Molecular Determinants of the Cardiometabolic Improvements of Dietary Flavanols Identified by an Integrative Analysis of Nutrigenomic Data From a Systematic Review of Animal Studies

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Abstract

Flavanols are important polyphenols of the human diet with extensive demonstrations of their beneficial effects on cardiometabolic health. They contribute to preserve health acting on a large range of cellular processes. The underlying mechanisms of action of flavanols are not fully understood but involve a nutrigenomic regulation. To further capture how the intake of dietary flavanols results in the modulation of gene expression, nutrigenomics data in response to dietary flavanols obtained from animal models of cardiometabolic diseases have been collected and submitted to a bioinformatics analysis. This systematic analysis shows that dietary flavanols modulate a large range of genes mainly involved in endocrine function, fatty acid metabolism, and inflammation. Several regulators of the gene expression have been

predicted and include transcription factors, miRNAs and epigenetic factors. This review highlights the complex and multilevel action of dietary flavanols contributing to their strong potential to preserve cardiometabolic health.

1. Introduction

Polyphenols constitute a very large group of plant-derived bioactive compounds that include multiple families structurally distinct, such as stilbenes, phenolic acids, lignans and flavonoids. The flavonoid group can be further divided into six different subclasses based on their chemical structure: flavones, isoflavones, flavanones, flavonols, anthocyanins and flavanols (also known as flavan-3-ols), with these latter being present as both monomeric (catechins) and polymeric (proanthocyanidins) compounds. The group of catechins includes catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Overall, flavanols represent the most commonly consumed flavonoids and are particularly abundant in (green) tea, cocoa, grapes, apples, red wine, and also in many whole and processed foods and dietary supplements.

A wealth of epidemiological studies has documented an inverse correlation between the intake of polyphenol-rich foods and the incidence of cardiometabolic diseases [1]. More recently, strong meta-analyses of prospective cohort studies have established that high consumption of flavonoids reduced the incidence of cardiovascular diseases (CVD) and type 2 diabetes (T2D), with associations remaining significant when considering individual classes of flavonoids [2, 3]. Regarding flavanols intake and cardiometabolic health, a recent systematic review and meta-analysis of randomized trials and prospective cohort studies concluded on a beneficial effect of flavanols, irrespective of dietary source, on a range of cardiometabolic outcomes [4]. However, this paper also underlined heterogeneity in the meta-analysis leading to a variable strength of evidence for the different outcomes. More

specifically the intake of flavanols can improve cardiometabolic health by increasing the production of vasoprotective agents, especially nitric oxide (NO), by reducing circulating lipids (triglycerides (TG), low-density lipoprotein (LDL)-cholesterol, free fatty acids (FFA)) and their accumulation in liver and adipose tissues, by preventing weight gain, improving glucose homeostasis (i.e., blood glucose and insulin levels) and lowering blood pressure [5-8]. These cardioprotective effects have been demonstrated to be linked to the activation of several pro-survival cellular pathways that involve metabolic intermediates, microRNAs (miRNAs), sirtuins and mediators of the reperfusion injury salvage kinases (RISK), and survivor activating factor enhancement (SAFE) pathways [9]. Based on single dosing of flavanols administered in animal models, it was shown that the enhancement of energy expenditure induced by flavanols might represent a plausible explanation for their anti-obesity effects [10], which appeared to be associated with increased catecholamine secretion [11]. Of note, the flavanol-induced effects are dependent on the dose and the background diet [12]. Grapes and grape juice (rich in catechin and epicatechin) seem capable of ameliorating endothelial function and reducing LDL oxidation via an antioxidant activity [13, 14]. Flavanols were also shown to have mitochondrial biogenesis-inducing effects in skeletal muscle [15], which might underlie part of their positive effects on glucose homeostasis observed in both animal models and humans [16-18].

Cumulatively, it appears that a plethora of different mechanisms have been identified underlying the flavanol-induced effects *in vivo*, urging us to review here those solely for the flavanols-induced effects in relation to cardiometabolic risk improvement. The use of preclinical animal studies is appropriate to investigate, under highly controlled conditions, the physiological effects following specific dietary intakes. These studies are of particular relevance as they allow to establish a causality relationship between flavanols consumption and cardiometabolic outcomes and subsequently contribute to help elucidate tissue-specific

cellular mechanisms. In particular, animal intervention studies offer a unique opportunity to address the systemic interaction of the underlying transcriptional and cellular regulatory networks. On this background, we decided to perform a systematic and integrative analysis of the nutrigenomic effects exerted by flavanols in different animal models of cardiometabolic disease (i.e., the effect of flavanols on gene expression) with the aim to identify the key molecular determinants involved in the cardiometabolic protective effects of these dietary components.

2. Methods

The present study complied with the Preferred Reporting Items for Systematic Reviews statement [19].

