



# One-week administration of hydroxytyrosol to humans does not activate Phase II enzymes



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## ABSTRACT

The notion that (poly)phenols act as direct free radical scavengers is being challenged by mere chemical and biochemical considerations such as bioavailability and intracellular concentrations. An alternative hypothesis that is gaining considerable traction is that (poly)phenols are processed by the body as xenobiotics via the Keap1/Nrf2/ARE signaling axis, leading to the induction of Phase II enzymes. However, there are no solid human data to confirm this interesting supposition. In this study, we tested the activities of hydroxytyrosol (HT) on Phase II enzymes' expression in a double-blind, randomized, placebo-controlled study. We tested two HT doses, i.e. 5 and 25 mg/d, vs. placebo following a Latin square design. We report that HT is well tolerated but does not significantly modify Phase II enzyme expression in peripheral blood mononuclear cells. Moreover, we were unable to record significant effects on a variety of surrogate markers of cardiovascular disease such as lipid profile and inflammation and oxidation markers. Available evidence indicates that the "hormesis hypothesis" that (poly)phenols activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

This study is registered at ClinicalTrials.gov (identifier: NCT02273622).

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## 1. Introduction

(Poly)phenols are the products of plants' secondary metabolism and are endowed with several botanical activities [1]. Notable examples include roles in pollination, color, insect repelling, and cellular signal transduction. In addition, since the Zutphen Study [2] (poly)phenol consumption by humans is being consistently associated with better cardiovascular prognosis and chemoprevention. Pharma-nutritionists are trying to explain the molecular mechanisms responsible for the purported healthful activities of (poly)phenols; major emphasis is being placed on their antioxidant actions, which would counteract the noxious effects of reactive oxygen species and free radicals. However, the widespread notion that (poly)phenols act as direct free radical scavengers is challenged by mere chemical and biochemical considerations

such as bioavailability and intracellular concentrations (namely, as compared with endogenous antioxidants), reaction kinetics, etc. [3].

An alternative hypothesis that is gaining considerable traction is that (poly)phenols are processed by the body as xenobiotics [3,4]. Therefore, they stimulate stress-related cell signaling pathways that result in increased expression of genes encoding cytoprotective genes. In particular, Nrf2 (NF-E2-related factor 2) is a transcription factor which binds to the Antioxidant Response Element (ARE) in cells and thus regulates enzymes involved in antioxidant functions or detoxification such as thioredoxin reductase-1 and glutathione peroxidases [3]. According to the hormesis theory, (poly)phenols paradoxically act on the Keap1/Nrf2/ARE signaling axis to produce additive increases in electrophilic signaling that results in the induction of Phase II enzymes and increased nucleophilic substrates, such as glutathione, thioredoxin, and NADPH. In brief, (poly)phenols likely exert indirect rather than direct antioxidant actions. However, there are no solid human data to confirm this interesting supposition.

