Carotenoids, mostly the xanthophylls, exchange between plasma lipoproteins

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Abstract

Carotenoids are exclusively transported by lipoproteins and in vitro studies suggest that they might protect these particles against oxidation. Yet, little is known on the factors that govern the distribution of these micronutrients among lipoproteins. The objective was to assess whether carotenoids exchange between lipoproteinsand the factors involved. In a first experiment different group of trouts were fed for 5 days with either a carotenoid-free diet or diets containing a single 80 mg pure carotenoid/kg feed. Lipoproteins were separated by ultracentrifugation and carotenoid-rich HDL were incubated for 2 h at 37°C with carotenoid-free VLDL, and *vice versa*. After incubation, lipoproteins were re-separated and carotenoids were quantified to measure the transfer. The same experiments were done in the presence of cholesteryl ester transfer protein (CETP) and lecithin cholesterol acyltransferase (LCAT) inhibitors. In a second experiment the exchange was measured between human VLDL and HDL. In trout, incubation of carotenoid-rich HDL with carotenoid-free VLDL resulted in the appearance of carotenoids in VLDL, and inversely. The higher the hydrophobicity of a carotenoid, the lower its proportion in HDL after incubation. CETP and LCAT inhibitors significantly increased the proportion of carotenoids in HDL after incubation. Results obtained with human lipoproteins showed that the xanthophyll lutein transferred between lipoproteins, but could not show any carotenes (α-carotene, β-carotene and lycopene) transfer. We conclude that carotenoids, chiefly the xanthophylls, exchange between lipoproteins. The transfer depends on plasma factor(s) sensitive to CETP and/or LCAT inhibitors.

Introduction

Epidemiological studies consistently associate diets rich in fruit and vegetables with a reduced risk of chronic diseases [1]. Among the components suspected to explain this effect, the lipid-soluble plant pigment carotenoids, whose antioxidant properties have been clearly demonstrated [2], are likely to play an important role [3-7].

Carotenoids are transported in human plasma exclusively by lipoproteins [8, 9]. Chylomicrons are responsible for the transport of carotenoids from the intestinal mucosa to the bloodstream, via the lymphatics, and then to the liver. VLDL and LDL are apparently responsible for the transport of carotenoids from the liver to peripheral tissues. The role of HDL in carotenoid transport is unknown. The relative distribution of carotenoids among lipoproteins has been reported [10, 11]. Hydrocarbon carotenoids (α-carotene, β-carotene and lycopene), are mainly recovered in LDL (58-73%), monohydroxy carotenoids (mainly β-cryptoxanthin) are distributed almost equally between LDL and HDL (about 40% in each class), and dihydroxy carotenoids (lutein and zeaxanthin), are found predominantly in HDL (53%). These micronutrients might participate in the protection of lipoproteins against oxidation [12-16], and thus might prevent the development of atherosclerosis [6, 17-21].

The transfer/exchange of lipids and vitamin E between lipoproteins has been extensively studied [22-24]. On the contrary the exchange of carotenoids among lipoproteins has not been investigated in depth. Although postprandial studies have suggested that some β-carotene can be transferred from chylomicrons to HDL [25], only three studies have been dedicated to study the transfer of carotenoids between other lipoprotein fractions. One has been done with insect lipophorins [26], one with

unilamellar vesicles as model of lipoproteins [27], and one with human lipoproteins [10]. The results were contradictory. Indeed, the team of Maekawa [26] found a transfer, while the two other teams did not. These different results, which probably depend on the model used, do not lead to any definite conclusion on the ability of carotenoids to exchange between human lipoproteins. Thus we have decided to re-assess this very important aspect of carotenoid transport.

Materials, Subjects and Methods

Animals and diets

A total of 80 immature rainbow trouts (*Oncorhynchus mykiss* Walbaum) of both sexes, with a mean weight of 200g, were used as experimental animals. The fishes were obtained from the INRA experimental fish farm of Donzacq (France), and kept in freshwater tanks at 17 ± 1°C during the experimental period. The following protocol complied with the Guide for the Care and Use of Laboratory animals. After an adaptation period (15 days), while fishes received a commercial fish diet (Trouw, Fontaine les Vervins, France), 6 groups of 10 trouts, were fed diets containing similar amount (80 mg carotenoid/kg feed) of single pure (>95%) carotenoids during 5 days. The carotenoids were either all-trans astaxanthin, or canthaxanthin, or zeaxanthin, or β-cryptoxanthin, or β-carotene, or lycopene (Roche Vitamines France, Neuilly-sur-Seine, France). During the same period, a group (20 trouts) received the same diet free of carotenoids. Feed distribution was withheld from fishes19 hours before they were killed by stunning. Blood was withdrawn in the caudal vein and plasma was immediately prepared on EDTA (Sigma, Saint Louis, USA) by centrifugation (910 g, 4°C, 15 min). After centrifugation, 10 µl/mL of a solution of sucrose (60%) and EDTA (10%) was added to preserve plasma lipoproteins. The plasma was stored under nitrogen at –20 °C until analysis.

Isolation of trout lipoproteins

Trout lipoproteins were isolated by ultracentrifugation (Kontron T2070, Zurich, Switzerland) (200000g for 22 hours, at 15°C) in a Swinging TST 41.14 rotor [28].

