Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins

Maria González-Santiago, Juristo Fonollá, Eduardo Lopez-Huertas

Abstract

There is growing interest in the health effects of olive oil polyphenols, particularly hydroxytyrosol (HT), for their potential application in the treatment of inflammatory conditions such as cardiovascular disease (CVD). As oxidative modification of low-density lipoproteins (LDL) plays a central role in the development of CVD, natural antioxidants are a main target for the nutraceutical industry. In this study we firstly investigated the absorption of pure hydroxytyrosol (99.5%) administered as a supplement in an aqueous solution (2.5 mg/kg BW) in the plasma and urine of healthy volunteers (n = 10).

Plasma Cmax for HT and homovanillic alcohol (HvOH) were detected at 13.0 ± 1.5 and 16.7 ± 2.4 min, respectively. The HT and HvOH levels were undetectable 2-h after the administration. HT, HvOH, homovanillic acid and 3,4-dihydroxyphenylacetic acid were found as free forms (44%) or as glucuronide (34.4%) or sulphate (21.2%) conjugates in the 24-h urine samples of the subjects.

In a second phase of the study, the same amounts of HT were administered to the subjects and the presence of HT in purified plasma lipoproteins was investigated in LDL fractions freshly isolated. 10 min after the ingestion of the HT supplement, more than 50% of the total amount detected was present in the LDL-purified fractions and its concentration declined in accordance with its presence in plasma but no changes were found in total antioxidant capacity, malondialdehyde or LDL lag time. These results indicate that pure HT transiently associates with LDL lipoproteins in vivo.

1. Introduction

There is strong evidence for an association between the Mediterranean dietary pattern and protection from cardiovascular disease where olive oil, high in monounsaturated fatty acids, constitutes the main source of fat [1]. In addition to oleic acid, virgin olive oil contains a wide range of “minor constituents”, like polyphenols, that contribute to the stability of the oil and exhibit potent antioxidant properties [2,3].

Several lines of evidence coming from human studies have suggested beneficial effects of phenolic compounds present in olive oil, including antioxidant effects in plasma [4–6], in the LDL [7], antithrombotic effects [5,8], increased HDL-cholesterol [7] and inhibition of platelet aggregation [9,10].

Whilst most human studies describing cardiovascular effects of olive oil polyphenols have been carried out with nutritional doses administered together with olive oil [11–14], less attention has been paid to its effects when administered in aqueous solutions or other food matrices, with a few exceptions [8,15]. Furthermore, the absorption profile and the effects of individual olive oil polyphenols have never been investigated in humans.

Hydroxytyrosol (3,4-dihydroxyphenyl-ethanol, HT) is one of the main components of virgin olive oil and olive mill waste [16] that has demonstrated the strongest radical-scavenging properties in vitro among all the olive oil polyphenols [4]. In a previous study carried out in our laboratory, we showed that supplementation with 4 mg/kg of purified HT improved blood lipids, the antioxidant status and reduced the size of atherosclerotic lesions in a rabbit model of diet-induced atherosclerosis [17]. These results suggested that HT could be a natural antioxidant with a possible role in the prevention of cardiovascular disease. We purified large amounts of HT from olive mill waste aimed to produce a water-soluble pharma.
colological formulation to be further studied in intervention trials. In this paper we report, for the first time, the absorption profile in humans of highly purified HT administered as a supplement in an aqueous solution, without the intervention of other nutrients. We also investigated the interactions of HT with plasma lipoproteins.

2. Subjects and methods

2.1. Subjects

Ten healthy volunteers (8 men and 2 women) who were resident in Granada (Spain) participated in the study after giving a written informed consent. The subjects were given a physical examination and their medical history was consulted before they were included in the study. The subjects had a mean age of 25.3 years (range: 22–34 years), a mean body mass index of 24.9 kg/m² (range: 18.3–28.2 kg/m²), fasting plasma triglyceride concentrations <2.2 mmol/L and total cholesterol <5.2 mmol/L. The subjects were not suffering from any chronic or metabolic disease, had no medical history of gastrointestinal, liver or kidney disease and were not taking any medication or antioxidant supplement. The study was conducted according to the Helsinki Declaration, the protocol was approved by the ethical committee of Puleva Biotech SA (Granada, Spain).