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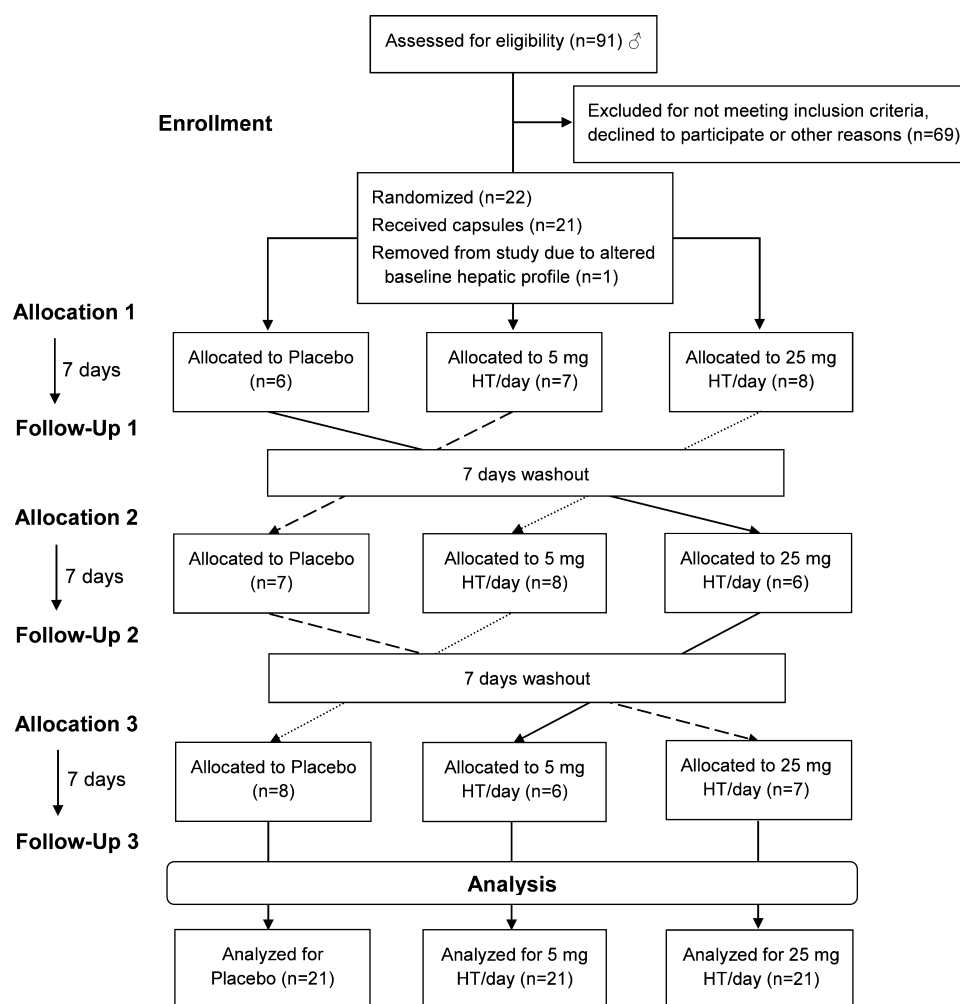


Fig. 1. Study flowchart.

In this study, we tested the activities of hydroxytyrosol (HT) on Phase II enzymes' expression in a double-blind, randomized, placebo-controlled study.

## 2. Materials and methods

### 2.1. Subjects and study design

The study protocol was approved by the local Ethics committee and was fully explained to the participants. Written informed consent was obtained by all subjects prior to starting the trial. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and is registered at ClinicalTrials.gov (identifier: NCT02273622).

This was a double-blind, randomized, placebo-controlled study. We tested two HT doses, i.e. 5 and 25 mg/d provided via administration of Hytolive® (Genosa, Madrid, Spain), an olive mill waste water (OMWW) extract selectively enriched in HT, i.e. devoid of oleuropein or other HT-containing secoiridoids as assessed by HPLC [5], via anion-exchange chromatography. Twenty-two apparently healthy volunteers were recruited from within the IMDEA-Food Genyal platform database. Inclusion criteria were: age between 20 and 40 years; adequate understanding of the study; willingness to complete the entire treatment. Exclusion criteria were: body mass index <19 or >26; diagnosis of diabetes mellitus, hypertension, dyslipidemia or other cardiometabolic disorders; impaired cognitive function; diagnosed hepatic, renal, or cardiovascular disease;

allergy to olives and their derivatives; pharmacological therapies; and habitual smoking. To our knowledge, there are no previous studies testing the activities of HT on Phase II enzymes' expression in humans. Therefore, we designed a pilot study and we could not calculate power. However, the use of repeated measures increases the power to detect treatment differences in mean levels of the outcome measure over time.

We followed a Latin square design: after one-week washout, i.e. olive-free diet, subjects were randomly assigned to either the placebo (maltodextrin), 5 mg/d HT, or 25 mg/d HT group (Fig. 1). The complete capsule composition is described in Supplementary Table 1. Administration of each treatment was carried out for one week, followed by a one-week washout after which treatments were switched. Volunteers were instructed not to consume any olive-based products or medication throughout the study and were instructed to write down any occurrences (consumption of prohibited foods, medication intake, etc.) and intolerance issues (diarrhea, acidity, nausea, abdominal distension, or halitosis) in questionnaires. Capsules were provided in bottles labeled A, B, or C and their contents were unknown to both the volunteers and the nurse who administered the bottles. Compliance was assessed by capsule count.

Blood samples were drawn and anthropometric data (height, weight, body mass index (BMI), bioelectric impedance analysis (BIA)) and vital constants (systolic (SBP) and diastolic blood pressure (DBP), and heart rate) were monitored at each visit. Morning urine was also collected at each time point.

