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Study material will be made available upon reasonable request.

Abstract

Background: Familial hypobetalipoproteinemias (FHBL) are rare genetic diseases characterized by lipid malabsorption. We focused on abetalipoproteinemia (FHBL-SD1) and chylomicron retention disease (FHBL-SD3), caused by mutations in MTTP and SAR1B genes, respectively. Treatments include a low-fat diet and high-dose liposoluble vitamin supplementations. However, patients are not supplemented in carotenoids, a group of lipid-soluble pigments essential for eye health. **Objective**: Our aim was to evaluate carotenoid absorption and status in the context of hypobetalipoproteinemia. **Methods**: We first used knock-out Caco-2/TC7 cell models of FHBL-SD1 and FHBL-SD3 to evaluate carotenoid absorption. We then characterized FHBL-SD1 and FHBL-SD3 patient status in the main dietary carotenoids and compared it to that of control subjects. **Results**: *In vitro* results showed a significant decrease in basolateral secretion of α- and β-carotene, lutein, and zeaxanthin (-88.8±2.2% to -95.3±5.8%, -79.2±4.4% to -96.1±2.6%, -91.0±4.5% to -96.7±0.3% and -65.4±3.6% to -96.6±1.9%, respectively). Carotenoids plasma levels in patients confirmed significant deficiencies, with decreases ranging from –89% for zeaxanthin to -98% for α-carotene, compared to control subjects. **Conclusion**: Given the continuous loss in visual function despite fat-soluble vitamin treatment in some patients, carotenoid supplementation may be of clinical utility. Future studies should assess the correlation between carotenoid status and visual function in aging patients and investigate whether carotenoid supplementation could prevent their visual impairment.

Introduction

Familial hypobetalipoproteinemias (FHBL) and related disorders (also known as primary hypocholesterolemia or monogenic hypobetalipoproteinemia disorders) represent a heterogeneous group of rare monogenic diseases characterized by very low lipids and apolipoprotein (apo) B-containing lipoproteins plasma levels. These diseases are classified in two categories according to the molecular mechanisms involved [1]. Here, we focus on class I disorders caused by defects in synthesis, assembly, and secretion of apoB-containing lipoproteins, such as chylomicrons and VLDL/LDL from enterocytes and hepatocytes, respectively. Assembly of these triglycerides-rich particles begins in the endoplasmic reticulum. It requires a lipid association step with apolipoprotein B (apoB), the main non-exchangeable structural component, through microsomal triglyceride transfer protein (MTP), a lipid transfer protein that assembles lipoproteins and acts as a chaperone for apoB [2]. Loss-of-function mutations in the *MTTP* gene cause Familial Hypobetalipoproteinemia due to lipoprotein assembly and Secretion Defects 1 (FHBL-SD1) commonly known as Abetalipoproteinemia (ABL, OMIM 200100) [3]. Mutations in the *APOB* gene result in FHBL-SD2 (FHBL or FHBL-SD2, OMIM 615558), with homozygous and heterozygous forms [4]. Lipoproteins are then transported to the Golgi apparatus for terminal maturation [5] through a mechanism requiring a proper activation of the small GTPase Sar1b [6]. Its loss of function affects chylomicrons secretion by enterocytes and causes FHBL-SD3, usually referred to as Chylomicron Retention Disease or Anderson disease (CMRD, OMIM 246700) [7]. Lack of MTP, ApoB or Sar1b activity leads to the accumulation of large lipid droplets in enterocytes and hepatocytes and is responsible for fat malabsorption and nearly complete absence of plasma apoB-containing lipoproteins [8].