2.2. Study design

The volunteers were instructed not to consume olive oil, olives or olive-derived products and to refrain from alcohol consumption for at least 14 days prior to the beginning of the study. The study was divided into two phases separated by a 1-month wash out. In phase 1, the absorption of HT was investigated. The HT supplement was administered as a single oral dose of 2.5 mg of HT per kg of body weight in an aqueous solution, after an overnight fast lasting 10 h at least. Peripheral blood (25 mL) was withdrawn from each volunteer at baseline and at times 10, 20, 30, 40, 50, 60 and 120 min after the oral dose. The blood extractions were made by qualified personnel under medical supervision. 24-h urine was collected in acid-washed containers the day before and on the test day itself. Urine aliquots were obtained and frozen at –80 °C until used. In phase 2, the association of HT with LDL lipoproteins was explored in the same subjects that received an identical dose of the HT solution. In this case, 50 mL of blood was collected at base line and at times 10 and 20 min, plasma was obtained and it was immediately processed to isolate LDL lipoproteins (see below).

2.3. Preparation of HT

The HT administered to the volunteers was obtained and supplied by the Process Engineering Department of Puleva Biotech SA, Granada (Spain). HT was purified from an olive mill wastewater extract using food grade solvents, preparative chromatography and further desiccation. The HT obtained was a white powder soluble in water with a purity of 99.5% (Fig. 1). The HT used for analytical determinations was synthesised by reducing 3,4-dihydroxyphenylacetic acid with LiAlH₄ in tetrahydrofuran under refluxing for 4 h. The reaction product was purified by chromatography on silica gel with CHCl₃:MeOH (7:1) as the eluting solvent. The identity of the HT obtained was verified by ¹H nuclear magnetic resonance and gas chromatography–mass spectrometry (GC–MS). The purity of the HT obtained using this method was 99%. The HT purified or obtained by synthesis was dissolved in sterile saline and stored at −80 °C until use. The concentrations of HT in the aqueous solutions used in the study were measured by high-performance liquid chromatography (HPLC) as described in [18].

2.4. Isolation of plasma and lipoprotein fractions

Blood was withdrawn in EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany). Plasma was obtained by centrifugation at 1600 × g for 10 min at 4 °C, and immediately frozen at −80 °C. For the isolation of lipoproteins, 10 mL of fresh plasma obtained from the volunteers was transferred to ultracentrifuge tubes and the density was adjusted to 1.30 g/mL by addition of solid KBr and continuous agitation. The tubes were then filled up dropwise with a 0.15 M NaCl solution and further centrifuged at 242,000 × g for 2.5 h at 4 °C in a VTi50 rotor, as previously described [19]. 36 fractions of 1 mL were obtained from each gradient tube by injecting a 66% sucrose solution from the bottom using a gradient collector (ISCO, USA) connected to a peristaltic pump. Fractions 1–36 were divided into groups of six and the volumes transferred to fresh Falcon tubes (pools A–F), each containing 6 mL of the pooled fractions, which were not subjected to dialysis. The LDL-containing fractions were pooled into tube C. HT and metabolite determinations in the fraction pools were carried out immediately after to avoid interferences produced by the freeze–thaw cycle. 1 mL from the fraction pools was set aside for lag time determinations and dialysed against PBS at 4 °C in the dark. The tubes were frozen at −80 °C under nitrogen atmosphere until needed. 4 μg of pure HT and 0.6 g of bovine serum albumin dissolved in 10 mL of saline were subjected to the same procedure and used as controls.

2.5. Analytical determinations in gradient fractions and electrophoresis of lipoproteins

The concentrations of triacylglycerols, cholesterol, Apo B and Apo A, total proteins and density were measured in all the gradient fractions and used as markers to identify the sedimented lipoproteins. The concentrations of triacylglycerols and total cholesterol were measured using commercial kits obtained from Biosystems® (Barcelona, Spain). Protein concentration was determined using a bicinchoninic acid kit (Pierce, USA) according to the manufacturer’s instructions. Apolipoprotein B (ApoB) and apolipoprotein A1 (ApoA1) were measured by immuno-turbidimetry kits obtained from Olimpus Diagnostica GmbH (Germany). Agarose gel electrophoresis was used to separate lipoproteins using a kit (Paragon 6®) obtained from Beckman Coulter (CA, US) and following the manufacturer’s instructions.
2.6. Determinations of hydroxytyrosol and metabolites in plasma, urine and lipoprotein samples