## 2.2. Biochemical analyses

Triacylglycerol (TG), total (TC), low-density (LDL-c), and high-density (HDL-c) cholesterol concentrations were measured by routine laboratory (Laboratorio CQS, Madrid, Spain, which follows the UNE-ISO 15189:2007 directives) methods. Urea, creatinine, hepatic enzymes: glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT) and gamma glutamyltransferase (GGT), bilirubin, and alkaline phosphatase (AP) were also measured by routine laboratory methods. The concentration of oxidized LDL was measured by sandwich enzyme-linked immunosorbent assay (ELISA) by using the monoclonal antibody mAb-4E6 (Merckodia AB, Sweden).

Urinary thromboxane B<sub>2</sub> and total isoprostanes were quantified by competitive ELISA (Enzo Biochem, Inc., NY, USA and Oxford Biomedical Research, MI, USA, respectively).

## 2.3. Multiplex bead immunoassay

In a subset of samples, plasma concentrations of Interleukin (IL)-6, IL-8, IL-10, IL-17, monocyte chemoattractant protein 1 (MCP-1); Tumor necrosis factor alpha (TNF $\alpha$ ), and vascular endothelial growth factor (VEGF) were measured using a magnetic bead-based immunoassay (MAGPIX-Luminex) kit from Millipore, following the manufacturer's instructions. A minimum of 50 beads per parameter were analyzed by the MAGPIX-Luminex system. Raw data (median fluorescence intensity, MFI) were analyzed with the xPONENT software 4.1.

## 2.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Subjects were instructed to fast overnight before each blood collection. Blood samples were collected between 8 and 10 AM to minimize circadian variations. Blood samples were collected in heparinized tubes (BD Vacutainer, Franklin Lakes, NJ, USA) at each visit, processed within 2 h after extraction and used to PBMCs. Isolation was carried out under sterile conditions to avoid monocytes activation. Whole blood was diluted (1:1) with phosphate buffer solution (PBS) and centrifuged by density gradient with Histopaque-1077 (Sigma–Aldrich, Madrid, Spain) according to the manufacturer's instructions. After collection, PBMCs were washed twice with PBS, homogenized in Qiazol (Qiagen, Madrid, Spain) and stored at  $-80^{\circ}\text{C}$  prior to RNA extraction.

## 2.5. RNA extraction and microarray samples preparation

Total RNA was extracted and purified from homogenized PBMCs with miRNeasy minikit (Qiagen, Valencia, CA, USA) following

the manufacturer's protocol. Recovered RNA's concentration and integrity were verified using a Nanodrop ND-1000 spectrophotometer (Nanodrop TechnologyR, Cambridge, UK) and an Agilent 2100 Bioanalyzer (Agilent, Madrid, Spain).

## 2.6. Gene expression analysis

After DNase I treatment (Invitrogen, Madrid, Spain), reverse transcription was performed with miScript<sup>®</sup> II Reverse Transcription kit (Qiagen, Germantown, MD) according to the manufacturer's guidelines. RT-qPCR reactions were performed in 384-well plates and gene expression was determined using the 7900HT Real-Time PCR System (Life Technologies, Spain). Reactions were performed with 5  $\mu\text{L}$  of miScript SYBR<sup>®</sup> Green qPCR Master Mix (Qiagen, Madrid, Spain). Cycling conditions were 15 min at  $95^{\circ}\text{C}$  for one cycle, then 40 cycles at  $94^{\circ}\text{C}$  for 15 s,  $58^{\circ}\text{C}$  for 30 s,  $70^{\circ}\text{C}$  for 30 s, for each gene. The dissociation stage was analyzed at  $95^{\circ}\text{C}$  for 15 s, followed by one cycle at  $60^{\circ}\text{C}$  for 15 s, and  $95^{\circ}\text{C}$  for 15 s. Gene expression analysis was carried out for twenty Phase II enzymes (primers can be found in Supplementary Table 2). Three potential reference genes–GAPDH, HPLPO, and ACTB–were tested in order to select the most stable to be used in target gene's normalization, which according to Normfinder was the latter. Reactions were run in triplicate and relative expression of Phase II enzymes was calculated by the comparative Ct method and presented as  $2^{-\Delta\Delta\text{Ct}}$ .