Chronic malabsorption of lipids leads to fat-soluble vitamin deficiencies (vitamins A, E, D, and K), which have direct effects on growth and development in infancy, and impact a wide

range of organ systems [8]. Untreated patients typically suffer from neurological and ophthalmological degeneration during the second or third decade of life. To limit the serious sequelae of these pathologies, treatment with large oral doses of vitamin A and E should be initiated at a young age [9]. However, despite early and appropriate supplementation, serum vitamin E levels are never completely restored [8]. Additionally, ophthalmologic protection remains incomplete in some patients. Ocular manifestations including a decrease in visual acuity, night blindness, and loss of dark adaptation are usually reported during adulthood and may progress to blindness with aging [10]. Eye abnormalities are variable, but most commonly evoke an atypical retinal degeneration described as retinitis-pigmentosa (RP)-like symptoms [11] with atrophy of the macula lutea [12]. This particular region of the retina contains high amounts of two carotenoids, lutein and zeaxanthin, also called macular pigments [13] which follow the process of absorption of lipids and fat-soluble vitamins as briefly described above [14].

Carotenoids are lipid-soluble red-yellow vegetal pigments divided into carotenes and xanthophylls. At least 600 carotenoids occur naturally, although about 20 of them, including β-carotene, α-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin, are detectable in the human blood [15]. Some carotenes, such as β-carotene, α-carotene, and β-cryptoxanthin, display a provitamin A activity, while non-provitamin A carotenoids or xanthophylls (lycopene, lutein and zeaxanthin), participate in physiological functions ranging from fetal development to vision [16]. Both carotenes and xanthophylls are important for eye health. Indeed, provitamin A carotenes can provide retinol to the visual system, while xanthophylls such as lutein and zeaxanthin were shown to protect the macula from damage by blue light, improving visual acuity and scavenging harmful reactive oxygen species [17]. Supplementation studies with xanthophylls showed promising results regarding the progression of Age-related Macular Disease (AMD) and other ocular disorders[18,19]. In particular, a long-term

epidemiologic follow-up study of the AREDS2 cohort suggested that compared to a β-carotene supplementation, a lutein/zeaxanthin supplementation had a potential beneficial association with late AMD progression without increasing the risk of lung cancer [20]. Even though rare articles, mainly case-reports, reported very low total carotenoid plasma levels in FHBL patients, carotenoids have never been considered in their treatment [21]. This is likely because they are not yet considered essential micronutrients and no recommendations for intake have formally been established.

As a chronic carotenoid deficiency might participate in the ophthalmic deterioration related to the macular pigment depletion observed in some patients, we first evaluated both carotene and xanthophyll absorption/secretion using intestinal cell lines mimicking patients enterocytes we previously established [22]. We then assayed the 6 main carotenes and xanthophylls found in human plasma in both patients with FHBL and in control subjects.

Materials and methods

Supplies

Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and trypsin-EDTA, non-essential amino acids, penicillin/streptomycin and PBS were purchased from Life Technologies (Illkirch, France). Fetal bovine serum (FBS) came from PAA (Vélizy Villacoublay, France). β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin and apo-8'-carotenal (used as an internal standard in cell samples) were from Extrasynthèse (Genay, France). α-carotene, echinenone (used as internal standard in plasma samples) was from Sigma-Aldrich Chimie (Merck, Saint-Quentin-Fallavier, France). All solvents used were HPLC grade from Carlo-Erba Reagents (Peypin, France).

Cell culture

Two Caco2 TC7 cell lines knock-out for MTTP (MTTP1-KO1 and MTTP-KO2), two Caco2 TC7 cell lines knock-out for Sar1B (Sar1b-KO1 and Sar1b-KO2), and the control Caco2 cell line were routinely cultured in DMEM containing 8% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids at 37°C in a 10% CO2 atmosphere [22].

Carotenoid absorption experiments

For delivery of each carotenoid to cells, mixed micelles were prepared as previously described [23] to obtain the following final concentrations: 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.3 mM monoolein, 0.1 mM free cholesterol, 0.5 mM oleic acid, 5 mM taurocholate, and either 1 µM α- or β-carotene or 5 µM lutein or zeaxanthin. Cell lines were grown on 12-well plates with inserts for 21 days.

Twenty-four hours prior to each experiment, cells were fasted using serum-free medium at both apical and basolateral sides. At the beginning of each experiment, the apical side of the cell monolayers received the carotenoid-rich micelles, whereas the other side received a serum-free complete medium. Cells were incubated for 6 hours at 37°C. At the end of the experiment, media from each side of the cell monolayer were harvested. Cells were washed twice in 0.5 mL ice-cold PBS to eliminate adsorbed micelles, then scraped and collected in 0.560 mL PBS. Scraped cell and basolateral media samples were stored at –80°C under nitrogen before tocopherol and lipid assays. Each experiment was repeated at least twice.