Analyses of HT, 4-hydroxy-3-methoxyphenylethanol (homovanillic alcohol, HvOH), 4-hydroxy-3-methoxyphenylethylacetic acid (homovanillic acid, HVA) and 3,4-dihydroxyphenylacetic acid (DHPA) were carried out by GC–MS as previously described in [20] with modifications. A solution of α-naphthol (1 g/L) was used as internal standard. For the analysis of un-conjugated phenols in plasma, i.e. HT, HvOH, HVA and DHPA, 1 μL of internal standard was added to 2 mL of plasma and it was extracted with two volumes of ethyl acetate. The organic phase obtained was transferred to fresh tubes and evaporated under vacuum at 45 °C for 1 h. The dried residue was dissolved with 30 μL of a mixture of bis-trimethylsilyl-trifluoroacetamide:pyridine:ethyl acetate (2:1:2). For the analysis of the fresh undialysed gradient fractions pools A–F, and urine samples, 4 and 2 μL of internal standard were added to 10 mL of pools A–F and 5 mL of urine, respectively, followed by the same extraction procedure. For the analysis of conjugated phenols, plasma and urine samples were pre-incubated with 800 U/mL of β-glucuronidase (Type L-II, Sigma) or 100 U/mL of sulfatase (Type H-I, Sigma) at 37 °C for 20 min before the extraction.

For GC–MS analyses, 2 μL of samples were injected onto a 30 m × 0.25 mm i.d. CP-Sil 8CB column connected to a GC–MS system model 3400, with a MS/MS detector model Saturn 2000 (Varian, USA). The split ratio was 1:10. Ultrapure helium was used as carrier gas at a flow rate of 1 mL/min. The injector temperature was set at 150 °C. The temperature conditions of the oven during sample run were as follows: initial temperature 45 °C for 2 min. Then it was increased to 150 °C at a rate of 20 °C/min and was maintained for 8.25 min. The temperature was further increased to 250 °C at a rate for 10 °C/min until the end of the analysis, which lasted 30 min in total. The temperatures used for the trap, manifold, and transfer line of the detector were 200 °C, 50 °C and 260 °C, respectively. The trimethylsilyl ether derivatives of α-naphthol, HvOH, HT, HVA and DHPA showed single peaks with retention times of 11.3, 12.9, 13.5, 13.6 and 14.1 min, respectively.

The detector operated in the selective ion monitoring mode. Ions at m/z 201 and 216 for α-naphthol, m/z 179, 267 and 370 for HT, m/z 209 and 312 for HvOH, m/z 296, 311 and 326 for HVA and m/z 384 for DHPA were selected for quantitative determinations on the bases of a mass spectra obtained from pure standards. The calibration curves were prepared using basal plasma (2 mL) or urine (5 mL) spiked with α-naphthol (0.4 μg/mL) and increasing amounts of the investigated phenols in the 0.5–10 μg/mL range. Good correlations (r² > 0.98) were obtained between intraday and interday analyses. To ensure analytical consistency, all the samples were analysed in the one batch for a single volunteer and calibration curves for the 4 investigated phenols were obtained before every batch.

2.7. Plasma malondialdehyde, total antioxidant capacity and lag time determinations

The total antioxidant capacity (TAC) was determined as described in [21], using TROLOX as standard. Plasma malondialdehyde (MDA) concentrations were measured using a HPLC separation described in [22] that is based on the thiobarbituric acid reaction and reverse-phase separation with fluorescence detection. For lag time determinations, 50 μg of dialysed LDL in 1 mL PBS were incubated with 10 μM CuSO₄ for several hours at 37 °C. The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm every 10 min. Lag time was determined according to [23].

2.8. Pharmacokinetic calculations

The maximum concentration (Cmax), the time to reach Cmax (tmax), the half-life (t1/2) and the area under the curve (AUC0–2h) were calculated in plasma for both HT and HvOH alcohol using specific software from Microsoft (Excel PK Functions spreadsheet).

2.9. Chemicals and reagents

Homovanillic alcohol, homovanillic acid, 4-dihydroxyphenylethylacetic acid, α-naphthol, β-glucuronidase and arylsulfatase were purchased from Sigma (St. Louis, US). All reagents and solvents were of analytical grade (Merck, Darmstadt, Germany).

2.10. Statistical analyses

All the data are expressed as means ± SEMs. Comparisons between groups at the different time points were assessed by a one-way analysis of variance (ANOVA). When this analysis indicated a significant difference (P<0.05), paired Student’s t-test analyses followed by Bonferroni corrections for multiple comparisons were performed. The data was analysed using SPSS statistical software package (SPSS for Windows 10.1; SPSS Chicago, IL, USA).