2.1 Literature search strategy

A comprehensive search on PubMed and Web of Science was conducted in December 2019. The search included keywords referring to bioactives (catechin, epicatechin, epigallocatechin gallate, and proanthocyanidin), type of study and animal species (*in vivo*, animal experimentation, animal model, mouse, mice, murine, musculus, rats, Rattus, rodent, rabbit, Cuniculus, dog, Canis, Guinea Pig, cat, swine, pigs, porcus), nutrigenomic analysis (gene expression, miRNA, transcripts, nutrigenomic, TaqMan low density array (TLDA), microarray, genomic, mRNA, western-blot), target tissues and cardiometabolic outcomes (aorta, vessel, vascular, heart, fat, adipose tissue, liver, muscle, circulating cells, blood cells, peripheral blood mononuclear cells (PBMC), blood pressure, hypertension, myogenic tone, vascular tone, atherosclerosis, LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, cholesterol, TG, body weight, inflammatory markers, insulin, Homesostatic Model Assessment of Insulin Resistance (HOMA-IR), glucose, glycemia, adipokines, anti-oxidant, homocysteinemia).

2.2 Study Selection and Data Extraction

Studies included in this analysis were limited to animal interventions investigating the effects of flavanols (pure compounds or extracts from tea, cocoa, grape or apple), which had a control group receiving placebo or no treatment and demonstrated the improvement of one or more of the predefined cardiometabolic outcomes. Two authors independently assessed all full-text papers and in the case of disagreement, a third author was contacted. Manuscripts written in any European language were included, whereas other manuscripts were excluded. Data extraction was performed using a template. Extracted data included: publication details (authors, year of publication, ID of the publication); animal model characteristics (species, strain/model, age, gender); elements of study design (tested compound, dose used for the supplementation, duration of the intervention); cardiometabolic outcomes (type and changes in the outcome); and gene expression data (type of tissue, official gene name, official gene symbol, modulation of gene expression). The final database was cross-checked by two authors.

2.3 Bioinformatics analysis of gene expression data

Differentially expressed genes were submitted to Gene Set Enrichment Analysis (GSEA) using EnrichR (https://amp.pharm.mssm.edu/Enrichr/) to identify significantly over-represented signalling pathways (referred by the Kyoto Encyclopedia of Genes and Genomes, KEGG) that can be modulated in response to flavanol intake. Fisher's exact test with Benjamini-Hochberg correction was used to determine the significance of the enrichment results. Only pathways related to cardiometabolic health with an adjusted-p-value < 0.01 were considered. A rank score was also computed using a modification to Fisher's exact test and used to build clustergram showing the association between the input genes and the

overlapping genes of the enriched term [20]. Only pathways related to cardiometabolic health with an adjusted-p-value < 0.01 were considered. A network analysis based on these enriched terms has been rendered using Cytoscape software [21] with ClueGO associated application [22]. Transcription Factor Enrichment Analysis (TFEA) was performed using Hypergeometric Optimization of Motif EnRichment (HOMER) to identify the significant transcription factors that may explain gene expression modulation in response to flavanols [23]. TFEA was performed on down- and up-regulated gene sets separately for each tissue. Gene sets were submitted to miRwalk 3.0 (http://mirwalk.umm.uni-heidelberg.de) for the search of miRNA regulators using a random-forest-based statistical approach [24]. Visualization of the interactions between mRNAs, mRNA-TFs and miRNAs-targets was performed in Cytoscape software (version 3.7.1; http://www.cytoscape.org/) [25].

3. Results

3.1. Description of the included studies

The process of selection applied in this systematic review is summarized in Figure 1. A total of 785 papers were initially identified in PubMed and Web of Science databases using our identified keywords. After the removal of duplicates and the first screening step by analysing the title and abstract, we used criteria defined in our search strategy to retrieve studies for the present analysis. As a consequence, 187 full-text papers were selected for data extraction. 27 papers were excluded during the detailed analysis of full text due to: the lack of relevant cardiometabolic outcome(s), analysis of gene expression restricted to the protein level, non-preclinical animal study, publication language, extracts from unexpected food source, use of animal model not relevant to cardiometabolic investigation (e.g., hepatotoxicity and surgically induced hepatic injury). Finally, significantly and differentially expressed genes at the mRNA level in response to flavanol supplementation and associated with improvement of

at least one of the predefined cardiometabolic outcomes, were extracted from 81 papers. 75 papers reported studies that had adopted a targeted approach to analyse the expression of genes (i.e., using mostly quantitative RT-PCR for a specific set of genes) in rodent models (37 in mouse, 38 in rat), whereas 6 had adopted a holistic approach using various whole genome array assays. Gene expression data obtained using a targeted approach (supplementary tables 1 and 2) have been consolidated, then submitted to bioinformatics analyses to decipher the underlying mechanisms of action by which dietary flavanols may impact on cardiometabolic health.

3.2. Overview of the beneficial effects of flavanols on cardiometabolic outcomes in rodent models

Cardiometabolic outcomes assessed in the studies included in the present work are presented in supplementary tables 1 and 2. Several well established rodent models of CMD, including genetic (e.g. LDLr-/- or ApoE-/-mice, Zucker rats) and induced CMD (e.g. chemically induced by NOS inhibitor, ouabain, angiotensin, or induced by cafeteria-, western-, high fat- or high sucrose-diets) have been employed to evaluate the impact of dietary flavanol supplementation on a large range of cardiometabolic outcomes. Acute supplementation with 250 mg/ kg BW / day (equivalent to a human equivalent dose (HED) of ~ 40 mg / kg / day in a 60 kg human) of grape seed procyanidin extract (GSPE) in healthy rats and mice decreases plasma triglycerides [26, 27], and hepatic triglycerides and cholesterol [26, 28]. Similarly, the long-term intake (between 3 to 20 weeks) of grape seeds extracts (25-1,000 mg / kg / day; HED: 4-160 mg / kg / day) [13, 29-36], tea (500-860 mg / kg / day) [13, 37-42], EGCG (50-300 mg / kg / day) [43-51] or apple flavanols extracts (0.5 % diet) [52] improves triglyceridemia [29, 32, 34, 39-41, 45, 46, 48-50], cholesterolemia [29, 32-34, 40, 46, 49, 50], plasma free fatty acids (FFAs) levels [33, 49], glycemia [34, 36, 41, 45, 46, 48, 50, 52],