Hypobetalipoproteinemia patients

We used plasma samples from eight patients with genetically proven HBL (3 FHBL-SD1 and 5 FHBL-SD3) and four controls from a study registered at clinicaltrials.gov (NCT01457690). During the follow-up of patients, ophthalmic function was evaluated with visual acuity, color vision, fundus, and visual evoked potentials as well as electroretinograms were obtained following ophthalmology consultation. The study protocol was approved by the local ethics committee (March 2011, Hospices Civils de Lyon) and the French National Agency for Medicines and Health Products (A110125-36). It was conducted in accordance to the ethical guidelines of the Declaration of Helsinki. Informed consent to participate in the study was obtained from all patients or from their parents.

Carotenoids extraction

Carotenoids were extracted either from 50 - 1000 µL aqueous samples from cell experiments or from 250 -500 µL plasma samples, as follows. When necessary, distilled water was added to samples to reach a final volume of at least 500 µL. Apo-8'-carotenal was added to the cell experiment samples in the same volume of ethanol. Echinenone was added to the plasma

samples in 500 µL ethanol. The mixtures were then extracted twice with two volumes of hexane. The hexane phases obtained after centrifugation (1200 × g, 10 min, 4 °C) were collected, pooled and evaporated to dryness under nitrogen, and the dried extracts were dissolved in 10 to 200 µL methanol/dichloromethane. A volume of 10-20 µL was used for HPLC analysis of the cell experiment samples, and a volume of 50-180 µL was used for HPLC analysis of plasma samples.

Carotenoid HPLC analysis

The chromatographic separation and determination of carotenoids from cell experiment samples was performed on an analytical column Kinetex® C-18 100 × 4.6 mm, particle size 2.6 µm (Phenomenex) and a guard column (SecurityGuard ULTRA Holder, Phenomenex), using a mobile phase consisting of 100% methanol (flow rate = 1.2 mL min^{-1}). The column oven temperature was kept constant at 40°C. Run time was 4 minutes for lutein and zeaxanthin and 15 minutes for α- and β-carotene.

The chromatographic separation and determination of plasma carotenoids was performed on an analytical column YMC Carotenoid 250 × 4.6 mm, particle size 5 µm (YMC) and a guard column (Carotenoids, YMC), using a mobile phase consisting of a gradient of methanol (A), methyl tert-butyl ether (B) and water (C). Flow rate was 1 mL/min. The gradient profile of the mobile phase (A:B:C) was set at 96:2:2 and changed linearly to 18:80:2 in 27 min, and then the mobile phase was changed back to 96:2:2 from 31 to 35 min. The column oven temperature was kept constant at 35°C. Run time was 35 minutes.

Detection was performed at 450 nm using a Thermo Scientific UltiMate 3000 Diode Array Detector (DAD). The HPLC system also comprised a Thermo Scientific UltiMate 3000 separation module (Pump LPG 3400SD, Column oven TCC-3000SD and Autosampler WPS-

3000TSL) with Chromeleon system manager as data processor. Carotenoids were identified by retention time compared with pure (>95%) standards. Quantification was performed using Chromeleon software (version 7.2, ThermoFisher Scientific, Villebon sur Yvette, France) comparing peak area with standard reference curves.

Statistical Analysis

Statistical tests were performed using GraphPad Prism software, version 8.4.3 (GraphPad Software, San Diego, CA, USA). For all tests, the bilateral alpha risk was $\alpha = 0.05$. A p-value < 0.05 was considered significant. Data were expressed as means ± SEM and tested, along with residuals (residual plot, homoscedasticity plot and QQ plot), for normal distribution and equality of variances before statistical testing. When appropriate, data with non-normal distributions was log-transformed before further parametric analysis was done. Differences between more than two groups of unpaired data underwent the ANOVA test. Post-hoc Dunnett's test was used to compare means of the different groups.