Discontinuous six-step density gradients were prepared with NaBr solutions (Sigma, Saint Louis, USA). Successive densities were, from the top to the bottom of the tube : 1.006 g/mL (1.1 mL) ; 1.019 g/mL (2.5 mL) ; 1.063 g/mL (3.5 mL) ; 1.210 g/mL (2.5 mL) ; 1.310 g/mL (1 mL plasma adjusted to this density with solid NaBr) and 1.386 g/mL (0.8 mL). Each density solution was controlled with a density meter (Digital Density Meter DMA 40, Anton Paar, Austria). After ultracentrifugation, lipoprotein fractions were removed; 1.7 mL for VLDL ($d<1.015$ g/mL); 2.1 mL for LDL ($1.015<d<1.085$ g/mL); 3.2 mL for HDL ($1.085<d<1.21$ g/mL) and 2.6 mL for Vitellogenin ($1.21<d<1.31$ g/mL). Immediately after the separation, VLDL and HDL were used to study the transfer of carotenoids (see below). The other lipoprotein fractions were stored under nitrogen, at –80°C, until analysis.

Humans

In order to study the transfer of carotenoids between human lipoproteins, we recruited two young ($18 \text{ y} < \text{age} < 35 \text{ y}$), non obese ($BMI<30$) male volunteers. These volunteers were chosen among a sample of 79 subjects who were enrolled in a previous study [29]. One had a relatively high plasma carotenoid status (sum of β-carotene + lycopene + lutein + zeaxanthin + β-cryptoxanthin + α-carotene = 2.5 μmol/L), the other had a relatively low plasma carotenoid status (sum = 0.8 μmol/L). Blood withdrawal was approved by the regional committee on human experimentation of the regional university hospital in Clermont-Ferrand (France). The two volunteers, who gave informed consent, were apparently healthy according to clinical examination and disease history. Their fasting plasma lipid parameters (total cholesterol and total

triacylglycerols) were in the normal range, suggesting that they had a normal lipid metabolism.

Isolation of human lipoproteins.

Human lipoproteins were separated by using a discontinuous KBr gradient (Sigma, Saint Louis, USA), subjected to ultracentrifugation (200 000g for 24 hours, at 10°C) in a Swinging TST 41.14 rotor [30, 31]. The successive densities of the discontinuous six-step density gradient were, from the top to the bottom of the tube : 1.006 g/mL (1 mL) ; 1.019 g/mL (2.5 mL) ; 1.040 g/mL (2.5 mL) ; 1.063 g/mL (2.5 mL) ; 1.21 g/mL (2 mL plasma adjusted to this density with solid KBr) and 1.34 g/mL (1 mL). After ultracentrifugation, lipoprotein fractions were removed; 2.5 mL for VLDL ($0.96<d<1.006$ g/mL), 5 mL for LDL ($1.03<d<1.063$ g/mL) and 2.5mL for HDL ($1.063<d<1.21$ g/mL). Immediately after the separation, VLDL and HDL were used to study the transfer of carotenoids (see below). The others lipoprotein fractions were stored at – 80°C, under nitrogen, until analysis.

Purity of lipoprotein fractions

The purity of the lipoprotein fractions was checked by using polyacrylamide gel electrophoresis (method adapted from Babin [32]). Gels were made with a stacking gel at 2.5 % (pH 6.8). The running gel was a linear gradient of 2.5-16% polyacrylamide adjusted to pH 8.8 with 1M tris(hydroxy-methyl)aminomethane (Tris) buffer. An aliquot of lipoproteins (which provided 35 to 40 µg proteins) was applied to each well and electrophoresis was carried out at 150 V for 10 h in a Tris/borate buffer (0.09M Tris / 0.08 M Boric acid / 3mM EDTA: 3mM NaN_3, pH 8.35). After migration, lipoproteins were revealed with Coomassie Blue R250 (0.025%) (Sigma, Saint Louis, USA). Results

showed that lipoprotein fractions were apparently pure (no detectable LDL and HDL band in the VLDL fraction and no detectable VLDL and LDL band in the HDL fraction).

Protocol to study the transfer of carotenoids between lipoproteins

The transfer was studied by incubating carotenoid-rich lipoproteins (either HDL or VLDL) with carotenoid-poor lipoproteins (either VLDL or HDL). Carotenoid-rich lipoproteins were either lipoproteins coming from trouts who were fed diets containing carotenoids, or lipoproteins coming from the human subject with a high plasma carotenoid status. Carotenoid-poor lipoproteins were either lipoproteins coming from trouts who were fed diets without carotenoids, or lipoproteins coming from the human subject with a low plasma carotenoid status. Immediately after separation of lipoproteins, 550 µL carotenoid-rich VLDL and 550 µL carotenoid-poor HDL (and inversely) were incubated together in the dark, and stirred at 37°C, under nitrogen, for 2h in presence of antioxidants: ascorbic acid (200 mmol/L) (Prolabo, Lyon, France) and pyrogallol (200 mmol/L) (Sigma, Saint Quentin Fallavier, France). A control experiment had previously shown that this combination of antioxidants preserve > 90% carotenoids during incubation. The same experiments were done with LCAT (lecithin cholesterol acyltransferase) inhibitors [33]: 5,5’ dithio-bis (2-nitrobenzoïc acid) (2 mmol/L) and p-chloromercuriphenyl sulfonic acid (2 mmol/L) (Sigma, Saint Quentin Fallavier, France) and a CETP (cholesteryl ester transfer protein) inhibitor [34]: N,N-dimethylsphingosine (0.1 mmol/L) (Sigma, Saint Quentin Fallavier, France). After incubation, VLDL and HDL were reseparated by ultracentrifugation as described above. Carotenoids were measured in the lipoprotein fractions before and after incubation.