insulinemia/HOMA-IR [30, 36, 40, 41, 43, 45, 46, 52], and circulating inflammatory markers (e.g. C-reactive protein (CRP)) [31], reduces hepatic cholesterol [29, 43] and triglycerides [29, 39, 43], and attenuates body weight gain [35-41, 43, 46, 48]. Similarly, procyanidin B2 at 50-150 mg / kg / day improves glucose homeostasis and decreases hepatic steatosis [53]. Catechin [54-56] and EGCG [57, 58] given at 30 mg / kg / day (HED: 2.5 mg / kg / day) and 25 mg / kg / day (HED: 2 mg / kg / day), respectively, present anti-inflammatory properties as observed by a decreased plasma level of interleukin-6 (IL-6) or tumor necrosis factor (TNF). These supplementations also preserve aorta relaxation and slow down the development of atherosclerotic lesion. EGCG (25-50 mg/kg/day for a week) also decreased systolic blood pressure and serum inflammation (CRP) in angiotensin II-induced hypertensive rats [59]. In addition, the systemic inflammation in N(G)-Nitro-L-arginine-methyl ester (L-NAME)-induced hypertensive rats is decreased by 4-week supplementation with 2-10 mg / kg / day epicatechin (HED: 0.3-16 mg / kg /day) [60]. In T2D rat models, a chronic intake of pure catechin (20 mg / kg / day; HED: 3.2 mg / kg / day) or EGCG (0.1 % in diet, that is equivalent to ~20 mg / day for a rat) reduced blood glucose [61] and improved plasma insulin level [62], respectively. All these aforementioned health effects were also observed in a large range of studies investigating the effects of flavanols supplementation on high fat diet (HFD)-induced cardiometabolic disorders [37-40, 42, 44, 46, 48, 49, 53, 63-92]. Taken together, these studies support that dietary flavanols contribute to improve cardiometabolic health in animals by reducing circulating lipids (TG, LDL-cholesterol, FFA) and their accumulation in the liver and adipose tissues, therefore preventing weight gain, and by improving glucose homeostasis.

3.3. Nutrigenomic impact of flavanols in cardiometabolic tissues in rodent models

A detailed analysis of studies using a targeted approach to assess the gene expression profiles retrieved a set of 110 and 77 differentially expressed genes, in response to flavanol(s) supplementation compared to the same un-supplemented diet, in cardiometabolic target tissues (liver, adipose tissues, muscle, aorta and immune cells) from mice and rats, respectively (Figure 2). Some genes were particularly investigated in identified studies (Table 1). The expression of the gene encoding the tumor necrosis factor (*Tnf*), the most investigated gene with 28 reported hits, was assessed in response to pure compounds or flavanol extracts reported here. Whatever flavanols given to animals and the target tissues assessed, *Tnf* expression is always observed as significantly decreased in comparison to the non-supplemented control [13, 37, 41, 46, 50, 52, 58, 63, 71, 72, 75, 81, 84, 92, 96, 100]. The same effect was reported for the second inflammatory marker mostly investigated i.e., interleukin-6 (*Il6*) [52, 58, 62, 63, 71, 72, 74, 78, 84, 92, 96]. The expression of the gene encoding the fatty acid synthase (*Fasn*), only measured in liver and adipose tissues, was reported to a large extend as significantly decreased in flavanol-supplemented animals [26, 29, 42, 46, 47, 64, 65, 73, 77, 80, 81, 86, 88, 90, 95]. The genes encoding for the acetyl-Coenzyme A carboxylase alpha, *Acaca* [13, 37, 46, 68, 71, 72, 77, 86] and for the transcription factor Sterol regulatory element-binding transcription factor 1 (*Srebf1)* [32, 68, 70, 73, 75, 80, 86], both involved in lipid synthesis are also significantly repressed in liver and adipose tissues. Conversely, flavanols supplementation enhance the expression of genes involved in fatty acid beta-oxidation (*Acox1*) [39, 68, 81, 82, 87, 93, 94], mitochondrial uncoupling protein 1 (*Ucp1*) [13, 39, 48, 72, 89] and the trans-membrane transport of proteins (*Abca1*) [26, 29, 47, 64, 65, 73]. Flavanol supplementation has been reported to exhibit a positive impact on the gene expression of metabolic regulators such as sirtuin 1 (*Sirt1*) [27, 46, 54, 55, 57, 71, 72] and peroxisome proliferator activated receptor (PPAR) members:

PPAR alpha (*Ppara*) [28, 43, 68, 71, 72, 75, 80, 81, 83, 93]; gamma (*Pparg)* [31, 34, 44, 59, 71, 72, 80]; and PPARg coactivator 1 alpha (*Ppargc1a)* [27, 44, 46, 48, 71, 72, 89].