Results

Carotenoids basolateral secretion is impaired in mutated clones

The impact of SAR1B or MTTP knock-out in Caco-2/TC7 cells on carotenoids basolateral secretion of enterocytes was studied by using KO-Caco-2/TC7 cell monolayers cultivated on filters. A significant decrease of α-carotene secretion ($p < 0.0001$) was observed after mixed micelles were added to the apical side of the cells for 6 h, with a mean reduction of -92.8 ± 3.7% for MTTP-KO1, -95.3 ± 5.8% for MTTP-KO2, -91.2 ± 4.5% for SAR1B-KO1 and -88.8 ± 2.2% for SAR1B-KO2 (Figure 1A) compared to control cells. The same was observed for β-carotene, under the same experimental conditions, with a mean reduction of -96.1 ± 2.6% for MTTP-KO1, -85.3 ± 8.7% for MTTP-KO2, -88.2 ± 3.8% for SAR1B-KO1 and -79.2 ± 4.4% for SAR1B-KO2, compared to control cells ($p < 0.0001$) (Figure 1B). Regarding xanthophylls, we report a significant decrease in basolateral secretion with a mean reduction of -91.0 ± 4.5% (MTTP-KO1), -92.7 ± 2.8% (MTTP-KO2), -95.1 ± 2.9% (SAR1B-KO1) and -96.7 ± 0.3% (SAR1B-KO2) for lutein ($p < 0.0001$) (Figure 1C), and a mean reduction of -96.6 ± 1.9% (MTTP-KO1), -87.8 ± 0.9% (MTTP-KO2), -65.4 ± 3.6% (SAR1B-KO1) and -72.0 ± 9.6% (SAR1B-KO2) for zeaxanthin ($p < 0.0001$) (Figure 1D), compared to control cells.

Carotenoids plasma levels are markedly depleted in FHBL patients

Patient characteristics are presented in Table 1 and Control characteristics in Table 2. Patients were relatively young (11-21 years-old). Their clinical ophthalmological exams including fundus, visual acuity and color vision, electroretinogram (ERG) measurement and visual evoked potential were in the normal range.

We then analyzed both patient and control subject plasma levels of α- and β-carotene, lutein, zeaxanthin (studied in our cell models), and assayed the concentrations of lycopene and β-cryptoxanthin, which are two other major carotenoids in human plasma (Figure 2, Table 1,

Table 2). Our results indicate strong deficiencies, in patients whatever the carotenoid considered. Our results show a decrease of -96.5 ± 1.3 % and – 91.3 ± 5.2 in patients compared to controls for α- and β-carotene concentrations, respectively ($p = 0.0058$ and $p = 0.0021$, Fig. 2A and 2B). A decrease of – 91.0 ± 2.8% was also observed for lycopene ($p < 0.0001$, Fig. 2F). Concerning xanthophylls, lutein and zeaxanthin concentrations were decreased in patients compared to controls by -97.4 ± 0.9 % and – 98.4 ± 0.8 %, respectively ($p < 0.0001$, Fig. 2C and 2D). Finally, β-cryptoxanthin concentration was decreased by – 95.0 ±1.5 % in patients compared to controls ($p = 0.0266$, figure 2E).

Carotene but not xanthophyll levels tended to be lower in FHBL-SD1 patients than in FHBL-SD3 patients, but more analyses are required to confirm this observation (Table 1).

Discussion

Carotenoids status in FHBL patients is poorly documented in international literature, and no data on xanthophyll levels in patients are available. A patient diagnosed with FHBL-SD1 (abetalipoproteinemia) was first reported with plasma concentrations of serum carotene of 11 µg/100 ml (normal range 50-200 µg/100 ml) [24]. Similarly, Illingworth and colleagues observed values of carotene of 5-8 µg/dL and 4 µg/dL (normal range: 50 – 300 µg/dL) for two other individuals diagnosed with FHBL-SD1 [25]. Chowers et al. (2001) investigated plasma β-carotene concentrations in 13 patients diagnosed with FHBL-SD1 or FHBL-SD2. The study found that the patients low plasma carotene values ranging from 15 to 151 mg/100 ml, while normal values ranged from 20 to 500 mg/100 ml [9]. Finally, two recent case-study confirmed low level of β-carotene in 5 FHBL-SD1 patients [26,27].

