Identification and validation of common molecular targets of hydroxytyrosol.

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Abstract:

Hydroxytyrosol (HT) plays healthful activities and is beneficial to lipid metabolism. Many investigations focused on finding tissue-specific targets of HT through the use of different omics approaches such as transcriptomics and proteomics. However, it is not clear which (if any) of the potential molecular targets of HT reported among different studies are concurrently affected in the various tissues. Following bioinformatic analyses of publicly available data from a selection of *in vivo* studies involving HT-supplementation, we selected differentially expressed lipid metabolism-related genes and proteins common to more than one study, for validation in rodent liver samples from the entire selection. Four miRNAs (miR-802-5p, miR-423-3p, miR-30a-5p, and miR-146b-5p) responded to HT supplementation. Of note, miR-802-5p was commonly regulated in liver and intestine. Our premise was that, in an organ crucial for lipid metabolism such as the liver, consistent modulation should be found for a specific target of HT even if different doses and duration of HT supplementation were used *in vivo*. Even though our results show inconsistency regarding differently expressed lipid metabolism-related genes and proteins among studies, we found *Fgf21* and *Rora* as potential novel targets of HT. Omics approaches should be fine-tuned to better exploit the available databases.

Keywords: Hydroxytyrosol, miRNAs, lipid metabolism, bioinformatic, transcriptomic, proteomic.

Abbreviations: HT: Hydroxytyrosol; GO: Gene ontology; GI: gene Interaction

1. Introduction

3,4-dihydroxyphenylethanol (hydroxytyrosol, HT), the main olive oil phenolic compound, is mostly found as part of complex (poly)phenols (secoiridoids) [1], which are easily hydrolyzed to yield HT after ingestion [2]. As digestion progresses, HT-derived metabolites (mainly its sulfate form) become the main compounds circulating in blood [3, 4] and recovered in urine [5]. The biological properties of HT have been widely investigated in different research areas including nutrition, medicine, pharmacology, chemistry and biotechnology [6]. This phenol is now considered as one of the most bioactive natural molecules [7]. In vitro, HT is an antioxidant [8] and its intake may beneficially influence cardiovascular disease (CVD) risk, via its potential to induce anti-atherosclerotic [9], hypotensive, antioxidant, anti-inflammatory [10], and hypocholesterolemic effects [11]. Of note, the European Food Safety Authority (EFSA) issued a health claim based on consistent results regarding the protective effects of olive polyphenols against the oxidation of blood lipids [11].

Investigations that use cutting-edge, high-throughput, techniques referred to as “omics” are increasingly popular [12]. Omics tools have allowed deepening the knowledge on metabolism changes and identifying new potential disease (such as CVD) biomarkers, in a way that was not possible by genetic techniques alone [13]. In this sense, dietary intervention studies have successfully used transcriptomics and proteomics to show the mode through which diet induces alterations in gene and protein expression, providing information about the mechanisms of action and pathways regulated by micronutrients and helping in the identification of new biomarkers [14]. However, in contrast to other fields of study [15], very few initiatives focused on the establishment of databases that integrate largescale nutritional and genomics or genetics data are being developed [15–17]. The majority of such actions are aimed at

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standardizing nutritional studies and some publications– sometimes - over-emphasize the results obtained via database interrogation. Indeed, the scientific literature only describes two examples of such largescale nutritional genomic data analysis: one related to functional genomics in chicken (Dhanasekaran, Bhattacharya, Chatterjee, Paswan, & Dyushanth, 2014) and the other one to genomic responses triggered by food bioactive compounds [12]. Indeed, it is complicated to extract the most relevant information from the large amount of data being produced worldwide, even if such approach would greatly strengthen scientific conclusions [19].

By integrating transcriptomic and proteomic data, our initial goal was to identify consistently modulated potential molecular targets of HT reported in different studies where this compound was supplemented *in vivo*. We then used liver samples, a key tissue in lipid metabolism, obtained from different HT rodent studies used to evaluate if the previously identified candidates could be considered solid targets of HT.

2. Material and methods

2.1 Data collection and gene selection

From the PubMed and Scopus scientific databases, we gathered studies of *in vivo* supplementation with HT or its phenolic precursors, where gene and protein differential expression were screened. Specific queries were launched with keywords such as "hydroxytyrosol AND proteomic", "hydroxytyrosol AND transcriptomic", "hydroxytyrosol AND gen", "hydroxytyrosol AND protein", "hydroxytyrosol AND miRNA", "hydroxytyrosol AND mRNA", "hydroxytyrosol AND genomic". We generated Venn diagrams of data from the selected studies to, by means of in-house R scripts, to find intersections among differentially expressed genes.

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Functional enrichment. Genecodis3 software was used for functional enrichment using default parameters and selecting GO biological processes as target annotations.

Statistical analysis. Moderated t-test statistics were applied to microarray features once a linear model was fitted. Statistical significance of the overrepresented GO biological processes in the target gene list was assessed through the chi-square test. The False discovery rate (FDR) method was employed to adjust the obtained p-values.

2.2 Ethics statements

All animal studies were approved by the respective Animal Ethics Committee of the institutions where each animal experimentation took place, namely: University Complutense of Madrid (CEA-UCM 93/2012); (CEEA 10-06/14, 31st July 2014 University of Lleida, Universidad Mixta de Investigación. Zaragoza. All procedures followed the Guide for the Care and Use of Laboratory Animals, published by the US National Research Council (Eight Edition, 2010), except for the study by Acin *et al.* (study 3, see below), which is previous to 2010 and followed the Ethical Committee for Animal research of the University of Zaragoza.

2.3 Brain and liver microarray analysis

Gene expression profiles in brain and liver tissues were analyzed using the Illumina MouseRef-8 v2 Expression BeadChip® platform with Ambion Labelling. Four biological replicates per group were included. This BeadChip targets approximately 25,600 well-annotated RefSeq transcripts, representing over 19,100 unique genes. Data were background-corrected and normalized using the GenomeStudioTM Software (Illumina, San Diego, CA, USA) and following the manufacturer's instruction.

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Differential expression was assessed using Limma's Bioconductor package on R statistical programming environment.

