04$), but non–HDL-cholesterol concentrations were significant in the additive model ($P = 0.009$). In the dominant model, participants with ≥1 copy of the rs6564851 T allele had lower plasma total cholesterol ($P = 0.011$) and non–HDL cholesterol compared with participants with 2 copies of the rs6564851 G allele ($P = 0.002$). There were no statistically significant associations in the recessive model (Table 3).

Discussion

The interaction between nutrients, bioactive food components, and gene variants is gaining increasing attention among scientists and physicians as a strategy to predict and modify the incidence of certain diseases. Among these food components, carotenoids are especially interesting for a few reasons. First, even though carotenoid content varies between foods, these compounds are present in all fruits and vegetables. Second, carotenoids accumulate in easily measurable quantities in the human body, including plasma, which facilitates their measurement. Third, carotenoids are cleaved by 2 carotenoid oxygenases, BCO1 and BCO2, BCO1 being the only enzyme

TABLE 3 Serum lipid concentrations by *BCO1*-rs6564851 genotype variants calculated using general linear models and adjusted for age, sex, BMI, retinol intake, and β-carotene intake[1]

Variables	Genotype			*P* value		
	GG	GT	TT	Additive model: GG vs GT vs TT	Dominant model: GG vs GT/TT	Recessive model: TT vs GG/GT
n	130	227	118		130 vs 345	118 vs 357
Serum TC, mg/dL	175 ± 3	165 ± 2	166 ± 3	0.04	0.011	0.47
Serum non–HDL-C, mg/dL	124 ± 3	114 ± 2	115 ± 3	0.009	0.002	0.46
Serum HDL-C, mg/dL	48.3 ± 1.0	49.7 ± 0.8	49.5 ± 1.1	0.96	0.79	0.96
Serum TGs, mg/dL	102 ± 4	96.4 ± 2.4	99.2 ± 3.5	0.39	0.23	0.82

[1]Values are means ± SEMs. Statistical significance was set to $P < 0.05$. After applying the Bonferroni correction to consider the measurement of 3 independent tests (TC, HDL-C, and TG), we established the Bonferroni critical value to $P < 0.017$. *BCO1*, β-carotene oxygenase 1; HDL-C, HDL cholesterol; non–HDL-C, non–HDL cholesterol; TC, total cholesterol; TG, total triglyceride.

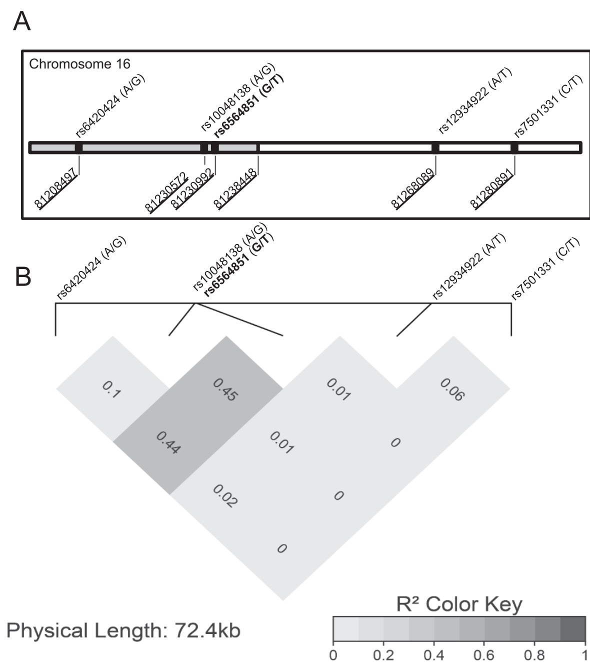

FIGURE 2 Localization and frequency of the 5 *BCO1* SNPs in the UP AMIGOS cohort. (A) Localization of the 5 SNPs analyzed in this study. Numbers at the bottom indicate the position of the SNP or starting site in the genome. A segment of the putative promoter region is represented in gray, and the coding region is shown in white. (B) Linkage disequilibrium map for the 5 loci in the *BCO1* gene. *BCO1*, β-carotene oxygenase 1; SNP, single nucleotide polymorphism; UP AMIGOS, Universities of San Luis Potosí and Illinois: A Multidisciplinary Investigation on Genetics, Obesity, and Social-Environment.

capable of producing vitamin A in mammals (7). Fourth, the carotenoid-derivative vitamin A, in its acidic form retinoic acid, regulates gene expression of nearly 700 genes affecting biological processes, including energy metabolism (4). Nonetheless, significant gaps remain unclear on the interaction between carotenoids, their cleaving enzymes, and predisposition to develop chronic diseases, including ASCVD.