The fact that no study assayed carotenoid individually might lead to misinterpretation in terms of carotenoid status as carotenoids can display very different absorption efficiency depending on their molecular structure. Indeed, carotenes such as lycopene are poorly absorbed while the absorption of xanthophylls (i.e. oxygenated carotenoids) such as lutein can be much more effective [28].

To explore individual carotenoid absorption in familial hypobetalipoproteinemia, we first used Caco-2/TC7 cells knocked-out for *MTTP* or *SAR1B* genes to measure the absorption and secretion of carotenoids by enterocytes affected by FHBL [22]. These Caco-2/TC7 cell knockout models have replace had previously been developed and validated by our laboratory. We successfully knocked out MTTP (identified as MTTP-KO1 and MTTP-KO2) and SAR1B (identified as SAR1B-KO1 and SAR1B-KO2). Gene knock-out was confirmed by DNA sequencing, revealing mutated sequences compared to control sequences, and Western blotting (WB) showing no protein expression. These clones demonstrate impaired basolateral vitamin E secretion, in agreement with the clinical presentation of these diseases. Results

showed that free α-tocopherol secretion was significantly lower in all KO clones, and to a greater degree in MTTP-KO clones compared to SAR1B-KO clones, according to what is observed in patients. Given these results, we studied the basolateral secretion of four carotenoids (α-carotene, β-carotene lutein and zeaxanthin,) using these models. Our results showed an impaired basolateral secretion for the four carotenoids studied. These findings align with the shared mechanisms of absorption for both carotenoids and vitamin E at the intestinal level. Indeed, as in the case of vitamin E, carotenoids are extracted from food and combined with other lipids in mixed micelles during duodenal digestion to be absorbed by enterocytes [28,29]. Most carotenoids and vitamin E are then packaged within chylomicrons and secreted by the enterocytes at their basolateral pole. This basolateral secretion is mainly mediated by chylomicrons whose formation and secretion depend on MTTP [30] and Sar1b [6].

We then evaluated the carotenoid profile in patients. Three young adults with FHBL-SD1 (one female, two males; age = 18.3 ± 2.5 years; BMI 19.9 ± 1.9 kg/m²) and five with FHBL-SD3 (four females, one male; age = 17.4 ± 4.3 years; BMI 19.5 ± 3.5 kg/m²) were included in the study. All were characterized with typical hypobetalipoproteinemia, except Patient #8 whose vitamin E status was almost restored after supplementation (Table 1). As expected, FHBL-SD3 patients had higher levels of apoB, triglycerides, and LDL-cholesterol than FHBL-SD1 patients. Except for some slight elevations in liver biochemical parameters and a classical increase of Creatinine Phosphokinase (CK) in patients with FHBL-SD3, all other biochemical values were relatively close to normal. Out of the three FHBL-SD1 patients, two had a decrease in hemoglobin levels, and their blood samples showed a significant number of acanthocytes. The plasmatic levels of liposoluble vitamins were normal after high-dose therapy of vitamin A (89,796 ± 85,675 IU/day), vitamin D (85,714 ± 24,397 IU/month) and vitamin K (14 ± 5 mg vitamin K/week) evaluated with the prothrombin ratio.

Carotenoid levels in control subjects were consistent with values observed in the general population according to previous studies and meta-analyses [31–33]. Conversely, carotenoid assays in regularly monitored patients without specific carotenoid supplementation indicate collapsed levels of the 6 main carotenoids compared to values measured in healthy volunteers. Carotene but not xanthophyll levels tended to be lower in FHBL-SD1 patients than in FHBL-SD3 patients, but more analyses are required to confirm this observation.

Despite high carotenoid deficiencies, the visual function of the patients was normal. However, patients were young (age < 21 years old) and received early vitamin A, vitamin E and omega 3 fatty acid supplementations, which can delay the appearance of ophthalmic complications.