3.4. Functional enrichment analysis of the modulated genes in response to flavanol intake

To get a better understanding of the molecular mechanisms underlying the cardiometabolic effects associated with the intake of flavanols, the differentially expressed genes (n=110 mouse genes, n=77 rat genes) were submitted to a Gene Set Enrichment Analysis (GSEA). The enriched terms and the overlapping input genes assessed in liver and adipose tissues were rendered in clustergrams presented in Figure 3. These clustergrams revealed that the consumption of flavanols affects the expression of genes that can be clustered in four major functional groups. The first group comprises about fifteen highly significant modulated genes associated with endocrine functions (e.g. adipocytokine, glucagon and insulin signalling pathways). A second group is linked to fatty acid metabolism (e.g., PPAR signalling pathway and FFA oxidation). In the mouse liver, GSEA also revealed a third cluster related to the absorption of fatty acids (e.g. TG digestion and absorption; bile secretion, bile acid biosynthesis). The fourth group, detected in all tissues and species, comprises down-regulated genes in response to flavanol intake which are involved in inflammatory pathways (e.g., TNF-, NFκB- and toll-like receptor- signalling pathways).

The expression of mouse and rat genes was next also investigated in muscles and aortas. However, the low number of items in the gene inputs did not allow to build clustergrams following GSEA (Figure 3E). Nevertheless, the analysis showed that PPAR signalling, FA oxidation, thermogenesis, or non-alcoholic fatty liver disease (NAFLD) pathways were significantly enriched as seen in liver and adipose tissues. In murine aorta, the main target tissue in vascular diseases, the modulation of gene expression profiles affected by flavanol

intake is related to inflammatory pathways (e.g., TNF-, IL-17-, and NF-κB-signalling pathway), to pathways controlling endothelial activation (cell adhesion molecules and leukocyte trans-endothelial migration), and vascular tone (metabolism of arginine, a precursor for NO production).

As clustergrams further highlighted that some reported genes overlap in several enriched terms, functionally organized networks were built to better capture and summarize the relationship between genes modulated in response to flavanol intake and their enriched terms (Figure 4). In mice, the consumed flavanols mainly modulate genes involved in the interconnected pathways regulating fatty acids metabolism and glucose metabolism (insulin and glucagon regulations). In cardiometabolic rat models, flavanols modulate the pathway associated with insulin and fatty acids metabolism.

3.5. Putative regulators explaining the gene expression profiles in response to flavanols

The variation in gene expression in response to the intake of flavanols may result from their action on the activity of transcription factors (TF), or an epigenetic regulation involving e.g. miRNAs and DNA methylation/histone acetylation. To explore the capabilities of flavanols to modulate transcriptional regulation, we first performed a transcription factor enrichment analysis (TFEA) by submitting our gene set to the HOMER software (Figure 5A). Secondly, we looked for miRNAs that may regulate the gene set (Figure 5B), and then searched for other regulators of the transcriptional activity (e.g., chromatin regulators) using RegulatorTrail.

TFEA aims at revealing transcription factors potentially modulated by flavanols that may explain differential gene expression profiles observed in rodent tissues (Figure 5A). This analysis was performed independently for each target tissue and rodent model. In mouse liver, the bioinformatic analysis identified TF the most likely targeted by flavanols, such as

the retinoic acid receptor (namely RXR), PPARg, transcription factor MafA, and activator protein-1 (AP-1) regulating respectively 14, 13, 10 and 9 genes over the 67 genes identified as differentially expressed by flavanols. In mouse adipose tissues, flavanols appears to modulate the activity of the T-box transcription factor 5 (TBX5), the homeobox protein TG-interacting factor 1 (TGIF1) and the DNA-binding protein regulatory factor X, 6 (RFX6) that controlled the expression of 39%, 34% and 28% of the input genes, respectively. The transcription factor AP-1, the activating transcription factor 3 (ATF3) and the basic leucine zipper transcriptional factor ATF-like (BATF) appeared as the main transcriptional regulators modulated by flavanols in aorta of mice. In rat tissues, TFEA identified ATF3 and AP-1 in the liver and muscle, RUNX1 (Runt-related transcription factor 1) and HOXD13 (Homeobox protein Hox-D13) in the adipose tissue, and the transcription factor Sp5 and MAZ (Myc-associated zinc finger protein) in aorta. Taken together, these results highlight that dietary flavanols may affect the activity of a large range of TFs, and this with a tissue specificity as suggested by the low overlap between tissues of enriched TFs.

The modulation of gene expression observed in tissues of animals fed with flavanols can also be mediated by changes in miRNAs expression. From the dataset of modulated genes, the bioinformatic prediction identified 41 mouse miRNAs and 45 rat miRNAs that can be modulated in response to flavanols and affect the expression level of genes identified from publications (Figure 5B). Among the mouse miRNAs, 15 candidates may participate in the gene modulation observed in liver, 12 in adipose tissue, 9 in muscle and 12 in aorta. In rats, 22 miRNAs may modulate the gene expression profile observed in liver, 14 in adipose tissues, 11 in muscle and 2 in aorta. A very low number of predicted miRNAs overlapped between tissues, further demonstrating a tissue specific action of the flavanols.