2.4 Liver samples used for transcriptomic and proteomic validation

Transcriptomic and proteomic validations were performed in liver samples obtained from a selection of four previously published studies where HT was administrated to rodents, as follows: 1) Study from Tomé-Carneiro et al. (2016), hereon referred to as **Study 1**. Briefly, in this study young C57BL/6 mice (2 months old, n=14) were fed a purified control diet alone (n=7) or supplemented with approximately 45 mg HT/kg bw/day (Seprox Biotech, Madrid, Spain) (n=7), for 8 weeks [20]. 2) A second cohort from the Tomé-Carneiro et al. (2016) study, hereon referred to as **Study 2**. Briefly, in this acute ingestion study, 15 mg of HT dissolved in water (Seprox Biotech, Madrid, Spain) were administrated (by gavage) to young C57BL/6 mice (10 weeks old), which were sacrificed immediately (control group, n=9) or 4 h after ingestion (n=9). 3) Study from Acín et al. (2006), hereon referred to as **Study 3**. Briefly, for 10 weeks, 14 homozygous apoE KO mice (2 months old, n=14) were given free access to drinking water (control group, n=7) or to an aqueous solution providing a dose of 10 mg HT/kg/day (n=7) [21]. 4) Study from Catalán et al. (2016), hereon referred to as **Study 4**. Briefly, female Wistar rats (300–350 g, n=8) were fed a standard diet (SD) (control group, n=4) or SD supplemented with 5 mg of secoiridoids/kg/day (n=4), for 21 days [22].

2.5 Transcriptomic validations

Commonly differentially expressed genes identified in the Venn diagram (Figure 1) were used for transcriptomic validations. Oligonucleotide primers were designed to amplify the selected genes in both mouse and rat species. Gene function and primers are

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listed in **Table 1**. Briefly, total RNA was isolated from liver samples using Trizol/Qiagen RNeasy kit. cDNA synthesis was performed with 1 µg of RNA using miScript II RT Kit (Qiagen) according to the supplier's instructions. qPCR was carried out in a ABI 7900 HT Real-time PCR system with a 384 well plate format, using FastStart Essential DNA Green Master mix (Roche, Switzerland) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 58°C for 1 min. Gene expression was normalized with respect to *Gapdh* expression, and relative quantification was calculated by the $2^{\Delta\Delta Ct}$ method.

2.6 Proteomic validations

For proteomic validations, protein selection was based on the commonly differentially expressed proteins identified in the corresponding Venn diagram (Figure 3). Antibodies used and function of the selected proteins are described in **Table 2**. Briefly, liver samples from the four above mentioned studies were homogenized in RIPA buffer (200 mM sodium orthovanadate, 1 mM Pefabloc SC, and 2 mg/mL protease inhibitor cocktail (Sigma, Madrid, Spain)), sonicated for 5 min and frozen overnight at – 80 °C. After centrifugation at 12,000 x*g* for 30 min at 4 °C, supernatant was collected for total protein quantification using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For Western blots, 30 µg of total protein were electrophoresed in 6–15% SDS-PAGE gels and then transferred onto nitrocellulose membranes. The membrane was blocked with LiCor blocking buffer at RT for 1 h. Overnight incubation at 4 °C was performed with primary antibody followed by incubation (1 h, RT) with LiCor fluorescent secondary antibody (goat anti-Mouse IRDye® 800 CW or goat anti-Rabbit IRDye® 680 CW). Membranes were visualized using a LiCor Odyssey scanner. Proteins were quantified with

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densitometry using Image Studio Lite 5.2.5 analytical software (LiCor, Lincoln, NE) and normalized to GAPDH.

2.7 miRNA Analysis

For miRNA analysis, an unbiased whole genome miRNA analysis was performed in mice liver samples (n= 5 per group) from Study 1 by small RNA sequencing. RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Following the manufacturer's protocol, a NEBNext® multiplex small RNA Library Prep Set for Illumina (New England BioLabs, Ipswich, MA) was used to prepare the libraries, and sequenced using the Illumina NextSeq 500 platform. After trimming adapter sequences, Bowtie2 was used for read alignment against high confidence mouse mature miRNA sequences, obtained from miRBase database. Finally, only reads showing a unique valid alignment against the reference sequences were considered for mature miRNA counting.

2.8. miRNA bioinformatic analysis

MicroRNAs' targets presenting hits on 3'UTR position and showing a Binding P-value score equal to 1 were obtained from the miRWalk 3.0 database [23] and used for further analysis. A functional enrichment of these genes, targeted by at least two of the differential expressed microRNAs, was performed in Panther database v.11 [24] using Gene Ontology (GO) and Panther pathway annotations. A subset of four significantly modulated miRNAs in response to HT supplementation were used for Gene Interaction (GI) analysis using the above-mentioned target genes. GI analysis was performed as previously described [20] including target genes targeted at least by two miRNAs. Target dot size was directly correlated with the number of interactions with the set of

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miRNAs. As for the functional analysis, only genes targeted simultaneously by at least two miRNAs are shown.

2.9. Statistical analysis

Comparisons between HT-supplemented groups and controls were performed by means of two-tailed t tests or Mann-Whitney tests when assumptions for parametric testing were not met, using GraphPad Prism 7.02 (La Jolla, CA). In all cases, a $p < 0.05$ was considered as statistically significant.

3 Results

3.1 Identification of common differentially expressed genes

Specific searches in public scientific databases for *in vivo* interventions involving hydroxytyrosol supplementation returned scarce results (**Table 3**). The GEO database, which contains high throughput genomic and proteomic data among others, only accounted for two studies concerning gene expression in humans (GSE75027 and GSE75026) after olive oil intake (where HT is preeminent). Moreover, only one study was found concerning an *in vivo* intervention with HT, where a breast tumor-induced model of Rattus norvegicus was used (GSE15944) [25]. Finally, two sets of data from a study carried out in our laboratory (**Supplementary tables S1 and S2**; not available in scientific databases), regarding microarray screening in brain and liver tissues from diet-HT supplemented mice [26] were included in this study. The aforementioned five sets of data were used to find mutual differentially expressed genes (**Figure 1**). *Efr3*, *Kctd2*, *Plscr1* and *Scl37a4* were identified as differently expressed after HT supplementation in all analyzed data sets.

3.2 Transcriptomic validations

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After the identification of differently expressed genes shared by at least three studies (Figure 1 and supplementary table S3), 18 genes associated with lipid metabolism were finally selected for validation. Validation was performed in liver samples from HT-supplemented animal models vs. controls (**Figure 2**) coming from the four selected studies (Study 1-4, see Materials and Methods for details). In liver samples from Study 1 a statistically significant rise was seen in *Tjp2*, *Top1*, *Rora*, *Sorl1*, *Fgf21* and *Ppp1cb* (Figure 2A). In liver samples from Study 2 a statistically significant rise was seen in *Slc37a4*, *Snx16*, *Rora* and *Fgf21*, whereas a statistically significant decrease was observed in *Sorl1* (Figure 2B). In liver samples from Study 3 a statistically significant rise was seen in *Slc37a4*, *Anks6*, *Plscr2* and *Lipe* (Figure 2C). In liver samples from Study 4 a statistically significant rise was seen in *Sorl1* (Figure 2D).