## 2.7. Statistical analyses

Continuous descriptive variables were expressed as mean  $\pm$  SEM. Two-way repeated measures ANOVA was used to evaluate the effects of time ( $t_1$  and  $t_2$ ), treatment (A, B, C) and the time  $\times$  treatment interaction. A Bonferroni correction for multiple analyses was applied and models were adjusted for age and sequence (ABC/CAB/BCA) as covariates. All statistical analyses were considered as bilateral and significance was set at  $p < 0.05$ . Data were analyzed with R Statistical Software version 3.1.1 ([www.r-project.org](http://www.r-project.org)).

## 3. Results

One volunteer was excluded from the study because he exhibited abnormal basal bilirubin concentrations (Fig. 1); attrition rate was, therefore, zero. Both 5 and 25 mg HT doses were well-tolerated and no adverse effect was reported.

No differences in anthropometric variables, such as weight or body mass index, were recorded and no significant variations in vital signs such as blood pressure were noted either (Table 1).

**Table 1**  
Anthropometric parameters and vital signs.

Measured parameters	A		B		C		Effect ( $p$ ) <sup>*</sup>		
	Initial	Final	Initial	Final	Initial	Final	Time	Treatment	Time $\times$ treatment
Weight (kg)	73.31 (1.58)	73.1 (1.61)	73.1 (1.58)	72.85 (1.57)	73.07 (1.62)	72.73 (1.64)	0.002 <sup>*</sup>	0.989	0.833
B.M.I (kg/m <sup>2</sup> )	23.69 (0.41)	23.62 (0.42)	23.62 (0.43)	23.55 (0.43)	23.61 (0.44)	23.49 (0.45)	0.002 <sup>*</sup>	0.984	0.721
FM (%)	19.99 (1.16)	20.01 (1.23)	20.1 (1.23)	20.02 (1.24)	19.87 (1.3)	19.82 (1.25)	0.787	0.991	0.944
MM (%)	39.81 (0.71)	39.72 (0.77)	39.69 (0.77)	39.67 (0.77)	39.78 (0.81)	39.82 (0.78)	0.805	0.993	0.821
AVF	5.9 (0.42)	5.95 (0.43)	5.86 (0.44)	5.76 (0.44)	5.86 (0.44)	5.76 (0.45)	0.263	0.973	0.287
SBP (mmHg)	130 (2.35)	131.1 (1.98)	129.62 (2.84)	131.19 (2.18)	130.14 (2.47)	128.71 (2.17)	0.681	0.920	0.424
DBP (mmHg)	70.1 (1.51)	72.48 (1.48)	71.57 (1.61)	71.71 (1.46)	70.29 (1.21)	71.24 (1.76)	0.1622	0.878	0.531
HR (Beat/min)	60.19 (3.96)	69 (2.91)	64.05 (2.15)	67.76 (2.5)	63.38 (2.01)	66.1 (2.75)	0.0013 <sup>*</sup>	0.893	0.214

A: 250 mg Hytovie (25 mg hydroxytyrosol); B: 50 mg Hytovie (5 mg hydroxytyrosol); C: Placebo. B.M.I, body mass index; FM, Fat mass; MM: Muscle mass measured by BIA: bioelectrical impedance analysis; AVF, abdominal visceral fat (measured by BIA); SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, Heart Rate. Effect ( $p$ ): Time: indicates the evolution in the same group from the beginning to the end of the intervention. Treatment: indicates differences between treatments (A, B, C) independently of time. Time  $\times$  treatment: indicates the differences in evolution between groups as a result of treatment received (A, B, C). The  $p$  value for interaction was adjusted for age and sequence of consumption as covariates. Data are means (SEM).

<sup>\*</sup>  $p < 0.05$ .