Carotenoid measurement

Plasma and lipoprotein carotenoids were extracted twice with ethanol and hexane, sample/ethanol/hexane (1:1:2, by vol). Echinenone (Roche Vitamines France, Neuilly-sur-Seine, France) was used as internal standard. Carotenoids were quantified by reverse-phase HPLC on a Kontron apparatus (Zurich, Switzerland) with detection at 450 nm. Carotenoids were separated using two columns set in series [29]: a Nucleosyl C18, 150 × 4.6 mm, 3 µm followed by a Vydac C18, 250 × 4.6 nm (Interchim, Montluçon, France). The mobile phase was a mixture of acetonitrile/methanol/dichloromethane/water (70:15:10:5, by vol). Quantification was conducted using the Kontron MT 2 software, by comparing peak areas to those of standard solutions of carotenoids. Echinenone allowed to calculate and overall recovery yield 75-100%.

Statistical analysis

Results are expressed as means ± SEM. The repartition of the different carotenoids between trout VLDL and HDL after incubation was compared by analysis of variance (ANOVA). When a significant difference was detected ($P<0.05$), means were compared using the post-hoc Tukey / Kramer's test. The repartition of carotenoids between human VLDL and HDL before and after incubation was compared by the Student's t-test ($P<0.05$).

Results

Distribution of carotenoids among trout and human lipoproteins

The distribution of carotenoids among trout and human lipoproteins is shown in Figure 1. Note that after supplementation with 80 mg lycopene/kg feed for 5 days, no lycopene was detected in trout lipoproteins. In the trout, the higher proportion of carotenoids was found in the HDL fraction, whereas in humans it was found in the LDL fraction. There was a relationship between the percentage of carotenoids in certain lipoprotein classes and carotenoid hydrophobicity. Indeed, the higher the hydrophobicity of a carotenoid (as estimated by the octanol-water partition coefficient of the molecule: Log P [35]), the lower was its percentage in the HDL fraction (r^2=0.742, *P*=0.061 for trout HDL and r^2=0.957, *P*=0.001 for human HDL). There was also a strong relationship between the percentage of carotenoids in human LDL and carotenoid hydrophobicity (r^2=0.970, *P*=0.001).

Exchange of carotenoids between trout lipoproteins

Figure 2 shows the distribution of carotenoids among trout lipoproteins after incubation of a lipoprotein class rich in carotenoids (either VLDL or HDL) with a lipoprotein class free of carotenoids (either HDL or VLDL). Note that the repartitions obtained after incubation of carotenoid-rich VLDL with HDL which contained no carotenoids were the same than those obtained after incubation of carotenoid-rich HDL with VLDL which contained no carotenoids. There was no relationship between the percentage of carotenoids found in VLDL or HDL after incubation, and either the molecular weight or the melting point of carotenoids. Conversely, there was a high significant relationship (r^2=0.939, *P*=0.007) between the percentage of carotenoid found

in VLDL or HDL and carotenoid hydrophobicity (Log P). The percentage of astaxanthin in VLDL was significantly ($P < 0.05$) lower than that of the other carotenoids. On the other hand, the percentage of canthaxanthin and zeaxanthin in VLDL were not significantly different, but they were significantly lower than that of β-cryptoxanthin and β-carotene. Finally, no significant difference was found between the distribution of β-cryptoxanthin and β-carotene among VLDL and HDL after incubation.

Effect of CETP and LCAT inhibitors on the transfer of carotenoids between trout lipoproteins

The exchange of carotenoids between trout VLDL and HDL, in presence of CETP and LCAT inhibitors, was studied for three carotenoids: astaxanthin, zeaxanthin and β-carotene (Figure 3). The presence of inhibitors changed the repartition of carotenoids between VLDL and HDL after incubation. The inhibitors reduced the percentage of carotenoids recovered in the VLDL fraction and consequently increased the percentage of carotenoids in the HDL fraction (compare Figure 2 and Figure 3). In the presence of LCAT and CETP inhibitors astaxanthin and zeaxanthin were recovered almost exclusively in HDL, while a significant proportion of β-carotene (27%) was recovered in VLDL.

Exchange of carotenoids between human lipoproteins

Figure 4 shows the repartition of several carotenoids between human carotenoid-rich HDL and carotenoid-poor VLDL before (left column) and after (right column) incubation of the two lipoprotein classes with each other for 2 h at 37°C. There was a significantly higher proportion of lutein in VLDL after incubation (+12%). There was a

1 higher proportion of the other carotenoids in VLDL after incubation (around 4%
2 increase), but this was not significant.
3 Figure 5 shows the results obtained when carotenoid-rich VLDL were incubated
4 with carotenoid-poor HDL. As observed in Figure 4, there was a transfer of lutein from
5 the lutein-richest lipoprotein (VLDL in that case) to the lutein-poorest lipoprotein
6 (HDL). The percentage transferred was equivalent to that observed in Figure 4 (+14%),
7 but it was not significant due to the higher variability of the measurements.
8