3.3 Identification of common differentially expressed proteins

There are few publicly available large-scale proteomics data concerning HT supplementation. To determine if HT consumption affects specific signaling pathways, we comprehensively analyzed publications involving *in vivo* HT supplementation to extract protein expression information from the reported data tables (Table 4). Then, the collected proteomic data were submitted to bioinformatic analyses to identify differently expressed proteins common to at least two of these studies (**Figure 3**). Bioinformatic analysis showed that ALDH2, SELENBP1, HSPD1, PPIA, VIM, YWHAG, RPL8, ACTN4, NPM1, ALB, HSP90AB1, CAR3, FASN, HBB-B1, PRDX1, CFL1, GLUD1, VCL, DLAT, and GPD1 proteins were common to at least 2 studies. Only two proteins, Hspd1 and Actn4, were common to three studies.

3.4 Proteomic validations

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After the identification of differently expressed proteins, common to at least two studies, validation was carried out in liver samples from HT-supplemented animal models and controls (Studies 1-4). Overall, we did not find significant differences in any of the proteins analyzed in the livers of HT- (or their secoiridoids precursors) supplemented animals as compared with controls (**Figure 4**). In liver samples from Study 1, HT-supplemented groups showed a slight, statistically non-significant, decrease in VIM and rise in HSPD1, compared to controls (Figure 4A). In samples from Study 2, involving an acute ingestion of HT, a decrease in expression was seen for PRDX1 and CAR3, and a rise in FASN, although statistical significance was not reached (Figure 4B). As for Study 3, in HT-supplemented ApoE KO mice, non-significant decreases in the expression of ACTN4, and a rise in VIM were observed (Figure 4C). Finally, a non-significant decrease in FASN was observed in the liver of female Wistar rats (Study 4) (Figure 4D). CAR3 was not analyzed in Study 4 samples as the anti-mouse antibody used had no cross-reactivity to rat.

3.5 Post-transcriptional regulation by miRNAs

Post-transcriptional regulation commonplace in biological systems and miRNAs bind complementary to the 3´UTR sequence mediating negative post-transcriptional regulation [27], in turn impacting on the proteome [28]. Thus, we next assessed the modulation of miRNAs levels and explored the potential impact it could have on the proteome. Liver samples from Study 1 were subjected to small RNAs sequencing and miRNAs were analyzed (**Figure 5**). From 247 miRNAs detected in mouse liver samples (Supplementary Table S4), only 4 were found to be differentially expressed in the HT supplemented group after FDR adjustment (Figure 5A). From these, miR-802-5p, miR-30a-5p and miR-146b-5p were up-regulated, whereas miR-423-3p was down-regulated.

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Because one gene can be regulated by different miRNAs, we also searched for validated targets likely to be modulated by more than one miRNA responding to HT treatment (Figure 5B). Gene Interaction (GI) analysis was performed (see Materials and Methods for details) generating a unique list of 279 genes potentially modulated by at least two miRNAs. Genes modulated by the four miRNAs included *Ccdc117*, *Ntrk2*, *Mrpl17*, *Timm22*, *Zfp945*, *Ubxn7*, *Tmem71*, *Slc30a7*, *Gucy1a2*, *4931406C07Rik*, *Zdhhc21*, and *Dclk1* (Figure 5B). In particular, *Gucy1a2* is involved in endothelin signaling pathway, *Zdhhc21* in metabolic processes (palmitoyltransferase), and *Timm22/Ntrk2* in cellular component organization. Gene ontology analysis of modulated miRNAs targets (by more than one miRNA) suggested their involvement in the regulation of major pathways, including the Wnt signaling pathway (P00057), CCKR signaling map (P06959) or the inflammation mediated by chemokine and cytokine signaling pathway (P00031), among others (Figure 5C). Finally, none of the genes matched the ones obtained after the analysis of the transcriptomic or proteomic datasets, suggesting that their levels are not directly controlled by these specific miRNAs.

4 Discussion

Many nutritional intervention studies [29, 30] demonstrated that food and its bioactive components affect the expression of genes, which can impact disease prevention [31–34]. High throughput transcriptome and proteome analysis can be very useful in the discovery of new biomarkers and pathways implicated in metabolic diseases [35]. Moreover, high throughput analyses aid in assessing the physiological effect that bioactive compounds exert on a wide variety of diseases such as diabetes [36, 37], obesity [38], and cancer [39, 40]. Hence, these techniques are useful to explore the mechanisms of action of nutrients and phytochemicals. Omics technologies are widely

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adopted to concomitantly study the expression of thousands of genes and proteins, generating a vast amount of data that accumulates over time and is generally available in public repositories. These data sets could potentially be exploited to establish functional connections among compounds triggering similar responses at the molecular level by using computational approaches involving machine learning tools such as hierarchical clustering [41]. For example, we can predict the pharmacological properties of many molecules across different biological systems and conditions solely based on their transcriptional profiles [41]. Yet, there are few applications of such approaches to the emerging field of nutrigenomics, which investigates the effects of food and nutrients on gene expression [12]. Here, we analyzed the available transcriptomic and proteomic data to 1) identify differentially expressed genes and proteins prevailing among studies addressing hydroxytyrosol supplementation *in vivo* and 2) validate the identified differentially expressed genes and proteins as robust targets of HT.

We first searched for *in vivo* experiments involving dietary supplementation with HT where high throughput gene and protein expression data were generated. Then, we identified a signature of dozens of genes shared among the selected studies which could related to the biological effects associated with HT consumption. Although several genes exhibited different expression in at least two tissues, only four transcripts were significantly modulated in the four tissues we analyzed, i.e. brain, adipose tissue, liver, and intestine. This finding seemed to be particularly relevant, considering that the data were obtained by two independent laboratories [25, 26] and microarrays platforms. Regarding differently expressed proteins, very few candidates (less than five) were identified as commonly modulated in at least two different tissues (heart, aortic, hepatic, and adipose tissues) and none was common to all tissues. Proteomic data were generated from three different laboratories [22, 42, 43]. *In silico* analysis allowed us to

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identify 18 genes and a reduced number of proteins, which were subsequently tested for validation in rodent liver samples. Knowing that the liver is crucial for lipid metabolism and that samples were available from all studies, our hypothesis was that consistent modulation of specific targets of HT could be found in this tissue in animals supplemented with HT.