3. Results

The administration of the HT solution increased the plasma concentrations of free HT and HvOH in all of the subjects (Fig. 2). The only HT metabolite detected in plasma was HvOH. The pharmacokinetic data calculated from the absorption curves obtained showed large inter-individual differences in the absorption profile (Table 1). The maximal concentration (Cmax) for HT and HvOH were detected at times 13.0 ± 1.5 min and 16.7 ± 2.4 min, respectively. The concentrations of those declined thereafter to reach undetectable levels 1-h after the administration. The estimated half-life for HT was 8.6 ± 0.7 min and for HvOH was 8.65 ± 1.76 min. Bioavailability of HT calculated from the area under the curve in plasma was 6.2 ± 1.1% (range 2.4–11.8%). Estimations over the curve obtained from the accumulated HT and HvOH concentration values showed an absorption of 9.4 ± 1.8% (range 2.4–19.6%). No changes were found in plasma total antioxidant capacity, MDA or LDL lag time after the intake of the HT supplement (not shown).

Twelve different metabolites of HT were detected in the 24-h urine collected from the volunteers: HT, HvOH, HVA and DHPA as free forms (44%), as glucuronide (34.4%) and sulphate (21.2%) conjugates, showing large variability among the subjects (Fig. 3). The main metabolites found in urine were free HVA (31%) and DHPA.
The amounts of HT detected in plasma were similar to the concentrations already obtained in the first phase of the study: 1.1 ± 0.54 μM at 10 min and 0.52 ± 0.14 μM at 20 min. Plasma lipoproteins were separated by density gradient centrifugation. The LDL particles were typically sedimented at a density range of 1.019–1.060 g/mL, whereas VLDL and HDL particles were located in fractions 1–7 at 1.000–1.020 g/mL and fraction 8 (pool C) which coincided with the peaks of Apolipoprotein B and cholesterol, mostly present in LDLs (Fig. 4). VLDL and HDL particles separated well from LDLs as judged by the density values and the markers used to identify lipoproteins in the gradient fractions. VLDL particles were located in fractions 1–7 at the top of the gradient (mainly in pool A) and HDLs were present in fractions 23–38 (pools D and E). Glucurone and methyl glucuronide (22.7%) whereas only 5% was detected as free or conjugated HT. The average urinary recovery of HT, calculated from the accumulated amounts of all the metabolites detected in 24-h urine, was 7.4 ± 3.3% of the total administered HT.

In the second phase of the study, the association of HT with LDL lipoproteins was explored. After the same oral dose of the HT supplement, the plasma from the volunteers was isolated only at times 10 and 20 min as the C_max was obtained between those times. The amounts of HT detected in plasma were similar to the concentrations already obtained in the first phase of the study: 1.1 ± 0.54 μM at 10 min and 0.52 ± 0.14 μM at 20 min. Plasma lipoproteins were separated by density gradient centrifugation. The LDL particles were typically sedimented at a density range of 1.019–1.060 g/mL in gradient fractions 13–18 (pool C) which coincided with the peaks of Apolipoprotein B and cholesterol, mostly present in LDLs (Fig. 4).

**Table 1**

Plasma pharmacokinetic parameters for hydroxytyrosol and homovanillic alcohol after the intake of an aqueous solution containing 2.5 mg/kg of body weight. Panel A, average values as means ± SEMs and range. Panel B, individual values. HT, hydroxytyrosol; HvOH, homovanillic alcohol; BMI, body mass index; nd, not detected.

Panel A

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>C_max (μmol/L)</th>
<th>t_max (min)</th>
<th>t_1/2 (min)</th>
<th>AUC(0–2h) (μmol·min/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT</td>
<td>1.11 ± 0.20 (0.47–2.23)</td>
<td>13.0 ± 1.5 (10–20)</td>
<td>8.60 ± 0.68 (4.78–12.53)</td>
<td>20.3 ± 3.6 (7.6–40.0)</td>
</tr>
<tr>
<td>HvOH</td>
<td>0.49 ± 0.14 (0.00–1.48)</td>
<td>16.7 ± 2.4 (10–30)</td>
<td>8.65 ± 1.76 (2.92–17.76)</td>
<td>10.6 ± 3.5 (0.0–34.78)</td>
</tr>
</tbody>
</table>