Discussion

The choice of trout lipoproteins as a model to study the transfer of carotenoids between lipoproteins was dictated by four reasons. First, as we wanted to be able to detect a transfer, even very low, and as we wanted to compare the transfer of different carotenoids without possible interactions between carotenoids [36], it was essential to use acceptor lipoproteins without carotenoids and donor lipoproteins with only one class of carotenoid. Such lipoproteins cannot be obtained in humans. Indeed, this would have required the consumption of fruit and vegetable free diets for several weeks/months, followed by the consumption of purified carotenoids for several weeks. Second, we wanted to test a variety of carotenoids to assess whether there is a relationship between characteristic physical properties of carotenoids and their transfer. This was also not feasible in humans because not all carotenoids are allowed as diet additives. Third, we wanted to work with lipoproteins that had naturally incorporated carotenoids. Thus we did not want to incorporate carotenoids by using organic solvents. Indeed, it is highly probable that such an incorporation would not lead to a natural distribution of carotenoids between the core and the surface of lipoproteins [37]. Finally, despite the large evolutionary distance between trout and human, major elements of the structure of their plasma lipoproteins are shared [38, 39] and trout, as human, displayed substantial CETP and phospholipid transfer protein (PLTP) activities [23].

The results obtained with trout lipoproteins demonstrate for the first time that a carotene (β-carotene) as well as several xanthophylls (astaxanthin, canthaxanthin, lutein, and β-cryptoxanthin) can transfer between vertebrate lipoproteins. They also demonstrate that the transfer is bi-directional, from HDL to VLDL, and *vice versa*. The

fact that the proportion of carotenoid found in each lipoprotein class after exchange was similar suggests that there was an apparent equilibrium after 2 h incubation, and thus that all the carotenoids were transferred within the 2 h. The fact that the repartition of carotenoid after exchange was related to carotenoid hydrophobicity suggests that carotenoids distribute as a function of their relative solubility in the different lipid classes which constitute the lipoproteins. This hypothesis elegantly explain the relationship observed between carotenoid hydrophobicity and carotenoid proportion in trout and human HDL and in human LDL (see Figure 1 results).

Results obtained with human lipoproteins demonstrated a transfer for the xanthophyll (lutein) but not for the carotenes (α-carotene, β-carotene and lycopene). This result is in concordance with those obtained in the trout which showed that the xanthophylls were better transferred than the carotenes. This difference between the xanthophylls and the carotenes, with regard to the transfer, also explains the discrepancy between a result found with an insect model [26], i.e. a transfer of carotenoid was measured, and a result found with an unilamellar vesicle model [27], i.e. no transfer of carotenoid was detected. Indeed, in the insect model most (>90%) carotenoids were lutein, while in the unilamellar vesicle model the carotenoid studied was β-carotene. The fact that a significant transfer of the carotenes could not be significantly measured with human lipoproteins can be explained by three hypotheses. First, it is possible that in humans the carotenes do not transfer between lipoproteins. However, the demonstrated exchange of β-carotene between trout lipoproteins, which have a close lipid composition to that of human [38, 39], suggests that this is probably not the case. Another explanation could be the difference between trout and human with regard to PLTP activity. Indeed, if PLTP is involved in the transfer of carotenoids, its lower

activity in human than in trout [23], and the lower transfer of the carotenes as compared to the xanthophylls, might explain that it is more difficult to detect a transfer of the carotenes in humans. Finlly, the fact that we were able to detect a transfer of lutein between human lipoproteins while Romanchik et al [10] did not, can be explained by the fact that these authors have incubated lipoproteins which came from the same fasted volunteers. Indeed, carotenoids were probably already distributed in equilibrium between lipoproteins before incubation and therefore it is logical that they did not transfer.

The transfer of carotenoids between lipoproteins raises the question on the role of the plasma proteins involved in lipid metabolism (CETP, PLTP, LCAT) on this phenomenon. In fact, the protocol used to study the transfer could not differentiate between spontaneous transfer and protein mediated transfer. Indeed some proteins involved in lipid metabolism could have remained associated with lipoproteins. We have therefore performed an additional experiment with CETP and LCAT inhibitors in order to check whether these proteins are involved in carotenoid transfer. We used CETP inhibitors because we hypothesized that this enzyme that exchanges cholesterol esters between lipoproteins might exchange carotenoids as well. We used LCAT inhibitors because we hypothesized that modification of lipoproteins lipid metabolism might indirectly affect the transfer of the carotenoids by changing the lipid composition of lipoproteins. Addition of the inhibitors to the incubation medium led to a higher proportion of carotenoids in trout HDL after exchange. To explain this finding we suggest that a factor which was responsible for the transfer from HDL to VLDL, but not from VLDL to HDL, was partially impaired. Thus a factor sensitive to LCAT and/or to CETP inhibitors is directly or indirectly involved in the transfer from HDL to VLDL. Note that we state « directly or indirectly », because it is possible that carotenoids

simply follow the fate of certain lipid classes. Thus the fact that the exchange of lipids was probably affected (due to the presence of lipid transfer protein inhibitors), may have led to indirectly affect the distribution of carotenoids. It is also possible that the inhibitors had inhibited a protein which is specifically responsible for the transfer of carotenoids between lipoproteins.