The transcriptomics studies included in this work were performed in five different tissues: breast, adipose tissue, intestine, liver, and brain. Among the four commonly differentially regulated genes selected for validation, *Efr3a* was down-regulated in all tissues, except for the intestine. *Plscr1* was downregulated in all tissues, whereas *Kctd2* was only downregulated in intestine and liver and *Slc37a4* was upregulated in brain and adipose tissue. Validation of these four genes in liver samples from selected studies (Studies 1-4) confirmed the upregulation of *Slc37a4* in studies 2 and 3. The other genes did not change significantly, suggesting a large inter-study variability. None of the 14 additionally selected genes for validation (Figure 1) changed in all four studies. While some genes only changed in one study (*Anks6*, *Plscr2*, *Lipe*, *Snx16*, *Ppp1cb*, *Top1* and *Tip2*), others changed in two studies (*Sort1*, *Slc37a4*, *Rora* and *Fgf21*). According to our results, Study 4 showed the most reduced changes in gene expression, but we do not know whether this is related to the fact that this study was performed in rats rather than mice. Moreover, in contrast with the other three mice studies receiving HT, in this study rats received secoiridoids [22]. Secoiridoids are major precursors of HT, after their *in vivo* digestion [2]. Regarding the function of these genes, *Sort1* influences plasma lipid concentration [44]. *Slc37a4* is a glucose-6-phosphate translocase (G6PT), which transports G6P from the cytoplasm into the endoplasmatic reticulum (ER) lumen, and is involved in glucose metabolism [45]. *Rora* is a nuclear receptor involved in multiple biological processes, including lipid

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metabolism [46]. *Fgf21* is a metabolic gene that influences plasma glucose and triglyceride levels [47], and is a critical regulator of liver lipid homeostasis [48]. Moreover, FGF21 is induced directly by PPARalpha in liver in response to fasting [49] and its induction is required for the normal activation of hepatic lipid oxidation and triglyceride clearance [48]. As such, induced expression of *Fgf21* by HT feeding could be beneficial against metabolic diseases. Because *Fgf21* and *Rora* are important contributors to metabolic diseases, we further validated their response to HT supplementation in a different cohort [20]. Unexpectedly, C57Bl6J mice receiving a single ingestion of HT dramatically increased their hepatic expression of *Fgf21* at 1, 2, and 4 h post-ingestion. This effect was also observed for *Rora*, but to a lower degree 4 h post-ingestion (**Figure 6**). In this sense, our bioinformatic approach and further validation uncovered novel possible molecular targets of the beneficial effects of HT consumption. A previous study performed in a different mouse model showed that the repression of *Fgf21* produced by a high fat diet was reverted by HT supplementation [50]. These and our current data suggest *Fgf21* as a *bona fide* candidate target of HT. Whether this effect occurs in humans is unknown and deserves further investigation.

In the study by Tome-Carneiro et al, 2017 (Study 1), a decrease in the expression of FASN and PRDX1 was recorded, by both high throughput proteomics and WB, in mice supplemented with HT for eight weeks (Tomé-Carneiro et al., 2017). However, WB revealed only non-significant changes for these proteins in liver samples from the studies tested for validation. WB analysis also revealed non-significant changes for CAR3 in the studies used for validation, despite it being reported as upregulated by high throughput proteomics in Study 1.

By high throughput proteomics, VIM was significantly changed in adipose tissue samples from Study 1 and aortic tissue from Study 4. Here, however, WB

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analysis showed non-significant changes for these proteins in the liver samples used for validation. The statistically significant changes seen for ACTN4 proteins in liver (Study 1), aortic, and heart tissues (Study 4), by WB, were not confirmed in liver samples from the studies used for validation. Likewise, the statistically significant changes seen for HSPD1 in adipose (Study 1), hepatic (Study 3) and aortic (Study 4) tissues were not confirmed, by WB, in liver samples from the studies used for validation. Overall, we found inconsistency regarding differently expressed proteins among studies, as we were unable to validate neither in liver samples from independent studies involving HT supplementation.

It is important to note that the transcriptional data analysis was performed independently of the protein data levels and that correlations between transcripts and proteins were not intended during the validation process. Also, it is relevant to mention that the list of common genes or proteins to be validated were common to at least two different studies, regardless if tissues subjected to high throughput analysis matched or not. Indeed, in most cases, tissues from where transcripts (intestine, liver, adipose, brain or breast tumor) and proteins (liver, heart, aortic, or adipose) levels were selected did not match. Thus, the lack of consistent validation of potential targets of HT in response to dietary supplementation should be seen with caution.

Other aspects of the complex regulatory variation from RNA to protein may account for the lack of common tissue features in response to HT supplementation. For instance, studies in model organisms and humans have shown that variations in mRNA and protein expression levels are often uncorrelated [51, 52]. Few transcripts are exclusive to a particular tissue and varies more across tissues than individuals [53], while genetic variation can also influence the heterogenicity of protein expression in a diverse set of human tissues [52, 54]. Moreover, differences may also arise from

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alterations in posttranslational regulation. Regulation by ncRNAs, particularly miRNAs, is among the plethora of posttranslational controlling pathways.

Although in the last years increasing evidence suggests that food bioactive compounds can modulate the expression of miRNAs *in vitro* [55], in animal models [56], and in humans [57], very few have specifically focused on the action of HT. For example, specific miRNAs, miR-9 [58] and miR-146a [59], were evaluated *in vitro*, whereas only one study evaluated the whole miRNome in mouse small intestine [20]. Among liver modulated miRNAs in response to HT supplementation, miR-802-5p has been previously described as being obesity-induced and as being involved in glucose metabolism impairment and in angiotensin signaling regulation [60, 61]. As for miR-423-3p, its levels have been positively associated to cell growth in liver, colon or other types of cancers [62, 63]. Induction of miR-30a-5p has been previously described to ameliorate liver fibrosis [64] or to suppress breast tumor growth and metastasis [64, 65]. miR-146b has been shown to attenuate non-alcoholic steatohepatitis [66], although its down-regulation has been shown to promote cancer growth and metastasis [67].

Sustained intake by mice of HT at dietary doses resulted in altered miRNA expression in intestine (assessed in Study 1) and liver (assessed here). Of note, HT supplementation resulted only in a consistent ($p < 0.05$) regulation of miR-802-5p in both tissues (**Supplementary Figure S1**). The reduced number of common modulated miRNAs found could be explained by different aspects of miRNAs biogenesis, function, and technical analysis. For example, some miRNAs are tissue-specific [68]. Also, though the processing pattern of miRNAs in tissues and cell lines may differ, it has been reported that, especially in cell lines, several transcribed miRNAs are not processed to mature miRNA [69]. While miRNAs might play a role in the biological action of HT, the very reduced number of HT studies evaluating miRNAs precludes any

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conclusion regarding their regulatory potential at this point. However, the consistent induction of miR-802-5p in two different tissues, in response to dietary HT supplementation, seems to support a miRNA modulating action of small natural molecules, which could be exploited as a potential therapeutic alternative or adjuvant to the current pharmacological arsenal targeting endogenous miRNAs.