Panel B

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Sex</th>
<th>BMI</th>
<th>Metabolite</th>
<th>C_max (μmol/L)</th>
<th>t_max (min)</th>
<th>t_1/2 (min)</th>
<th>AUC(0–2h) (μmol·min/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>30.0</td>
<td>HT</td>
<td>2.24</td>
<td>10</td>
<td>9.0</td>
<td>40.0</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>20.6</td>
<td>HT</td>
<td>0.25</td>
<td>10</td>
<td>4.6</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>22.1</td>
<td>HT</td>
<td>1.91</td>
<td>10</td>
<td>4.8</td>
<td>23.4</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>21.1</td>
<td>HT</td>
<td>0.77</td>
<td>20</td>
<td>12.5</td>
<td>13.3</td>
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<tr>
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<td>M</td>
<td>22.6</td>
<td>HT</td>
<td>1.39</td>
<td>10</td>
<td>8.3</td>
<td>31.1</td>
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<tr>
<td>6</td>
<td>M</td>
<td>20.9</td>
<td>HT</td>
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<td>20</td>
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<tr>
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<td>M</td>
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<td>HT</td>
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<td>10</td>
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<td>1.53</td>
<td>20</td>
<td>7.5</td>
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**Fig. 3.** Average amounts of hydroxytyrosol and metabolic forms (free and conjugated with glucuronic acid and sulphate) in 24-h urine, after the intake of the HT supplement. The data is expressed as means ± SEM and range, in μmol per liter of urine. The amounts of the different forms are also shown above the bars as total percentages. HT, hydroxytyrosol; HvOH, homovanillic alcohol; HVA, homovanillic acid; DHPA, 3,4-dihydroxyphenylacetic.

**Fig. 4.** Hydroxytyrosol is one of the main components of virgin olive oil and olive mill waste with a strong antioxidant potential [16]. The characterisation of the absorption profile of HT was a preliminary step before considering further human intervention trials. The dose used in the study (2.5 mg/kg or 175 mg for a 70 kg subject), is well above the nutritional amounts of HT present in virgin olive oil, but in line with the estimated intake of polyphenols in the diet from foods like coffee (200 mg/day) [24] or 100 g of apples (141 mg) [25]. In this study we investigated, for the first time, the absorption profile of highly purified HT administered as a supplement in an aqueous solution, without the intervention of other nutri-
Fig. 4. Characterisation of the gradient used to purify LDL lipoproteins. After ultracentrifugation, fractions of 1 mL were eluted with a gradient collector and density (△), proteins (▲), Apo B (●), Apo A1 (□), cholesterol (□) and triacylglycerols (■) were measured in the fractions as described in the methods section.

Fig. 5. Agarose gel electrophoresis of fraction pools A–F (lanes 2–7). Lane 1 is purified LDL.

HT detected in the pools of fractions A–F of the lipoprotein gradients obtained at 10 min (white bars) and 20 min (shadow bars), after the intake of the HT supplement. Pool C contains LDL. Values are expressed as mean ± SEMs. *Different from A, B, D, E, F; P = 0.000.

The absorption of phenols from olive oil has been investigated in humans by several research groups [26–34,15], but the studies have mainly been performed with virgin olive oils naturally rich in phenols or with olive oils enriched with phenolic extracts. We previously investigated in rats the absorption profile of HT dissolved in water to more accurately select the blood sampling times in the study subjects. The absorption of the HT from the supplement was very fast as the plasma concentration showed maximum levels at 10 min after the administered dose. In line with our results, the oral absorption of synthesised HT dissolved in water administered to rats also showed a peak at 10 min [20]. Two other studies in humans have shown absorption curves of HT administered in olive oil with other naturally present polyphenols [28,34]. They also indicated the presence of HT and HvOH in plasma but not as free forms. This may be due to the lower amounts of polyphenols (administered in olive oil), which may render the more accessible to the metabolic enzymes. Besides, the pharmacokinetic parameters obtained were rather different to our study: \( C_{\text{max}} \) for HT was obtained between times 32 min and 54 min depending on the amounts of polyphenols present in the olive oil, whilst the half-life for HT was within the 2.4–3 h range. These results underline the differences in bioavailability of HT administered as aqueous and oil solutions, already described in [35]. Indeed, in a trial published by Visioli et al. [15], the excretion of HT differed depending on the vehicle of administration as follows: when 3.2 mg of total HT were given as a natural component of virgin olive oil, the excretion measured in urine was 44% of the total. However, it was 23% when 9 mg of total HT were added to refined olive oil and only 5.8% when 20 mg of synthetic HT were administered in a yoghurt. The bioavailability of HT obtained in our study was below 10%, in line with those results.