**Table 2**  
Biochemical parameters evaluated in each of the visits.

Measured parameters	A		B		C		Effect (p) <sup>*</sup>		
	Initial	Final	Initial	Final	Initial	Final	Time	Treatment	Time × treatment
TC (mg/dl)	169.3 (6.54)	169.7 (6.51)	168.6 (6.39)	173.9 (6.8)	170.8 (6.77)	169.4 (5.97)	0.388	0.975	0.258
HDL (mg/dl)	52.83 (2.57)	51.04 (2.06)	52.17 (2.66)	52.14 (2.44)	51.84 (2.26)	50.87 (2.48)	0.229	0.960	0.644
LDL (mg/dl)	99.47 (5.47)	103.0 (5.41)	101.3 (5.47)	106.2 (5.7)	102.5 (5.78)	103.7 (5.17)	0.031 <sup>*</sup>	0.923	0.559
TG (mg/dl)	85.1 (7.26)	78.24 (6.15)	75.81 (4.2)	77.6 (6.57)	82 (8.05)	74.6 (4.96)	0.163	0.793	0.371
hs-CRP (mg/dl)	0.07 (0.02)	0.16 (0.06)	0.09 (0.02)	0.05 (0.01)	0.09 (0.02)	0.05 (0.01)	0.47	0.117	0.049 <sup>*</sup>
TXB <sub>2</sub> (ng/ml)	1.55 (0.2)	1.71 (0.14)	1.97 (0.26)	1.72 (0.16)	2.17 (0.18)	1.83 (0.3)	0.253	0.316	0.222
TXB <sub>2</sub> /Creat (μg/g)	0.87 (0.06)	0.86 (0.05)	1.03 (0.10)	0.9 (0.06)	1.01 (0.06)	0.94 (0.08)	0.097	0.373	0.502
LDL-ox (U/l)	42.6 (2.32)	41.34 (2.04)	42.19 (2.31)	43.95 (2.56)	41.28 (2.84)	41.34 (2.31)	0.849	0.814	0.445
Isop (ng/ml)	2.99 (0.34)	3.59 (0.41)	3.71 (0.41)	3.78 (0.42)	4.78 (0.63)	3.38 (0.56)	0.477	0.298	0.052 <sup>*</sup>
Isop/Creat (μg/g)	1.64 (0.08)	1.75 (0.1)	1.92 (0.13)	2.05 (0.17)	2.42 (0.33)	1.74 (0.14)	0.257	0.128	0.020 <sup>*</sup>
GOT/AAT (U/l)	19.71 (1.26)	19.95 (1.18)	18.05 (0.79)	18.9 (0.96)	19.52 (0.77)	19.33 (1.09)	0.525	0.520	0.662
GPT/AT (U/l)	19.33 (1.9)	19.67 (1.79)	18.38 (1.49)	18.24 (1.38)	20.0 (1.6)	18.62 (1.17)	0.458	0.744	0.403
GGT (U/l)	21.07 (1.99)	21.48 (2.44)	22.38 (2.27)	21.89 (2.0)	21.69 (2.07)	20.66 (1.78)	0.260	0.909	0.196
TB (mg/dl)	1.1 (0.08)	1.16 (0.1)	1.16 (0.11)	1.12 (0.09)	1.06 (0.08)	1.21 (0.1)	0.148	0.994	0.143
AP (U/l)	55.95 (2.92)	64.33 (3.89)	58.86 (3.31)	62.86 (3.23)	57.76 (3.6)	61.76 (3.3)	0.001 <sup>*</sup>	0.967	0.204
Urine creatinin (mg/dl)	177.5 (16.9)	202.2 (15.9)	190.2 (14.5)	193.6 (14.0)	215.7 (14.8)	184.8 (14.1)	0.910	0.814	0.024 <sup>*</sup>

A: 250 mg Hytolute (25 mg hydroxytyrosol); B: 50 mg Hytolute (5 mg hydroxytyrosol); C: Placebo. TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: Triglycerides; hs-CRP: high-sensitive C-reactive protein; TXB<sub>2</sub>: Thromboxane B<sub>2</sub>; TXB<sub>2</sub>/Creat: TXB<sub>2</sub> ratio and creatinine in urine; LDL-ox: Oxidized Low Density Lipoprotein; Isop: Isoprostanes, Isop/Creat: Isoprostanes ratio and creatinine in urine; GOT: glutamyl oxaloacetic transaminase; GPT: glutamic-pyruvate transaminase; GGT: Gamma Glutamyltransferase; TB: total bilirubin; AP: alkaline phosphatase. Time: indicates the evolution in each group from beginning to end of the intervention. Treatment: differences between treatments (A, B, C) independently of time. Time × treatment: differences in evolution between groups as a result of treatment received (A, B, C). Age and sequence of consumption were used as covariates. Data are means (SEM).