By submitting the gene datasets to the RegulatorTrail software, some regulators other than TFs and miRNAs were identified as potential mediators in the nutrigenomic effect of

flavanols. This analysis shows that the activity of proteins altering the chromatin structure such as the helicase SMARCA4 (p-value $_{\text{mouse liver}}$: 1.12e-13; p-value $_{\text{mouse AT}}$: 3.96e-7; p-value $_{\text{rat}}$: 6.57e-14) and the histone deacetylase HDAC3 (p-value $_{\text{mouse liver}}$: 4.56e-22) may also contribute to the gene expression modulation in response to dietary flavanols. In addition, the lysine methyltransferase KMT2D known to methylate the lysine 4 position of histone H3, has been revealed as a potential epigenetic mediator of the effect of flavanols on gene expression. Another potential regulator identified is the CWC15 protein, a component of the spliceosome involved in pre-mRNA splicing (p-value $_{\text{mouse AT}}$:1.0e-7, p-value $_{\text{rat}}$: 6.07e-16). All these non-transcriptional regulations may contribute to change the chromatin conformation and consequently may alter the gene expression profiles observed in response to dietary flavanols. We than performed integration of all genomic data by combined analysis of interactions between mRNA, transcription factors and miRNA targets (Figure 6). This analysis showed an inner core network, as present highest number of interactions within the mouse network, that involves the nuclear receptor PPARG, 3 miRNAs namely miR-25, miR-32, miR-363 and the protein coding gene PRDM16. In the network observed for rat data analysis, the transcription factor AP-1, miR-25, miR-92a and miR-367 constitute the major nodes of interactions.

4. Discussion

The present systematic review of studies conducted in rodent models clearly highlights that dietary flavanols exert nutrigenomic effects in several target tissues involved in the development of cardiometabolic diseases. From the changes in gene expression observed, these nutrigenomic effects could largely mediate the reported beneficial effects of these compounds on a range of biomarkers associated with cardiometabolic risk. By analysing data obtained from quantitative real-time PCR, considered as the 'gold standard' method to assess gene expression, we selected 155 rodent genes (mouse + rat) as significantly

modulated in response to the consumption of dietary flavanols. The nutrigenomic response to dietary flavanols may result from a multilevel regulation involving transcriptional and non-transcriptional mediators.

The complexity of these nutrigenomic effects has been raised previously in a few animal intervention studies that have used holistic approaches [101-106]. It results from these studies that the hundreds of genes shown as modulated by dietary flavanols in several tissue targets (liver, aorta and adipose tissue) were notably involved in fatty acid metabolism, cell chemotaxis and accumulation of myeloid cells. Despite the high efficiency and performance of these holistic approaches to discriminate gene expression variation in response to a dietary intervention, the weak redundancy between these studies failed to provide enough robust and consolidated data to support further investigation of the molecular mechanisms by which dietary flavanols regulate gene expression. To overcome this limitation, we used the dataset of 155 genes identified as modulated by dietary flavanols in our systematic analysis of PCR data and submitted them to bioinformatics analyses. The functional enrichment analysis based on this gene dataset highlights that hereafter exerting a nutrigenomic effect flavanols may affect metabolism regulating several pathways involved in endocrine functions, in fatty acid metabolism and in inflammation. This result fits with the improvement of triglyceridemia, cholesterolemia, plasma FFA levels, glycemia, insulinemia and circulating inflammatory markers observed in cardiometabolic rodent models supplemented with flavanols. Several randomized controlled trials (RCT) support similar benefits of flavanols on cardiometabolic health. A meta-analysis collecting the RCTs-based evidence of the effects of flavanol-rich tea, cocoa, and apple products on biomarkers of cardiometabolic risk provides robust evidence of significant reductions in total cholesterol and triglycerides [107]. The nutrigenomic approach associated to enrichment analysis from animal studies give new

insights to understand the underlying mechanism of action that can be triggered in human in response to a nutritional intervention with flavanols.

Bioinformatic analysis (e.g., TFEA) predicts that dietary flavanols may affect the activity of a wide range of transcription factors resulting in a modulated gene expression profile. This direct transcriptional effect has been reported for different flavanol sources and compounds on transcription factors involved in different signalling pathways [108]. According to our TFEA analysis, one of the major TFs modulated appeared to be AP-1 both in rats and mice. AP-1 is made up of heterodimeric protein complexes of different protein families, including the c-Jun, c-Fos, Maf families, and it is closely related to activation transcription factor (ATF) subfamilies, some of which are defined in our study [109]. AP-1 plays a critical role in the progress of vascular dysfunction and atherogenesis [110]. Inhibition of AP-1 is one of the key targets to prevent atherosclerosis. Hexameric procyanidins isolated from cocoa protected Caco-2 cells [111] and procyanidin B2 elicited an anti-inflammatory effect in human umbilical endothelial cells through inhibition of AP-1 activation [112]. SREBP-1 is a major regulator of fatty acid synthesis, lipids, and lipoproteins. SREBP-1 may be a target transcription factor for grape seed procyanidin extract [113], EGCG [47, 114] to exert their anti-lipogenic, anti-inflammatory and hypotriglyceridemic effects in mice. Nuclear factor erythroid-2-related factor (Nrf2) controls genes encoding proteins that function in reactive oxygen species detoxification, cell survival, metabolism, inflammation, cell growth, cell adhesion, and adipocyte differentiation [115]. In cardiometabolic diseases NRF2 induces the expression of antioxidant genes, impairs atherosclerosis, reduces inflammation, modulate migration and proliferation of vascular smooth muscle cells [116]. In hepatic HepG2 cells, green tea EGCG upregulated Nrf2 dependent gene expressions of antioxidant and detoxification enzymes [115]. Similarly, cocoa phenolic extract and epicatechin upregulated antioxidant gene expressions and proteins