This study provides for the first time preclinical and clinical evidence that BCO1 activity, and not β-carotene concentration per se, acts as a modulator of non–HDL cholesterol, affecting total cholesterol concentration. We examined whether any of the 5 common SNPs in the *BCO1* gene, 3 of them in the promoter region and 2 of them in the coding region (Figure 2A), were associated with plasma lipid concentrations in a cohort of young Mexican adults. From these variants, only rs6564851 was significantly associated with plasma cholesterol. These results were not surprising since rs6564851 showed the strongest association with plasma β-carotene concentrations among all the studied variants (14). Our results demonstrate that individual carriers of the major T allele of *BCO1*-rs6564851 variant display lower concentrations of total cholesterol and non–HDL cholesterol, independently of age, sex, BMI, retinol intake, and total carotenoid consumption (Table 2) or β-carotene consumption (Table 3).

Since rs6564851 is located in the promoter of *BCO1*, we hypothesized that the presence of the different G or T alleles could affect BCO1 activity by generating a transcription factor–binding site that could affect protein expression, as observed in similar situations (30). Indeed, when we investigated the flanking regions of the rs6564851 with PROMO, a program designed to identify putative transcription factor–binding sites (31, 32), we observed that the allele variants could bind various transcription factors (**Supplemental Figure 2**). Among the different transcription factors, a putative binding site for the heterodimer peroxisome proliferator-activated receptor α:rexinoid X receptor α (PPARα:RXRα) in the rs6564851 G allele caught our attention, for several reasons. First, this heterodimer showed the highest similarity threshold among all the transcription factors. Second, it is a regulator of lipid metabolism (33). Third, this heterodimer is activated by 9-*cis* retinoic acid, a vitamin A metabolite that can derive from provitamin A carotenoids. Whether the PPARα:RXRα heterodimer actually occupies this binding site will require further investigation.

Lobo et al. (17) demonstrated that the transcription factor intestine-specific homeobox (ISX) binding site coincides with the rs6564851 G locus (Supplemental Figure 2). They later confirmed the flanking nucleotide sequence in the promoter region of the scavenger receptor class B type 1 (SR-B1) (34), which is involved in the uptake of carotenoids, including β-carotene (35, 36). The findings reported by these authors, therefore, describe the presence of an ISX binding site in the promoter of *Scarb1* (gene encoding SR-B1) and *Bco1* murine genes. Hence, the presence of allele variants corresponding to the rs6564851 locus of *BCO1* could modulate the binding affinity and transcriptional activity of ISX in the intestine (37).

In agreement with our results, Lietz and colleagues (13) showed that European subjects carrying the *BCO1*-rs6564851 T allele accumulate less β-carotene in plasma than carriers of the G allele. They found that subjects with the T allele were more efficient at converting provitamin A carotenoids to vitamin A, indicating greater BCO1 activity. This finding is in line with both clinical and preclinical studies published in the past. Greater circulating retinoic acid concentration correlates with a reduction in metabolic syndrome and myocardial infarction risk (38, 39), in agreement with studies where the dietary supplementation with provitamin A carotenoids reduces plasma cholesterol and delays atherosclerosis progression in various animal models (40, 41). Thus, the conversion to retinoic acid may mediate these positive effects of provitamin A carotenoids (41–43). Studies using retinoic acid supplementation support these findings and reached similar conclusions by affecting cholesterol metabolism and reducing cholesterol content in cultured cells (44, 45).