5 Conclusions

High throughput transcriptomics and proteomics are powerful tools that greatly contribute to the knowledge of how nutrition affects the expression of a wide number of genes and proteins. *In vivo* studies where these techniques are employed to investigate the molecular effects of hydroxytyrosol are scant yet increasing. Therefore, we think there is a growing need to integrate the accumulating data in order to identify consistent targets of this bioactive compound. Most of the genes and proteins identified and tested here as potential HT targets showed inconsistent modulation by HT. These results are, at least in part, due to the limited number of *in vivo* studies available, with heterogenous HT doses and supplementation times, where different tissues were used for transcriptomic/proteomic analysis. However, our transcriptomic analysis uncovered only two novel potential HT target candidates, i.e. *Fgf21* and *Rora*. While we certainly do not want to depreciate the important role of omics and related database, we think that more attention should be paid to the current pitfalls of this approach to nutritional research. Over-emphasis should be avoided and more HT-supplementation studies employing high throughput transcriptomics and proteomics tools are needed for potential HT targets to be identified and validated.

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Conflict of interest

The authors declare no conflicts of interest related to this work.

Authors´ contribution

MCLH, RMH and MCC contribute equally to this work. AD, MCLH and FV contributed to the conception or design of the work. RMH, MCC, JT-C, MB R-R and LdP contributed to data collection. LR, MJM, JO and MN contributed with samples from different studies. AD, FV, JAM and MPP obtained financial support. MCC, JT-C, MCLH, RMH, AD and FV drafted the article. JAM, JO, JCE-G and MPP revised the manuscript for important intellectual contribution. All authors reviewed and approved the manuscript.

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Figure legend

Figure 1. Venn diagram showing the common differentially expressed genes identified through bioinformatic analysis. Transcriptomic data available in public repositories from *in vivo* dietary supplementation with hydroxytyrosol. Gene differential expression was analyzed using LIMMA models.

Figure 2. Validation of common transcripts predicted to be modulated by hydroxytyrosol supplementation. A set of transcripts were chosen from bioinformatic analysis and validated in liver samples of different intervention studies. Gene expression was analyzed by RT-qPCR. (A) Male young C57BL/6 mice (n=7 per group) fed with a control or HT diet (45 mg HT/kg bw/day), for 8 weeks (Study 1). (B) Male young C57BL/6 mice were administered (gavage) an acute dose of 15 mg of HT (dissolved in water) and sacrificed 4h after ingestion (n=9) (Study 2). (C) Male young homozygous apoE KO mice (n=7 per group) fed an aqueous solution of 10 mg HT/kg/day (n=7), for 10 weeks (Study 3). (D) Female Wistar rats (300–350 g, n=4 per group) fed with a standard diet (SD) or SD supplemented with 5 mg HT/kg/day, for 21 days (Study 4). HT; hydroxytyrosol.

Figure 3. Venn diagram showing the common differentially expressed proteins identified by means of bioinformatic analysis. Proteomic data available in the scientific literature from *in vivo* dietary supplementation with hydroxytyrosol.

Figure 4. Validation of common proteins predicted to be modulated by HT supplementation. A set of proteins were chosen after bioinformatic analysis of proteomic data and validated in liver samples of different intervention studies. Protein expression was analyzed by Western blot. (A) Male young C57BL/6 mice (n=7 per group) fed with a control or HT diet (45 mg HT/kg bw/day), for 8 weeks (Study 1). (B)

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Male young C57BL/6 mice administrated (gavage) with an acute dose of 15 mg of HT dissolved in water and sacrificed 4h after ingestion (n=9) (Study 2). (C) Male young homozygous apoE KO mice (n=7 per group) fed an aqueous solution of 10 mg HT/kg/day (n=7), for 10 weeks (Study 3). (D) Female Wistar rats (300–350 g, n=4 per group) fed with a standard diet (SD) or SD supplemented with 5 mg HT/kg/day, for 21 days (Study 4). HT; hydroxytyrosol.

Figure 5. **Liver miRNA analysis**. (A) Scatter plot of RNA-seq data of liver miRNAs from mice supplemented with HT, for 8 weeks. (B) Genetic interaction analysis between miRNAs and their likely miRNAs targets. Target point sizes are directly correlated with the number of interactions within the set of miRNAs. (C) Functional enrichment analysis of differentially expressed miRNAs targets. HT, hydroxytyrosol.

Figure 6. Hydroxytyrosol target liver mRNA expression of *Fgf21* and *Rora*. Effects of hydroxytyrosol on *FGF21* (A) and RORA (B) relative gene expression in liver samples of C57Bl6J mice at different time points (1, 2 and 4 hours). Data shown as mean ± SEM. * $p < 0.05$ compared to Control group; ** $p < 0.0001$ compared to Control group. (n=9 per group).

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Table 1. Function of the genes and list of primers designed for the validation of the studies.

Gene	Primers		Function
	Forward	**Reverse**	
B-*Efr3a*	CTTTGCGTCCTCG CTACAAAC	CCATATCAGCTTTA ACAAGGCCA	Involved in the functional maintenance of sensory and motor nervous tissues.
B-*Kctd2*	CCTACTTCGTGAC CACCAGAC	GAGTTTTCCATGGC GGAGGT	Potassium channel tetramerization domain containing protein 2.
B-*Plscr1*	GGTCCGTGTGTTG TGTGTAG	TGCTCCTCGTTTCC AGTTCTT	Involved in Phosphatidylserine externalization regulation during cell activation.
B-*Slc37a4*	AACCGCAAAACC TTCTCCTT	TACGTTGACCAGA CCAACCA	Regulates glucose-6-phosphate transport and maintain glucose homeostasis.
B-*Ppp1cb*	CAGAAGTCCGAG GGTTGTGTA	CAGATGGTTTCCAA AGACTGCTT	Involved in the regulation of cell division, glycogen metabolism, muscle contractility.
B-*Tjp2*	GTTTGCCGTTCAG CAGCTTAG	CTTCAAAACCTCGG TCGTCAT	Component of the tight junction barrier in epithelial and endothelial cells.
B-Top1	GCCAAGGTGTTCC GTACCTA	TCAGGTCCTTTCGA GCATCT	Essential for cell growth and division *in vivo*.
B-Snx16	CCAGAAGAAAGC TGGGTAGTTTT	GGAAGTGCTAATC GAAAGCCTG	Involved in cholesterol transport, and transport of tetraspanin CD81.
B-Anks6	GGAGCTGGGGAT TAAGACGG	TAGAATCTGCCTCT CACGCC	Plays a role in renal and cardiovascular development.
B-Plscr2	CTGGGTATGCCCC TCAGTATC	GGGAACTTGGTAG TTAGTCTGGA	Plays active role in altering lipid asymmetry at the plasma membrane.
B-Soat1	GAAGGCTCACTC ATTTGTCAGA	GTCTCGGTAAATA AGTGTAGGCG	Catalyzes the formation of fatty acid-cholesterol esters.
B-Fgf21	CAGATGTGGGTTC CTCCGAC	AAGATGCATAGCT GGGGCTT	Secreted endocrine factor that functions as a major metabolic regulator.
R-*Crot*	AAGCCGGGTGCA GGAGTTTT	CCACTCTTCCAGCC AGTTTCT	Plays a role in lipid metabolism and fatty acid beta-oxidation.
M-*Crot*	GAACGGACATTT CAGTACCAGG	CTTCATTTGCGAAT GGTTTCACT	
B-Rora	GTGGAGACAAAT CGTCAGGAAT	GACATCCGACCAA ACTTGACA	Controls lipid homeostasis by negatively regulating transcriptional activity of PPARγ, that mediates hepatic lipid metabolism.
B-Sorl1	CCCAGCCTATCCA GGTGTATG	CGGGCTAATGCCA CGATCA	Binds LDL and transports it into cells by endocytosis.
B-Elovl1	GAAGAAGGACGG GCAAGTGA	TTGCAGCTGGGCAT GAAGTA	Involved as precursors of membrane lipids and lipid mediators.
B-Acsl4	CTCACCATTATAT TGCTGCCTGT	TCTCTTTGCCATAG CGTTTTTCT	Plays a key role in lipid biosynthesis and fatty acid degradation.
B-Lipe	GTTACCACCCTGC	AAGTGTCTCTCTGC	Converts cholesteryl esters to free