In conclusion, our study demonstrates that carotenoids, chiefly the xanthophylls, exchange between lipoproteins. It shows that, at equilibrium, the repartition of carotenoids between lipoproteins is related to carotenoid hydrophobicity. It also suggests that the exchange depends on factor(s) sensitive to inhibitors of LCAT and/or CETP.

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Figures

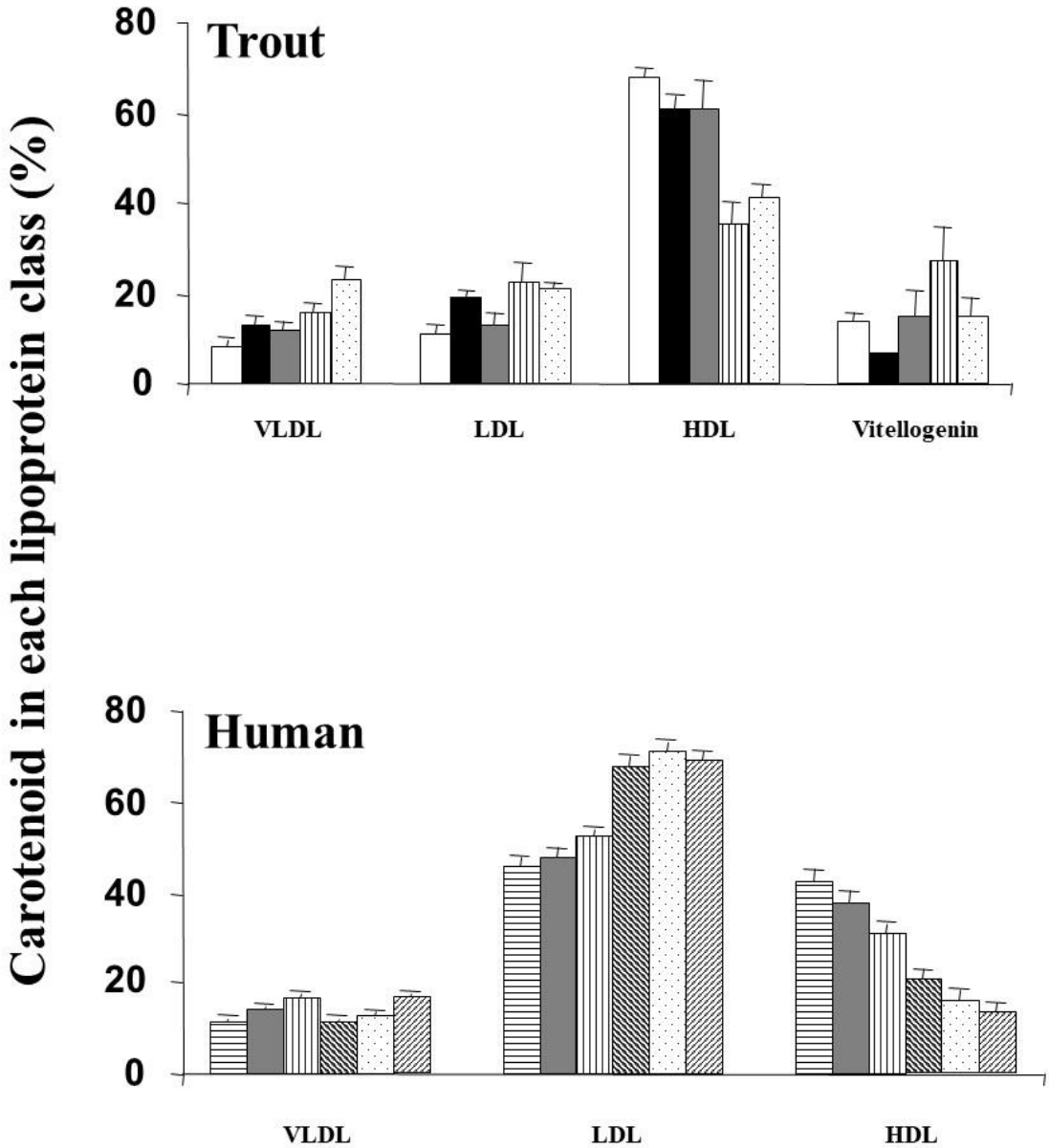
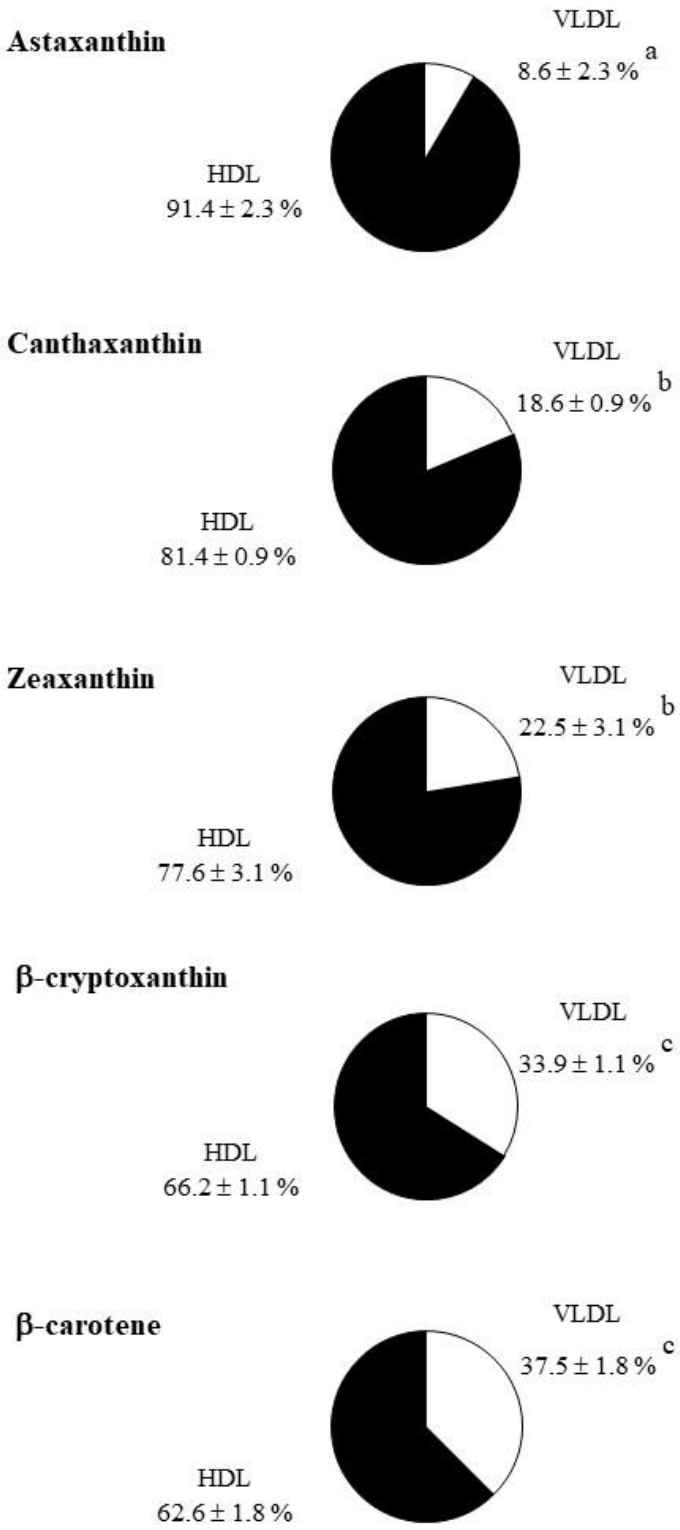