thus protected HepG2 cells against high glucose induced oxidative stress via Nrf2 pathway [117]. Epicatechin increased Nrf2 and its target genes in aortas of hypertensive rats resulting in lowered blood pressure, decreased oxidative stress and restored endothelial function [118]. In human aortic smooth muscle cells (HASMCs), EGCG dose dependently induced Nrf2 and inhibited IL-1β-induced HASMC proliferation and oxidative stress [119]. The human forkhead box (FOX) TFs are involved in multiple cellular processes such as cellular homeostasis, cell survival, cell proliferation, angiogenesis, immune regulation and cell death. In our study FOXM1 was detected in the liver of mice. FOXM1 and FOXO3a are two TFs that compete for binding to the same gene targets. FoxO3a is a major TF in cholesterol homeostasis in hepatic cells. Overexpression of FoxO3a improves hypercholesterolemia. FoxO3a deficiency leads to increased hepatic and plasma cholesterol levels. It has been shown that EGCG lowered LDL levels of rats [120]. Liver X Receptor (LXR) is an important regulator of cholesterol homeostasis and lipid metabolism. Besides it decreases the transcription of pro-inflammatory genes. In rats epicatechin decreased LXR dose dependently and improved blood lipid profile when fed high-fat diet [90]. In addition, EGCG upregulated LXR in ApoE Knock-Out mice [47]. Next to regulating TFs, flavanols have been demonstrated to modulate mRNA maturation acting on the spliceosome [121]. Thus, a large body of evidence sustains a direct effect of flavanols on transcriptional activities that can explain their nutrigenomic effects and their beneficial health effects.

Dietary polyphenols have been shown to modulate miRNAs profiles [122, 123]. miRNAs are small non-coding RNAs identified as a crucial regulatory layer in the control of transcription. Bioinformatics analysis performed suggests that flavanols may also exert a transcriptional regulation acting on the expression of miRNAs. Among the most scored miRNA is miRNA-187 that in our analysis was identified using the list of differentially expressed genes in mouse and rat models of disease in liver and adipose tissue. Interestingly,

and in agreement with our research aim, miRNA-187 is overexpressed in human subjects with T2D versus matched controls [124]. Our data reinforce the pathogenetic role for this miRNA in metabolic disease and suggest for polyphenols a complementary way of action in curbing glucose dysmetabolism. More and more data from the last years provide evidence that miRNAs have a potential to be diagnostic as well as prognostic markers for several cardiovascular diseases [125]. According to Wang et al. [126], miRNA-720, that was identified by us as potential actor in the nutrigenomic effect of flavanols in both rat and mouse, might be a useful and promising biomarker for the early detection of coronary artery disease. The downregulation of miR-150-5p (defined in mouse) has been reported as a prognostic marker for advanced heart failure [127]. In addition to regulate gene expression through a potential action on circulating miRNAs, flavanols could counteract some key actors in the onset of cardiovascular diseases.

Other molecular actors involved in the regulation of the epigenome can also participate in the nutrigenomic effects of dietary flavanols. These are proteins in charge of the modification of chromatin structure (e.g., HDAC, lysine methyltransferase, Helicase) [128]. Several studies report that an intervention with dietary flavanols modifies DNA methylation [129-131]. In cancer, epigallocatechin gallate has been shown to inhibit methyltransferase [132] and histone deacetylase activities [133] that participates to slow down cancer progression. These scientific evidence support the hypothesis that flavanols can drive transcriptional profiles through an epigenetic regulation and by consequence stress the need of new investigations to explore this further.

To exert any beneficial effects including nutrigenomic effects, flavanols need to be bioavailable. Several factors such as bioaccessibility, food matrix and also metabolizing enzymes may affect flavanol bioavailability and as consequence the bioactivity of dietary flavanols. After absorption, flavanol monomers undergo extensive phase II conjugation by

the gut microbiota and are found in the circulation in the low µmol/L range and as e.g., O-methylated, sulphated and glucuronidated conjugates [134]. These compounds can also be degraded into smaller phenolic compounds by the gut microbiota and thereafter absorbed [135-138]. The demonstration that circulating metabolites are responsive to the health effects of flavanols has been provided by *in vitro* studies. Claude et al. demonstrated that circulating flavanol metabolites (e.g., methylepicatechin) used in nutritionally relevant conditions preserve vascular endothelial function preventing monocyte adhesion to endothelial cells [139]. The same research group showed that these flavanol metabolites also exert nutri(epi)genomic changes in endothelial cells [140, 141] that contribute to regulate several signalling pathways already reported, as an anti-atherosclerotic effect of a flavanol supplementation [101]. These data further support that the mechanism of action of dietary flavanol monomers may be mediated by the metabolite present in the systemic circulation and that are likely to represent the physiological active forms. Moreover, our systematic analysis and integrated bioinformatic analysis of the genomic data allowed us to obtain a more precise and coherent molecular mechanisms of these molecules. In particular, we report that the potential underlying impact of the flavanols on cardiometabolic health is multi-modal and involves both transcriptional and post-transcriptional regulations