	AGTCCTC	ACCAGC	cholesterol for steroid hormone production.
B-Lpin2	GAAGTGGCGGCT CTCTATTTC	AGAGGGTTACATC AGGCAAGT	Plays a role in triglyceride metabolism.

Efr3a: EFR3 homolog A; *Kctd2*: potassium channel tetramerization domain containing 2; *Plscr1*: phospholipid scramblase 1; *Slc37a4*: solute carrier family 37 member 4; *Ppp1cb*: protein phosphatase 1 catalytic subunit beta; *Tjp2*: tight junction protein 2; *Top1*: DNA topoisomerase I; *Snx16*: sorting nexin 16; *Anks6*: ankyrin repeat and sterile alpha motif domain containing 6; *Plscr2*: phospholipid scramblase 2; *Soat1*: sterol O-acyltransferase 1; *Fgf21*: fibroblast growth factor 21; *Crot*: carnitine O-octanoyltransferase; *Rora*: RAR-related orphan receptor alpha; *Sorl1*: sortilin related receptor 1; *Elovl1*: ELOVL fatty acid elongase 1; *Acsl4*: acyl-CoA synthetase long chain family member 4; *Lipe*: hormone sensitive type lipase E; *Lpin2*: lipin 2; B: Primer designed for both species, mus musculus and Rattus norvegicus; R: Rattus Norvegicus; M: Mus musculus.

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Table 2. Selected proteins and type of antibodies used.

Proteins	**Company**	**Molecular Weight (kDa)**	**Host**	**Function**
CAR3	Thermo Fisher	29.6	Rabbit	Involved in oxidative stress.
FASN	Cell Signaling	273	Rabbit	Main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA.
PRDX1	Cell Signaling	21	Rabbit	Belongs to a family of antioxidant enzymes. Reduction of hydrogen peroxide and alkyl hydroperoxides.
VIM	Cell Signaling	57	Rabbit	Involved in neurogenesis and cholesterol transport.
GAPDH	Sigma	37	Mouse	Housekeeping protein.
HSPD1	Bethyl	60	Rabbit	Involved in stress response.
ACTN4	Bethyl	110	Rabbit	Transcriptional coactivator, stimulating transcription mediated by the nuclear hormone receptors PPARG and RARA.

CAR3: carbonic anhydrase 3; FASN: fatty acid synthase; PRDX1: peroxiredoxin 1; VIM: vimentin; HSPD1: heat shock protein family D (Hsp60) member 1; ACTN4: actinin alpha 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

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Table 3. *In vivo* studies involving supplementation with hydroxytyrosol where transcriptomic analyses were performed.

Model	Dose & time	Analysis	Aim of study	Reference
Male C57BL/6J mice	5 mg/kg bw HT.	qRT-PCR	Evaluate the molecular adaptations in liver involved in the anti-lipogenic, anti-inflammatory, and anti-oxidant effects of HT	[8]
Male C57BL/6J mice	20 mg HT/kg bw/day 21 days.	qRT-PCR	Identify early, predictive biomarkers for WAT expansion.	[70]
Male db/db mice	10 or 50 mg HT/kg/day. 8 wk	qRT-PCR	Evaluate the neuroprotective effects of HT in db/db mice and SH-SY-5Y neuroblastoma cells.	[71]
Sprague–Dawley rats	10 or 50 mg HT/kg/day. During gestation.	qRT-PCR	Investigate HT effect on prenatal stress	[72]
Male C57BL/6 mice	~45 mg HT/kg bw/day. 8wk	Microarray qRT-PCR	Nutrigenomic effects of HT with specific reference to the adipose tissue and glutathione metabolism.	[26]
Female Sprague–Dawley rats	0.5 mg/kg 6wk	Microarray qRT-PCR	Hydroxytyrosol inhibits growth and cell proliferation and promotes high expression of sfrp4 in rat mammary tumours.	[25]
C57BL/6 male mice	0.03 g% HT 8wk	Microarray qRT-PCR	Chronic hydroxytyrosol feeding modulates glutathione-mediated oxido-reduction pathways in adipose tissue: A nutrigenomic study	[26]
Male C57BL/6 mice	0.03 g% HT 8wk	Microarray qRT-PCR	Hydroxytyrosol supplementation modulates the expression of miRNAs in rodents and in humans.	[20]

HT: hydroxytyrosol;

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Table 4. *In vivo* studies involving supplementation with hydroxytyrosol where high throughput proteomic analyses were performed.

Model	**Dose & time**	**Aim of study**	**Ref.**
Male C57BL/6 mice	~45 mg HT/kg bw/day. 8 wk	Impact of long-term HT supplementation on the proteome in metabolically active tissues (adipose tissue and liver)	[42]
Female Wistar rats	5 mg/kg/day 21 days	Proteomic analyses in cardiovascular tissues (aorta and heart)	[22]
Male Rowett Hooded Lister rats	10 mg/kg diet. 12 wk	Effects in liver through proteomics and network analysis	[43]

HT: hydroxytyrosol; ACTN4: alpha-actinin -4; RPL8: 60S ribosomal protein L8; ALDH2: mitochondrial aldehyde dehydrogenase

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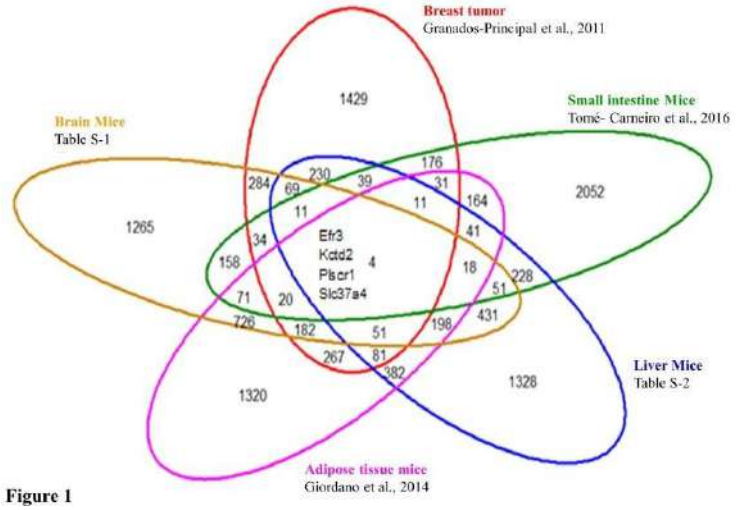

Figure 1. Venn diagram showing the common differentially expressed genes identified through bioinformatic analysis. Transcriptomic data available in public repositories from in vivo dietary supplementation with hydroxytyrosol. Gene differential expression was analyzed using LIMMA models.