Surprisingly, the average consumption of retinol in our cohort was ~1200 RAE/d, from which participants obtained only ~200 RAE from provitamin A carotenoids (Table 1). Hence, participants in our study consumed mostly retinol or other forms of vitamin A, suggesting that provitamin A carotenoids only contributed to approximately one-sixth of the total intake of vitamin A. Further experiments in subjects fed a diet depleted of vitamin A would be necessary to probe the independent impact of provitamin A carotenoids.

Although previous preclinical studies with provitamin A carotenoids suggest that BCO1-mediated vitamin A production reduces circulating cholesterol concentrations, none of them could confirm the direct impact of BCO1 activity on cholesterol concentrations due to the lack of an adequate experimental model (4). In the current work, we evaluate for the first time whether congenic wild-type and $Bco1^{-/-}$ mice respond differently to a purified Western-type diet containing β-carotene as the only source of vitamin A. Under these experimental conditions, we observed a reduction in total cholesterol and

non–HDL cholesterol in wild-type mice in comparison to $Bco1^{-/-}$ mice. These results recapitulate those described above in subjects with greater BCO1 activity, such as occurs in those with the rs6564851 T variant (Tables 2 and 3). This reduction was independent of circulating β-carotene, which was 20-fold higher in $Bco1^{-/-}$ mice than in wild-type mice, indicating that enzymatic conversion to vitamin A mediates its beneficial effects, as we have reported previously (46, 47).

A panel of experts recently concluded that the percentage reduction in LDL-cholesterol concentration is a reliable indicator of risk of ASCVD, such that risk is ~1% lower with every 1% decrease in LDL cholesterol (48). Considering that the current study was carried out in a cohort of healthy, very young adults (average: 18.8 y old), we could not evaluate any long-term clinical outcome related to ASCVD. Hence, we used the Friedewald formula to calculate the LDL-cholesterol concentrations using the data from Table 3 (49). Subjects carrying ≥1 copy of the rs6564851 T allele showed an ~9% reduction in LDL cholesterol when compared with GG-homozygous subjects (97 ± 2 vs 107 ± 2 mg/dL; $P = 0.007$). Although speculative, this decrease may be clinically meaningful if maintained through adulthood.

In summary, our data suggest that BCO1 activity may be a more meaningful indicator of circulating cholesterol concentration than circulating β-carotene. Our preclinical data, where BCO1-deficient mice show 20-fold higher plasma β-carotene accompanied by increased non–HDL-cholesterol concentrations than congenic wild-type mice, support this conclusion. Additionally, we report for the first time an association between the individual genetic variation in *BCO1*-rs6564851, which affects BCO1 activity in humans (13), and plasma cholesterol concentrations in young subjects independently of vitamin A, total carotenoid, and β-carotene intake, as determined by our FFQ assessment. Differences in total cholesterol were attributed to non–HDL cholesterol, a measurement that integrates all forms of cholesterol transported by atherogenic lipoproteins, a good indicator of apoB concentrations, and considered by some experts to be a better predictor of ASCVD than LDL cholesterol (50, 51). Future studies should determine the molecular mechanisms underlying BCO1 activity and ASCVD risk and whether vitamin A formation could modulate plasma lipids by affecting cholesterol absorption, hepatic lipoprotein secretion, or lipoprotein clearance in tissues (52). Additional studies to quantify the dietary intake of carotenoids will be necessary to validate the self-reported data obtained by FFQ, which is a limitation in our study design.

Acknowledgments

We thank Dr. Tessa J Barrett for proofreading the manuscript and her insightful comments and Marco Toc for his help in preparing the figures. We also thank Dr. Sa Shen (Biostatistics, Epidemiology, and Research Design Core, Interdisciplinary Health Institute, University of Illinois Urbana-Champaign) for her statistical advice and suggestions during the preparation of this manuscript. The authors' responsibilities were as follows—JA, CA-G, JMVM, FCDA, JWE, and MT-G: designed the research; JA, JC, CM, and MT-G: conducted the research and analyzed data; JA, JWE, and MT-G: wrote the manuscript; JA and MT-G: have primary responsibility for final content; and all authors: read and approved the final manuscript.

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