<sup>\*</sup> p < 0.05.

**Table 3**  
Cytokine levels after the consumption of the study products (A and C).

Measured parameters	A		C		Effect (p) <sup>*</sup>		
	Initial	Final	Initial	Final	Time	Treatment	Time × treatment
IL-10 (pg/ml)	2.22 (0.97)	1.36 (0.41)	0.79 (0.16)	1.71 (0.97)	0.96	0.46	0.17
IL-17 (pg/ml)	30.7 (10.2)	29.2 (8.18)	14.3 (2.61)	15.3 (3.98)	0.91	0.12	0.58
IL-6 (pg/ml)	10.6 (4.99)	11.5 (5.73)	6.20 (2.80)	5.20 (1.51)	0.96	0.18	0.45
IL-8 (pg/ml)	30.4 (9.57)	28.1 (8.56)	14.3 (4.43)	21.4 (9.92)	0.50	0.31	0.20
MCP-1 (pg/ml)	486 (54.0)	472 (54.1)	412 (37.0)	446 (43.5)	0.52	0.36	0.14
TNF-α (pg/ml)	8.99 (1.02)	8.31 (0.88)	7.37 (0.64)	6.74 (0.54)	0.04 <sup>*</sup>	0.12	0.92
VEGF (pg/ml)	605 (164)	682 (182)	577 (110)	497 (98.2)	0.98	0.53	0.10

A: 250 mg Hytolute (25 mg hydroxytyrosol); C: Placebo. n = 10 in both groups, randomly selected. IL-10: Interleukin 10; IL-17: Interleukin 17; IL-6: Interleukin 6; IL-8: Interleukin 8; MCP-1: Monocyte chemoattractant protein 1; TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor. Data are means (SEM).

<sup>\*</sup> p < 0.05.

**Table 2** reports the plasma concentrations of selected surrogate markers of cardiovascular disease and hepatic enzymes. We only recorded significant time × treatment interactions for isoprostanes (markers of oxidation) and hs-CRP (marker of inflammation). Of note, hepatic function enzymes, which are – alas – rarely measured in nutraceutical studies, were not modified by either treatment.

In a subset of samples, namely those who received the highest HT dose vs. controls, we also quantified an array of plasma cytokines (**Table 3**) and did not find any significant variation induced by HT.

Phase II enzyme expression in PBMCs is reported in **Table 4**. We only documented a time effect on GSTO1 and GSTP1 levels, whereas all other genes were unaffected by the provision of HT.

#### 4. Discussion

The notion that (poly)phenols trigger stress signaling mediated by Nrf-2 and Phase II enzymes is, nowadays, predominant over that of their plain antioxidant actions [3,6]. However, this hypothesis has never been confirmed in humans (to the best of our knowledge), with the exception of Hofmann et al. [7], who reported modest yet significant increases of glutathione S-transferase P1 (hGSTP1) protein expression in leukocytes of healthy volunteers given fruit juice. In brief, while biochemical verification of Nrf-2 mediated hormesis is strong, human evidence is scant or non-existent.

In this paper we report that HT, administered at two different doses in a Latin-square design study was well tolerated (as shown

by the unaltered levels of hepatic enzymes, **Table 2**), but does not significantly modify Phase II enzyme expression in PBMCs. Moreover, we were unable to record significant effects on a variety of surrogate markers of cardiovascular disease such as lipid profile and inflammation and oxidation markers. Actually, we computed a non-significant increase in plasma hs-CRP and in urinary isoprostanes concentrations. The latter finding is in agreement with that of Leger et al. [8] and in disagreement with Visioli et al [9], who, however, administered extra virgin olive oils rather than HT. Because these differences were non-significant it is difficult to comment on their true physiological relevance; however, these data reiterate the concept that (poly)phenols and other minor food components exert biological effects whose nature and extent depend on the matrix, i.e. as part of food or provided as pharmaceutical formulations.