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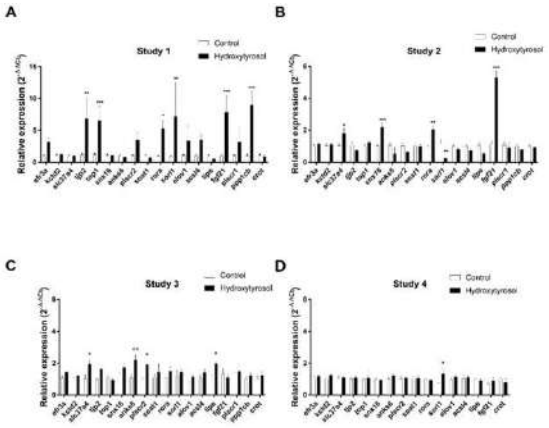

Figure 2

Figure 2. Validation of common transcripts predicted to be modulated by hydroxytyrosol supplementation. A set of transcripts were chosen from bioinformatic analysis and validated in liver samples of different intervention studies. Gene expression was analyzed by RT-qPCR. (A) Male young C57BL/6 mice (n=7 per group) fed with a control or HT diet (45 mg HT/kg bw/day), for 8 weeks (Study 1). (B) Male young C57BL/6 mice were administered (gavage) an acute dose of 15 mg of HT (dissolved in water) and sacrificed 4h after ingestion (n=9) (Study 2). (C) Male young homozygous apoE KO mice (n=7 per group) fed an aqueous solution of 10 mg HT/kg/day (n=7), for 10 weeks (Study 3). (D) Female Wistar rats (300–350 g, n=4 per group) fed with a standard diet (SD) or SD supplemented with 5 mg HT/kg/day, for 21 days (Study 4). HT; hydroxytyrosol.

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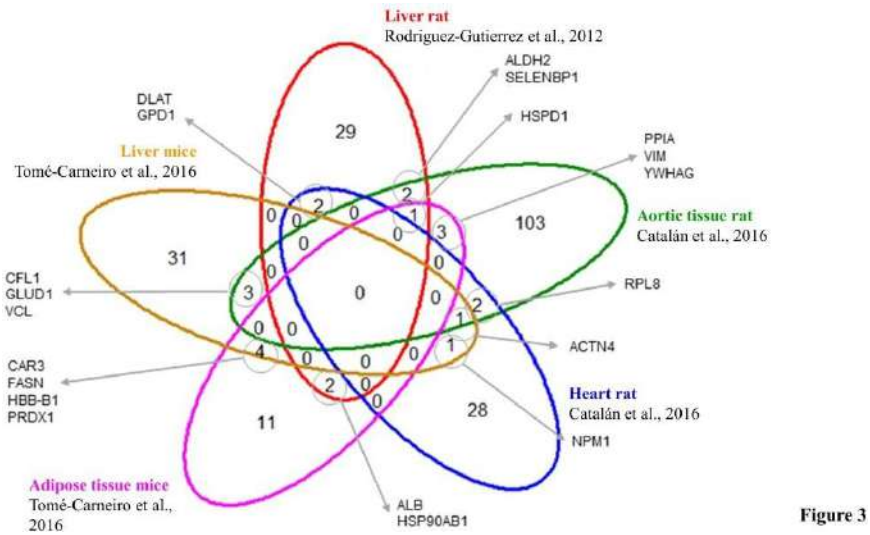

Figure 3. Venn diagram showing the common differentially expressed proteins identified by means of bioinformatic analysis. Proteomic data available in the scientific literature from in vivo dietary supplementation with hydroxytyrosol.

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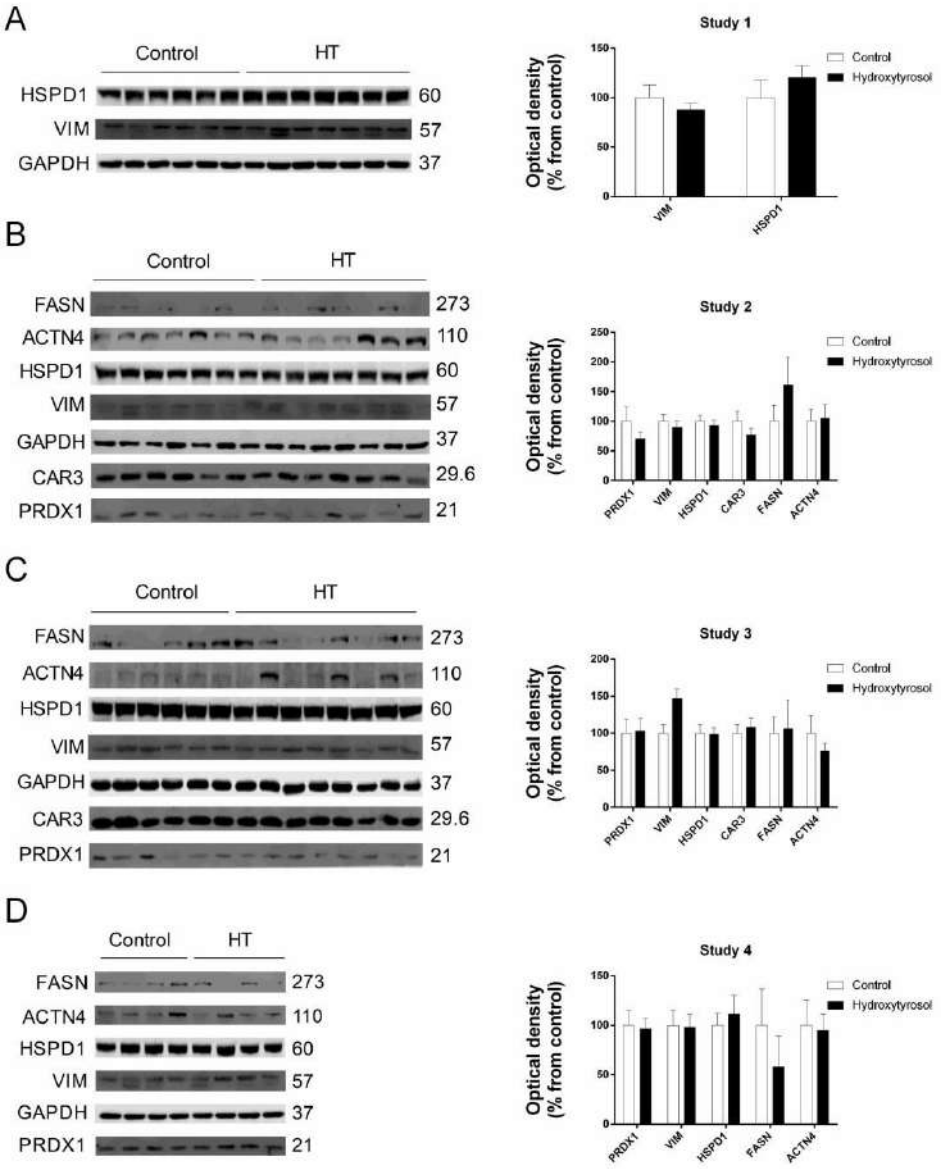