Figure 1: Distribution of carotenoids among trout and human lipoproteins. Values represent the percentage of carotenoid found in each lipoprotein fraction, relative to the amounts recovered in all lipoprotein fractions. Means ± SEM.

□ astaxanthin, ■ canthaxanthin, ▤ lutein, ▩ zeaxanthin, ▥ β-cryptoxanthin, ▨ α-carotene, ⁘ β-carotene, ▧ lycopene.



1

2 **Figure 2**: Distribution of carotenoids among trout VLDL □ and HDL ■ after

3 incubation of carotenoid-rich VLDL with HDL which contained no carotenoids for 2 h

at 37°C, and *vice versa*. For a better clarity, the distribution before incubation are not shown, but remember that 100% carotenoids were either in VLDL or in HDL. Values represent the percentage of carotenoid found in each lipoprotein class. Note that, because the repartitions obtained after incubation of carotenoid-rich VLDL with HDL which contained no carotenoids were the same than those obtained after incubation of carotenoid-rich HDL with VLDL which contained no carotenoids, the values obtained after the two incubations have been pooled. Thus values represent mean ± SEM of 10 measurements. A factorial ANOVA showed a significant effect ($P<0.05$) of the type of carotenoids on the repartition after incubation. A post-hoc Tukey / Kramer's test was used to compare the means (Different letters indicate significant differences).