This review presents a potential complex multilevel action of dietary flavanols on gene expression contributing to health benefits. Based on observed functional modulations, flavanols are suspected to act as signal molecules, agonists or else antagonists interacting directly with proteins. In 2018, Lacroix et al. identified the polyphenol interactome and showed that polyphenols and their metabolites interact with over 5,500 proteins [142]. However, this interactome is partly built with data mining tools and only a small number of crystal structures of polyphenol-protein complexes is currently available. Other research groups used computational docking approaches to screen pertinent targets of polyphenols

metabolites that are of major importance in mediating the health effects of polyphenols [140, 143].

An increasing number of controlled clinical trials have been conducted out over the last decades to shed light on the effects of flavanol-containing products on cardiometabolic health. However, factors intrinsic to the study (design, duration, dose or products) and factors inherent to the individuals (epi-genetic, health status, age, ethnicity, gut microbiota) introduce heterogenicity in the findings [107, 144, 145]. Similarly, the weak redundancy between the animal studies does not provide us with sufficiently robust and consolidated data and therefore does not allow us to estimate the most effective doses of dietary flavanols for a giving health effect. However, the identification of the main determinants in the nutrigenomic effects of the flavanols can be useful to understand the variability in response to the consumption of dietary flavanols. To this end, this review supports that *a posteriori* computational analyses of the nutrigenomic data can lend support on the identification of potential molecular mediators responsive for the bioactivity of flavanols. One critical question that still remains is related to the identification of the flavanol metabolites driving the nutrigenomic response in the target organs. Answering these pending questions will contribute to optimize the beneficial health effects of dietary bioactives.

Conflict of interest

The authors have no conflict of interest.

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Author contribution

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Figure and Table legends

Figure 1: Flow diagram of the study selection process.

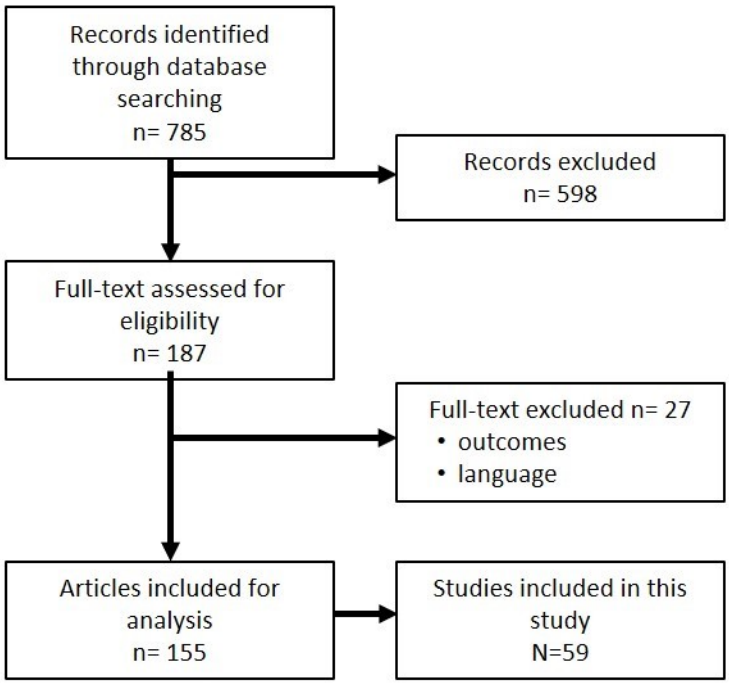


Figure 2: Differentially expressed genes in mice, rats and rodents after oral treatment with flavanols – pure compounds or extracts from tea, cocoa, apple or grape seeds.

*The number of differentially expressed genes in rodent target tissues was obtained by pooling together the differentially expressed genes in each specific tissue in mice and rats, and subsequent removal of duplicate genes. $All kinds of adipose tissues, including both brown and white adipose tissues of visceral, subcutaneous, and epididymal origin.