The large differences observed in the absorption of HT when administered in virgin olive oil (with other polyphenols) compared with its pure form as an aqueous solution could be explained by the number of olive oil phenols like oleuropein, that can release HT in the gut by hydrolysis [15]. In addition, as olive oil phenols are mostly absorbed in the small intestine and the colon [29], the rate of gastric emptying and the slow release of HT from the oil matrix which generates prolonged absorption curves [28] may be other factors. The possible protection from binding to proteins in the gut [15] or the presence of other olive oil antioxidants which prevents the breakdown of phenols [35], may also contribute to increase the bioavailability of HT in oil compared with aqueous solutions.

HT was excreted in urine mainly as sulphate and glucuronide conjugates. This was expected as olive oil phenols undergo extensive enterocyte and liver metabolism [27,29–31,36] and high doses of olive oil phenols increase the rate of conjugation [28]. Although the presence of DHPA has been previously described in rat plasma
after an oral dose of synthetic HT, we report for the first time the urinary excretion in humans of DHPA as free form (13.2%) and as conjugate derivatives, which represents more than 40% of the total HT metabolites in urine. The effects of this metabolite may be relevant and deserves further research as it seems to induce apoptosis of human HL60 cancer cells [37]. We could not find DHPA or HVA in plasma but their presence cannot be ruled out as the detection limit of our method was above 100 nmol/mL for those metabolites.

In spite of the high dose of HT administered to the subjects, no significant antioxidant effects were observed in plasma or LDL lipoproteins during the 2 h period. We believe this is too short time to be able to measure an antioxidant effect, as described in [6,33,38], besides the low bioavailability values.

The results from this study indicated a transient association of HT with low-density lipoproteins, which coincided in time and $C_{\text{max}}$ with the absorption curve obtained in plasma. A limited association with HDL cannot be ruled out as ca. 15% of the HT co-sedimented with the pool of fraction mainly containing HDLs.

To detect these associations we had to use fresh plasma and undialysed LDL fractions. Due to the high amounts of salts needed to generate the gradient that separates the lipoprotein bands during the ultracentrifugation, it is normal procedure to dialyse the resulting fractions before further analysis. In this occasion, the fractions were not subjected to dialysis as our previous experiments showed that HT quickly diffused through the dialysis membrane into the dialysis buffer. Indeed, 90% of the HT present in fraction pool C disappeared within the first 10 min into the dialysis. After the plasma and LDL isolation and during the analytical procedures, we also avoided the freeze–thaw cycles to rule out the possibilities of unspecific associations due to changes in the conformation of proteins or lipids and to minimise the effects of the high salts. We carried out a thorough characterisation of the gradient to confirm the purification of the different lipoprotein fractions. We also ran an HT solution as non-lipoprotein controls which sedimented with the bulk of salts at the bottom of the gradient.

Three human studies with virgin olive oil have suggested the presence of olive oil polyphenols in LDL but their results are controversial. In one study [39], administration of 12 mg of polyphenols present in olive oil during 1 week produced an increase in the amounts of total phenols of isolated LDL. However, no enrichment of polyphenols in LDL was observed when the same amounts were administered and the LDL content was analysed for the following 6 h. In contrast, in a later study [34], 13 mg of polyphenols in virgin olive oil were given to a group of subjects and the total phenolic content in purified LDL was found to increase by 57% compared with baseline. 1.5 h after the administration. In a recent paper [40], the same research group detected 5 different conjugates of HT in LDL upon the administration of 30 mg of olive oil polyphenols, using a new LDL purification method developed in their laboratory. In all these reports, the presence of ApoB was used as a marker of LDL but no other protein or lipid marker or even density values were used to discard the presence of other plasma components. Furthermore, the high presence of salts used in the purification, which in our experiments sedimented with HT in the absence of plasma, maybe a confounding factor.

The association of HT with LDL, although brief, may be relevant in the context of the physiopathology of atherosclerosis, as oxidised/modified LDL trapped in the arterial intima seem to trigger early events of the process [41]. This association is likely to be prolonged in the case of HT administered in olive oil due to its higher bioavailability.

In conclusion, the absorption of high amounts of purified HT administered as an aqueous solution is very low and produces a large number of metabolites. The transient association with LDL observed in plasma may have physiological implications but more research is needed using larger intervention trials with different HT and formulations that render HT more bioavailable before any conclusion can be made. Natural antioxidants are a main target for the nutraceutical industry. These results should provoke discussion concerning the number of commercially available products in the form of capsules of olive oil extracts, or olive oil antioxidant supplements, which try to mimic the antioxidant effects of virgin olive oil.

Conflict of interest

J. F. is an employee of Puleva Biotech SA. MG-S and EL-H state no conflict of interest.

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