Figure 4. Validation of common proteins predicted to be modulated by HT supplementation. A set of proteins were chosen after bioinformatic analysis of proteomic data and validated in liver samples of different intervention studies. Protein expression was analyzed by Western blot. (A) Male young C57BL/6 mice (n=7 per group) fed with a control or HT diet (45 mg HT/kg bw/day), for 8 weeks (Study 1). (B) Male young C57BL/6 mice administrated (gavage) with an acute dose of 15 mg of HT dissolved in water and sacrificed 4h after ingestion (n=9) (Study 2). (C) Male young homozygous apoE KO mice (n=7 per group) fed an aqueous solution of 10 mg HT/kg/day (n=7), for 10 weeks (Study 3). (D) Female Wistar rats (300–350 g, n=4 per group) fed with a standard diet (SD) or SD supplemented with 5 mg HT/kg/day, for 21 days (Study 4). HT; hydroxytyrosol.

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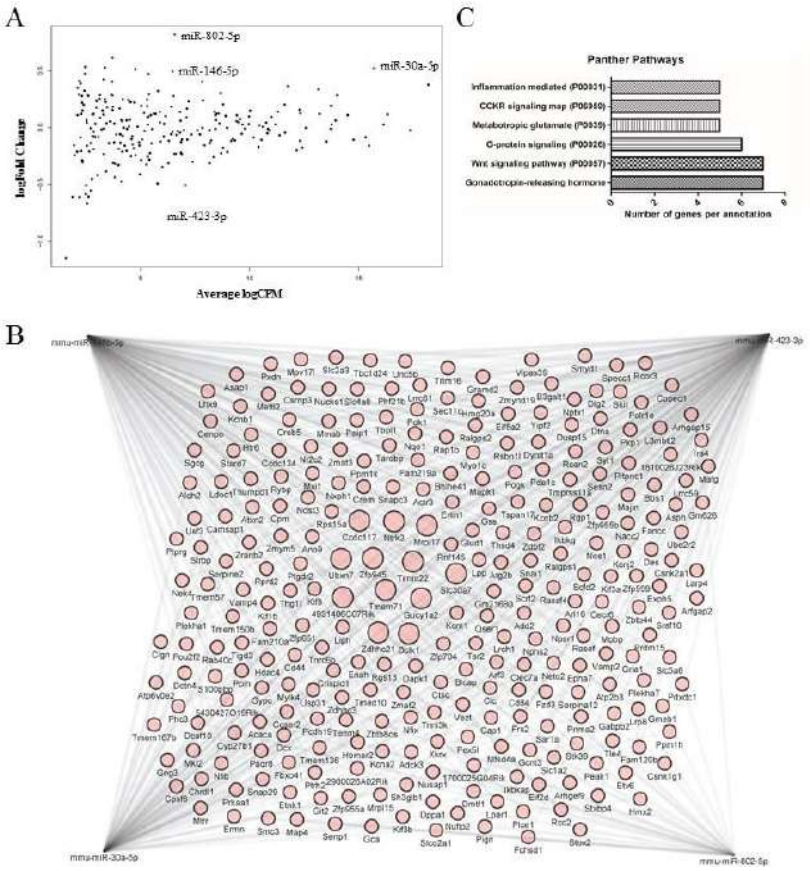

Figure 5. Liver miRNA analysis. (A) Scatter plot of RNA-seq data of liver miRNAs from mice supplemented with HT, for 8 weeks. (B) Genetic interaction analysis between miRNAs and their likely miRNAs targets. Target point sizes are directly correlated with the number of interactions within the set of miRNAs. (C) Functional enrichment analysis of differentially expressed miRNAs targets. HT, hydroxytyrosol.

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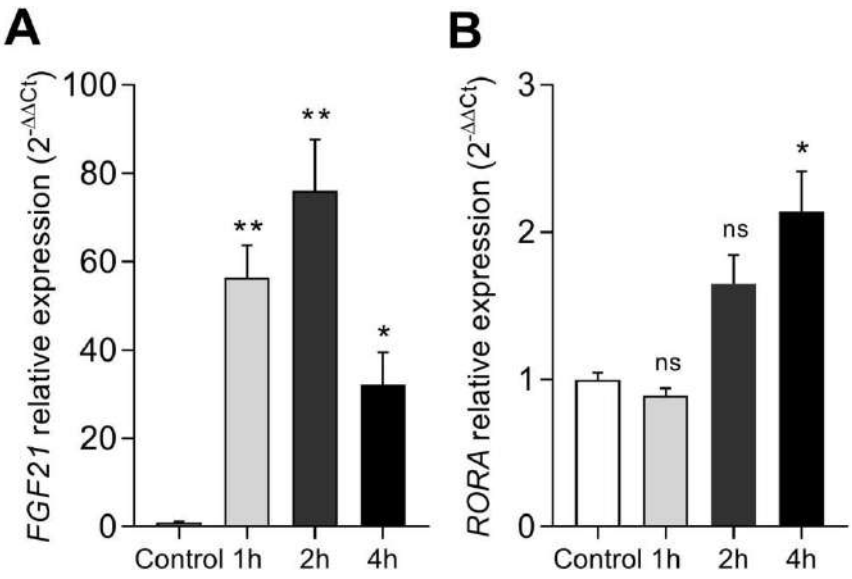

Figure 6. Hydroxytyrosol target liver mRNA expression of Fgf21 and Rora. Effects of hydroxytyrosol on FGF21 (A) and RORA (B) relative gene expression in liver samples of C57Bl6J mice at different time points (1, 2 and 4 hours). Data shown as mean ± SEM. * $p < 0.05$ compared to Control group; ** $p < 0.0001$ compared to Control group. (n=9 per group).

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