We chose hydroxytyrosol (the foremost component of extra virgin olive oil [10]) as paradigmatic example of (poly)phenols because (1) it is the only phenolic molecule that received a European Food Safety Authority (EFSA) health claim [11]; (2) it has been shown to increase Nrf-2 expression in vitro [12,13] albeit at non-physiological doses [14]; (3) a “xeno-hormesis” postulation has also been proposed to explain the salubrious actions of extra virgin olive oil phenolics [15]; and (4) a previous human study with an olive mill waste water (OMWW) preparation reported increased glutathione levels short-term in human volunteers [16]. A glutathione effect was also recorded in rats [17] and a short-term

**Table 4**

Gene expression of Phase II enzymes after the consumption of the study products (A, B and C).

Phase II enzymes	Initial	Final						Effect (p) <sup>a</sup>		
		A		B		C		Time	Treatment	Time × treatment
NQO1	1	1.11	(0.11)	0.81	(0.07)	1.50	(0.61)	0.79	0.58	0.35
NQO2	1	1.79	(0.87)	1.04	(0.08)	1.05	(0.09)	0.74	0.66	0.95
GSTA1	1	1.28	(0.32)	1.32	(0.24)	1.01	(0.17)	0.38	0.93	0.45
GSTA4	1	1.01	(0.10)	1.11	(0.09)	1.10	(0.13)	0.73	0.79	0.58
GSTK1	1	1.27	(0.35)	0.99	(0.07)	1.03	(0.12)	0.87	0.42	0.96
GSTM1	1	1.22	(0.13)	0.89	(0.06)	1.19	(0.24)	0.40	0.89	0.16
GSTM2	1	1.73	(0.37)	1.20	(0.15)	1.54	(0.44)	0.12	0.92	0.97
GSTM3	1	1.16	(0.17)	1.15	(0.12)	1.06	(0.15)	0.96	0.92	0.46
GSTM4	1	1.22	(0.17)	0.97	(0.07)	1.19	(0.29)	0.63	0.89	0.29
GSTM5	1	1.39	(0.39)	1.07	(0.15)	1.18	(0.24)	0.36	0.88	0.95
GSTO1	1	0.90	(0.09)	1.18	(0.15)	0.90	(0.08)	0.03 <sup>*</sup>	0.65	0.25
GSTO2	1	0.97	(0.08)	0.93	(0.08)	1.11	(0.11)	0.30	0.82	0.69
GSTP1	1	1.24	(0.09)	1.28	(0.16)	1.46	(0.25)	0.02 <sup>*</sup>	0.85	0.99
GSTT1	1	0.94	(0.07)	1.06	(0.09)	1.22	(0.21)	0.73	0.84	0.56
GSTT2	1	1.58	(0.26)	1.22	(0.15)	1.08	(0.14)	0.50	0.59	0.29
HNMT	1	0.97	(0.08)	1.02	(0.10)	1.02	(0.08)	0.56	0.94	0.63
INMT	1	1.00	(0.13)	1.13	(0.15)	1.03	(0.15)	0.09	0.54	0.52
MGST1	1	1.06	(0.13)	1.19	(0.13)	1.05	(0.10)	0.98	0.70	0.22
MGST2	1	1.01	(0.07)	1.02	(0.08)	1.06	(0.13)	0.31	0.89	0.55
MGST3	1	0.90	(0.08)	1.13	(0.08)	1.05	(0.11)	0.16	0.92	0.17

A: 250 mg Hytolive (25 mg hydroxytyrosol); B: 50 mg Hytolive (5 mg hydroxytyrosol); C: Placebo. NQO1: NADPH dehydrogenase, quinone 1; NQO2: NADPH dehydrogenase, quinone 2; GSTA1: Glutathione S-transferase alpha 1; GSTA4: Glutathione S-transferase alpha 4; GSTK1: Glutathione S-transferase kappa 1; GSTM1: Glutathione S-transferase mu 1; GSTM2: Glutathione S-transferase mu 2; GSTM3: Glutathione S-transferase mu 3; GSTM4: Glutathione S-transferase mu 4; GSTM5: Glutathione S-transferase mu 5; GSTO1: Glutathione S-transferase omega 1; GSTO2: Glutathione S-transferase omega 2; GSTP1: Glutathione S-transferase pi 1; GSTT1: Glutathione S-transferase theta 1; GSTT2: Glutathione S-transferase theta 2; HNMT: Histamine N-methyltransferase; INMT: Indoethylamine N-methyltransferase; MGST1: Microsomal glutathione S-transferase 1; MGST2: Microsomal glutathione S-transferase 2; MGST3: Microsomal glutathione S-transferase 3. Data are fold change means (SEM).