In conclusion, our data confirm the major impairment of carotenoid absorption-secretion in enterocyte KO for two genes of FHBL and the dramatically decreased carotenoid status in patients with familial hypobetalipoproteinemia.

Although not considered as micronutrients yet, carotenoids are suggested to have major effect on human health, and especially on vision. Attempts to provide dietary recommendations for carotenoids have recently been made [34]. Considering the continuous loss in visual function often observed in some of these patients [12,35–38], it is reasonable to suspect that carotenoid deficiency could participate in patient visual complication, raising the issue of potential benefit from appropriate carotenoid supplementation. It was reported that both HDL and LDL could deliver carotenoids to retinal cells *in vitro* [39]. However *in vivo,* Connor and colleagues reported that HDL was the prime transporter of carotenoids to the retina in normal chickens compared to mutant chickens with HDL deficiency [40], which was recently confirmed in transgenic mice [41]. Patients with hypobetalipoproteinemia disorders presenting low HDL cholesterol, this may aggravate retinal carotenoid deficiency. It thus seems urgent to develop studies to evaluate the correlation between carotenoid status and visual function in older patients, and if such carotenoid supplementation could prevent or improve retinopathy in

FHBL patients.

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Disclosure

Declarations of interest: none

Author contribution:

Claire Bordat: Formal analysis, Investigation, Visualization, Writing – Original draft;

Charlotte Cuerq: Investigation, Resources, Writing – Original draft;

Charlotte Halim: Methodology, Validation, Investigation;

Donato Vairo: Methodology, Validation, Investigation;

Emilie Blond: Resources;

Liora Restier: Supervision;

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Noël Peretti: Conceptualization, Methodology, Resources, Writing – Original draft, Writing – Review and Editing, Supervision; Project administration; Funding Acquisition;

Emmanuelle Reboul: Conceptualization, Methodology, Resources, Writing – Original draft, Writing – Review and Editing, Visualization; Supervision; Project administration; Funding Acquisition.

All authors have approved the final article.

I-assisted Technologies Statement: AI has not been used in the writing process.

Ethical Statement: Samples were from a study registered at clinicaltrials.gov (NCT01457690). During the follow-up of patients, ophthalmic function was evaluated with visual acuity, color vision, fundus, and visual evoked potentials as well as electroretinograms were obtained following ophthalmology consults. The study protocol was approved by the local ethics committee (March 2011, Hospices Civils de Lyon) and the French National Agency for Medicines and Health Products (A110125-36). It was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Informed consent to participate in the study was obtained from all patients or from their parents.

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Table legend

Table 1: d : day ; m : month ; Nal: normal result; NM : non-measurable ; w : week. *fundus, visual acuity, color vision. Chol = Total cholesterol.

Figure legend

Figure 1. α-carotene (A), β-carotene (B), lutein (C) and zeaxanthin (D) basolateral content after carotenoid micellar delivery. Cells were cultured on inserts for 21 days and were incubated with mixed micelles containing α-carotene (A), β-carotene (B), lutein (C) and zeaxanthin (D) for 6 hours. Carotenoid levels were measured by HPLC after liquid–liquid extraction with hexane. Data are expressed as means ± SEM (n = 4). For all figures, asterisks indicate a statistically significant difference in carotenoid concentrations between control and KO clones (* $p < 0.0001$). Values below the threshold of detectability were considered as zero for the calculation of means and statistical significance.

Figure 2

α-carotene (A), β-carotene (B), lutein (C), zeaxanthin (D), β-cryptoxanthin (E) and lycopene (F) plasma levels. Three patients with abetalipoproteinemia (FHBL-SD1) and five patients with chylomicron retention disease (FHBL-SD3) constitute the patient groups (n = 8). Control group is constituted by 4 individuals (n = 4). For all figures, asterisks indicate a statistically significant difference in carotenoid concentrations between control and KO clones (* $p < 0.05$, *** $p < 0.0001$). Values below the threshold of detectability were considered as zero for the calculation of means and statistical significance.