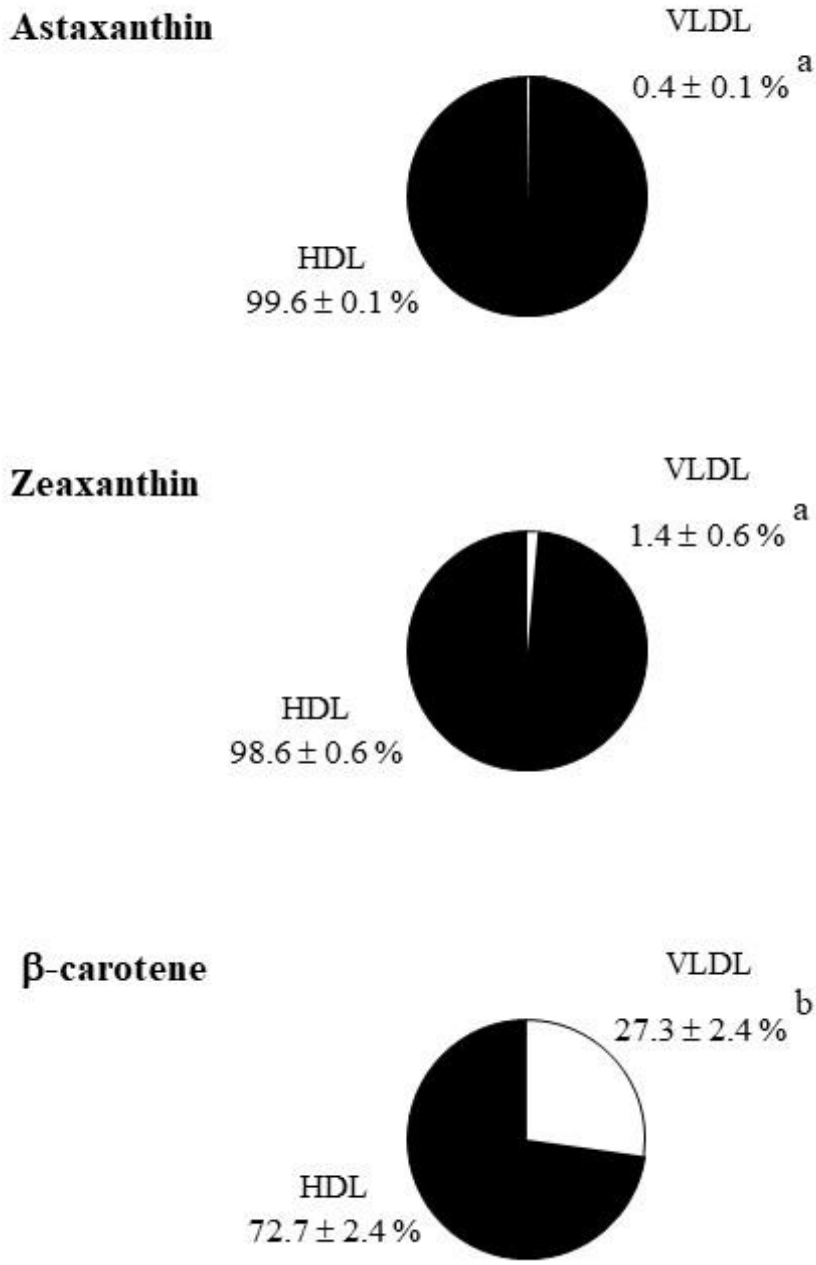
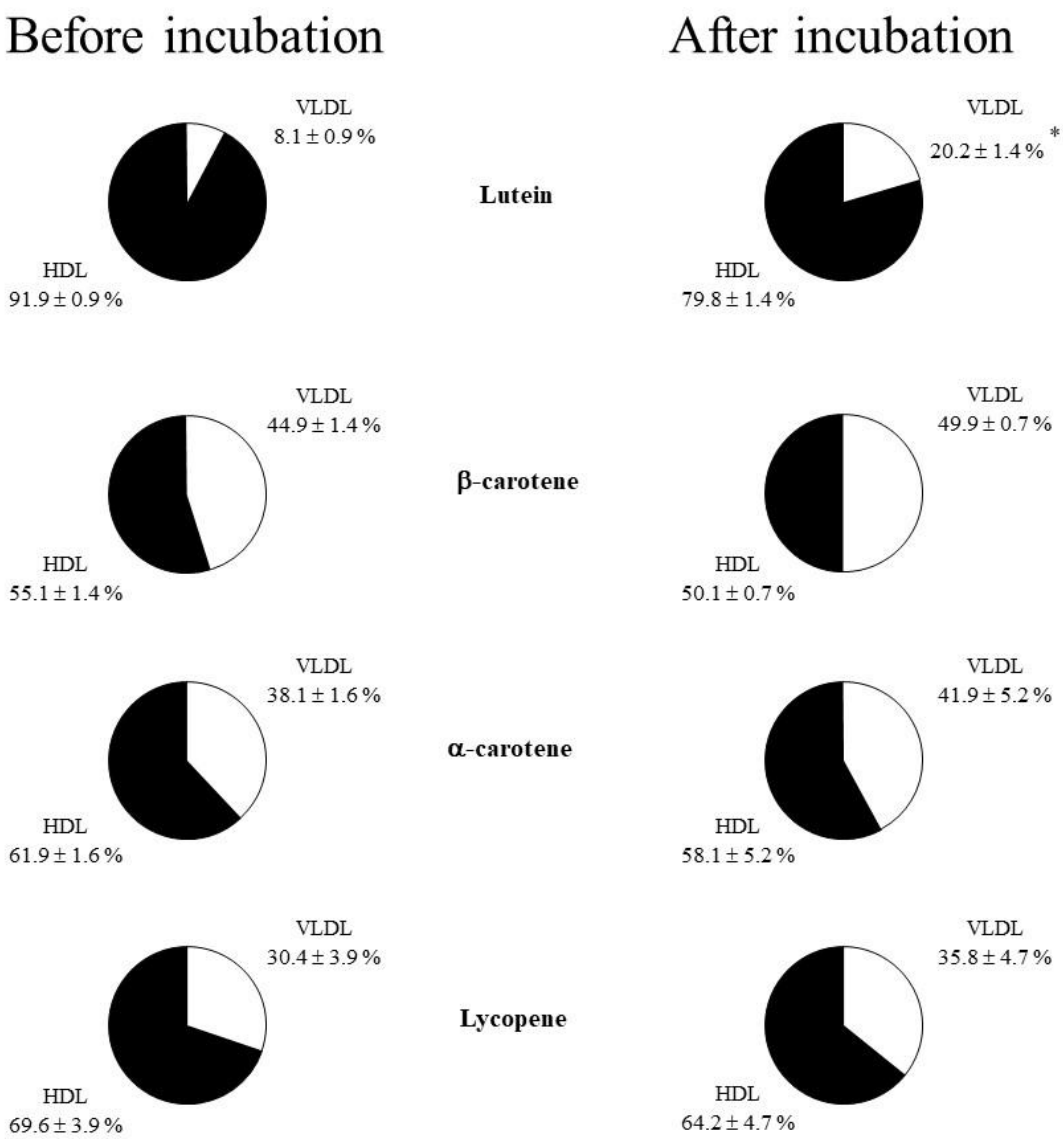


Figure 3: Distribution of carotenoids among trout VLDL □ and HDL ■ after incubation of carotenoid-rich VLDL with carotenoid-free HDL, and *vice versa*, in presence of CETP and LCAT inhibitors. For more informations see Figure 2 legend.