<sup>a</sup>  $p < 0.05$ .

increase in skeletal muscle glutathione concentrations after exercise was reported by Bast and Haenen, who administered an HT-rich olive extract (providing 200 mg of HT) to human volunteers [18]. Finally, a nutrigenomic study carried out in mice reported that HT – in nutritionally relevant amounts – is able to positively modulate the glutathione-driven antioxidant enzymatic machinery in adipose tissue [19].

Of note, Visioli et al. [16] previously suggested that part of the purported healthful activities of HT might be governed by the antioxidant response element (ARE)-mediated increase in Phase II enzyme expression. Therefore, we undertook the current study to verify this premise in a controlled setting. Indeed, we also wanted to verify in humans the now popular notion that describes (poly)phenols as activators of the stress response pathways. We used PMBCs because they are easily accessible, express many genes previously believed to be restricted to non-blood tissues responding to macro- or micro-environment alterations in organs, and have been shown to be suitable for nutritional studies, reflecting specific effects of diets or nutrients [20–22].

To date, there are two human studies that have been performed with OMWW and one where the authors administered pure HT [8,23,24]. The former reported anti-thrombogenicity [8,23], while the latter only addressed absorption and disposition and did not inform on biochemical data [24].

Several hypotheses can be formulated to explain the discrepancy between our current results and those obtained with high-polyphenol extra virgin olive oil or those of Visioli et al. [16], namely in terms of oxLDL [25] and platelet aggregation [8,23] or oxidation and inflammatory markers [9]. First and foremost, we administered two doses of HT, i.e. 5 and 25 mg/d for one week. Higher doses might produce the hypothesized effects and are supposed to be safe. However, it is noteworthy that the EFSA health-claim [11] relates to 5 mg/d of HT and HT-containing phenols as protectors of LDL from oxidation. From a practical viewpoint this implies that formulations that provide HT amounts higher than this dose might have to deal with some regulatory issues, even though the safety profile of HT is excellent [26–28] as confirmed

by the current study (Table 2). As a matter of fact, Visioli and Galli administered 50 mg of HT and recorded effects on TXB<sub>2</sub> production by serum short term, i.e. 1 h after intake [23]. These data were confirmed by Leger et al. [8], who provided five type I diabetic patients with 25 mg of HT the first day and 12.5 mg/day the following 3 days. A 46% decrease in the serum TXB<sub>2</sub> production after blood clotting at T (4d) was recorded [8]. Whether high doses of (poly)phenols trigger other short-term effects such as Phase II enzyme expression remains to be verified in humans. Also, the activation of Phase II enzymes might be organ-specific [29]; whether sustained hepatic Phase II enzymes activation occurs in humans would be very difficult to ascertain for obvious ethical and practical reasons.

Another important issue that the nutraceutical world is trying to address is that of healthy volunteers vs. patients [30]. Clinical trials of nutraceuticals, supplements, or functional foods in patients clash with obvious ethical guidelines; as a result, these preparations are often added to established, top-quality therapy, therefore making it difficult to ascertain their real contribution. One notable example is that of omega 3 fatty acids [31,32]. Our cohort of healthy volunteers exhibited normal-range concentrations of surrogate markers of cardiovascular disease at baseline: further reduction of such concentrations might be difficult to achieve. Conversely, HT might speculatively be beneficially used as medical food [33], i.e. as adjunct therapy of cardiovascular and other inflammation-based diseases [34] or to metabolic syndrome patients, pending confirmation by appropriate clinical trials [35].

Finally, it is worth reiterating that nutraceuticals and functional foods must exert modest (and often undetectable based on current methodologies) physiological effects which, however, could produce remarkable health activities if taking place long-term [36]. Indeed, epidemiological evidence of healthful activities is strong and corroborates the “sub-clinical, lifetime exposure” proposition.

In conclusion, the physiological activities of HT are – as of today – limited to inhibition of TXB<sub>2</sub> production by serum, leading to a possible prevention of thrombotic and micro-thrombotic processes. Most important, the “hormesis hypothesis” that (poly)phenols



activate Phase II enzymes requires solid human confirmation that might be provided by future trials.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2015.03.018>.

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