1

2 **Figure 4:** Exchange of carotenoids among human carotenoid-poor VLDL□ and
3 carotenoid-rich HDL■. Values represent the percentage of carotenoid found in each
4 lipoprotein class, relative to the amount recovered in the two classes. Left column:
5 repartition of carotenoids before incubation. Right column: repartition of carotenoids in
6 the same lipoproteins after incubation 2 h at 37°C. Means ± SEM, n=5. An asterisk
7 indicates that there was a significant difference in the repartition of a carotenoid before
8 and after incubation (Student's t-test, $P<0.05$).

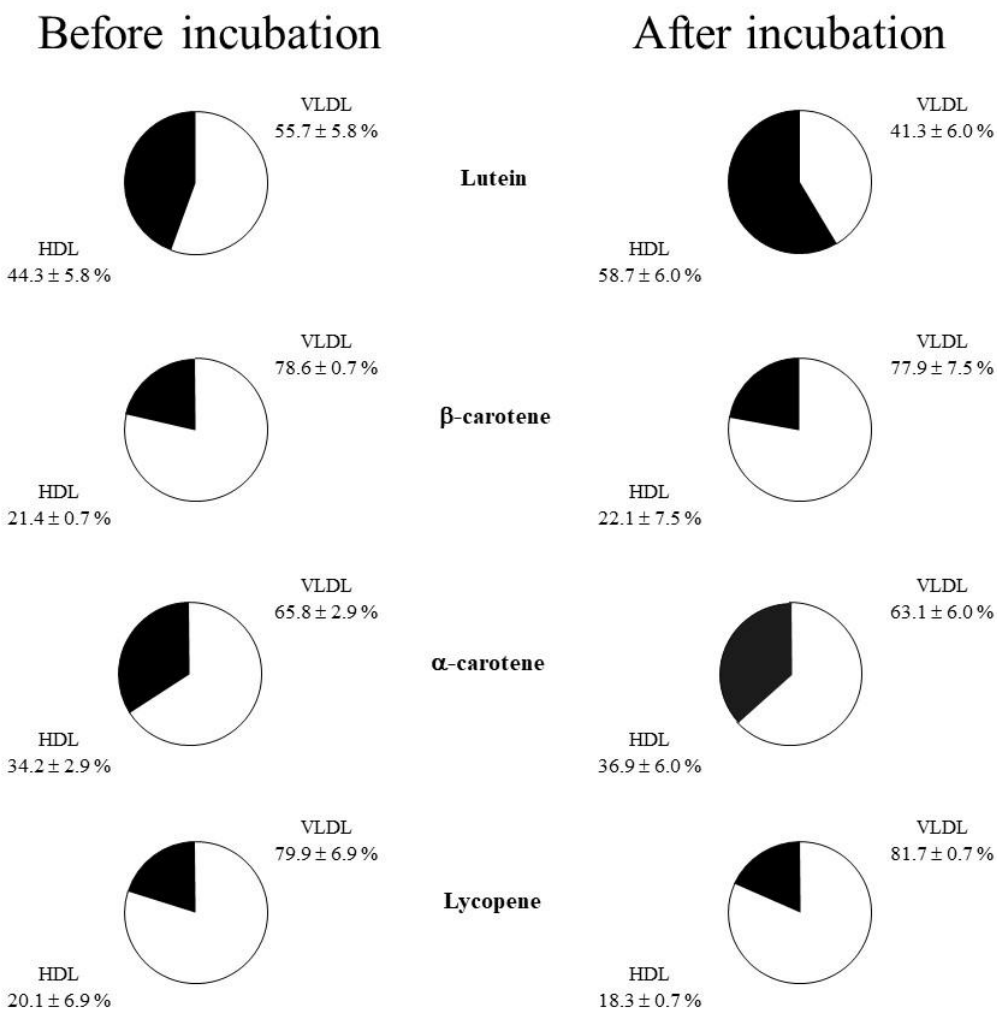


Figure 5: Exchange of carotenoids between human carotenoid-rich VLDL□ and human carotenoid-poor HDL ■ .Values represent the percentage of carotenoid found in each lipoprotein class, relative to the amount recovered in the two classes. Left column: repartition of carotenoids before incubation. Right column: repartition of carotenoids in the same lipoproteins after incubation 2 h at 37°C. Means ± SEM, n=5. No significant difference in the repartition of carotenoids before and after incubation was found (Student's t-test, $P<0.05$).

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Abbreviations

CETP: cholesteryl ester transfer protein

LCAT: lecithin cholesterol acyltransferase

PLTP: phospholipid transfer protein