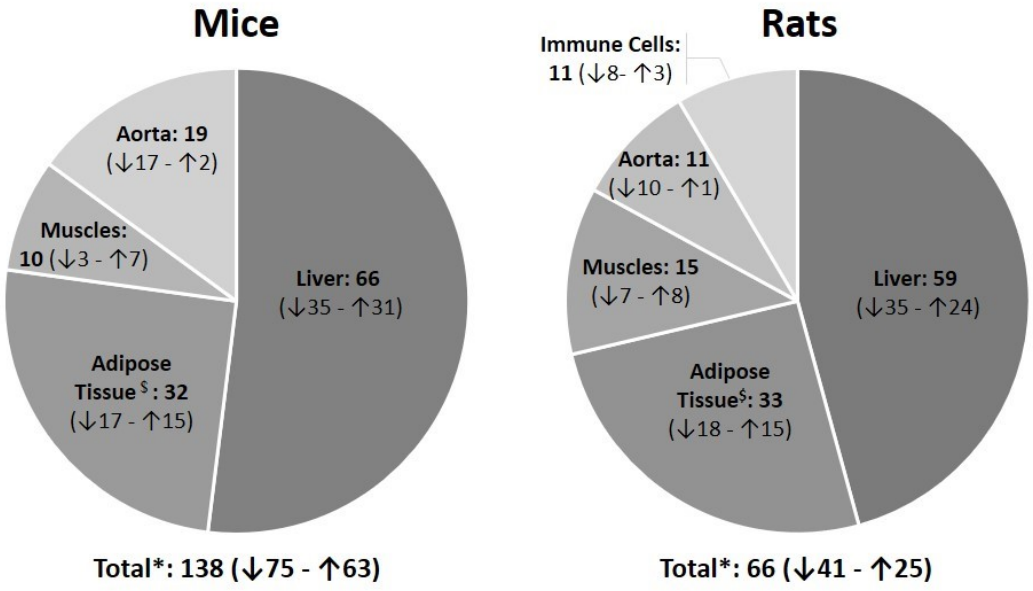
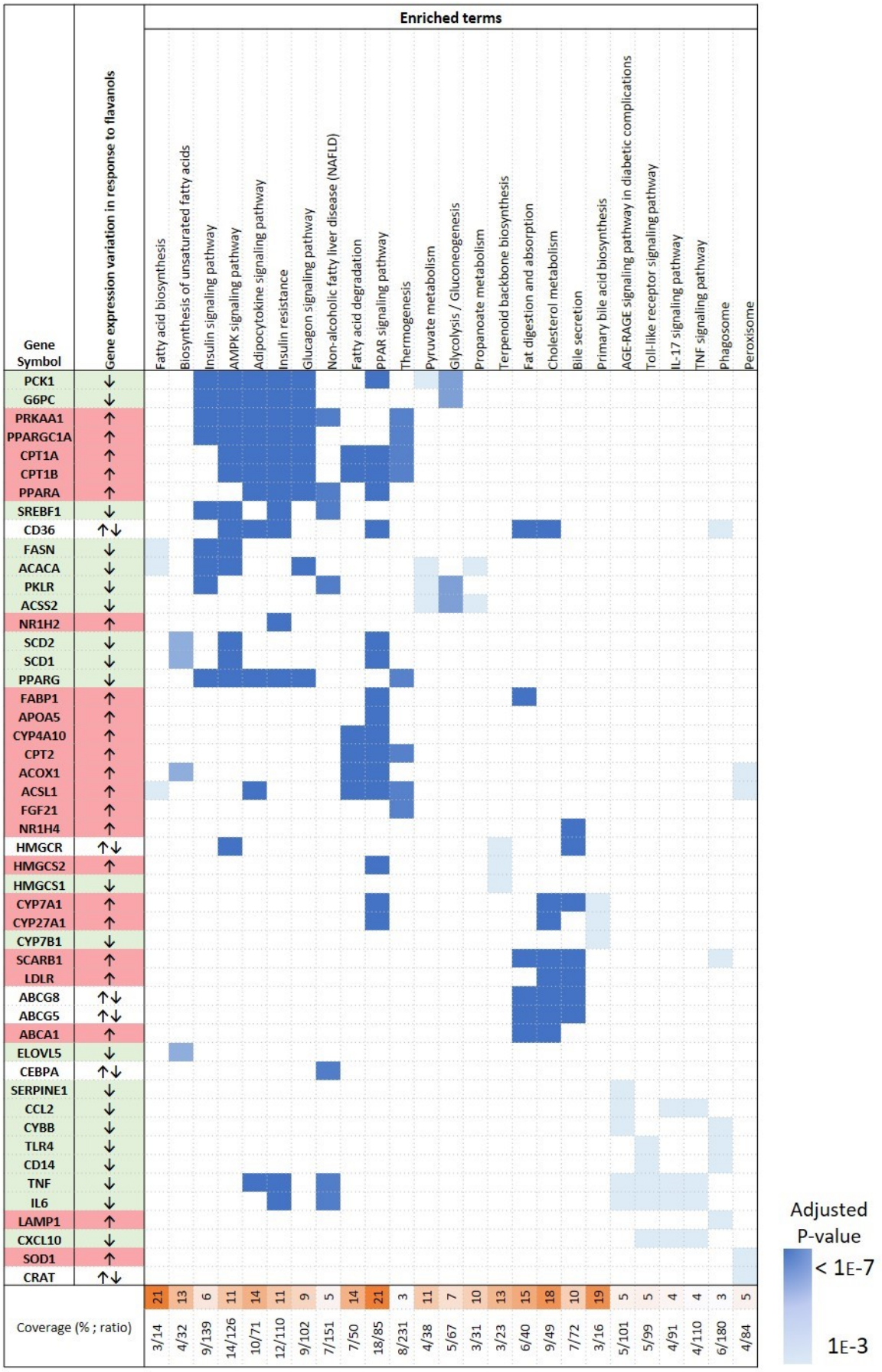
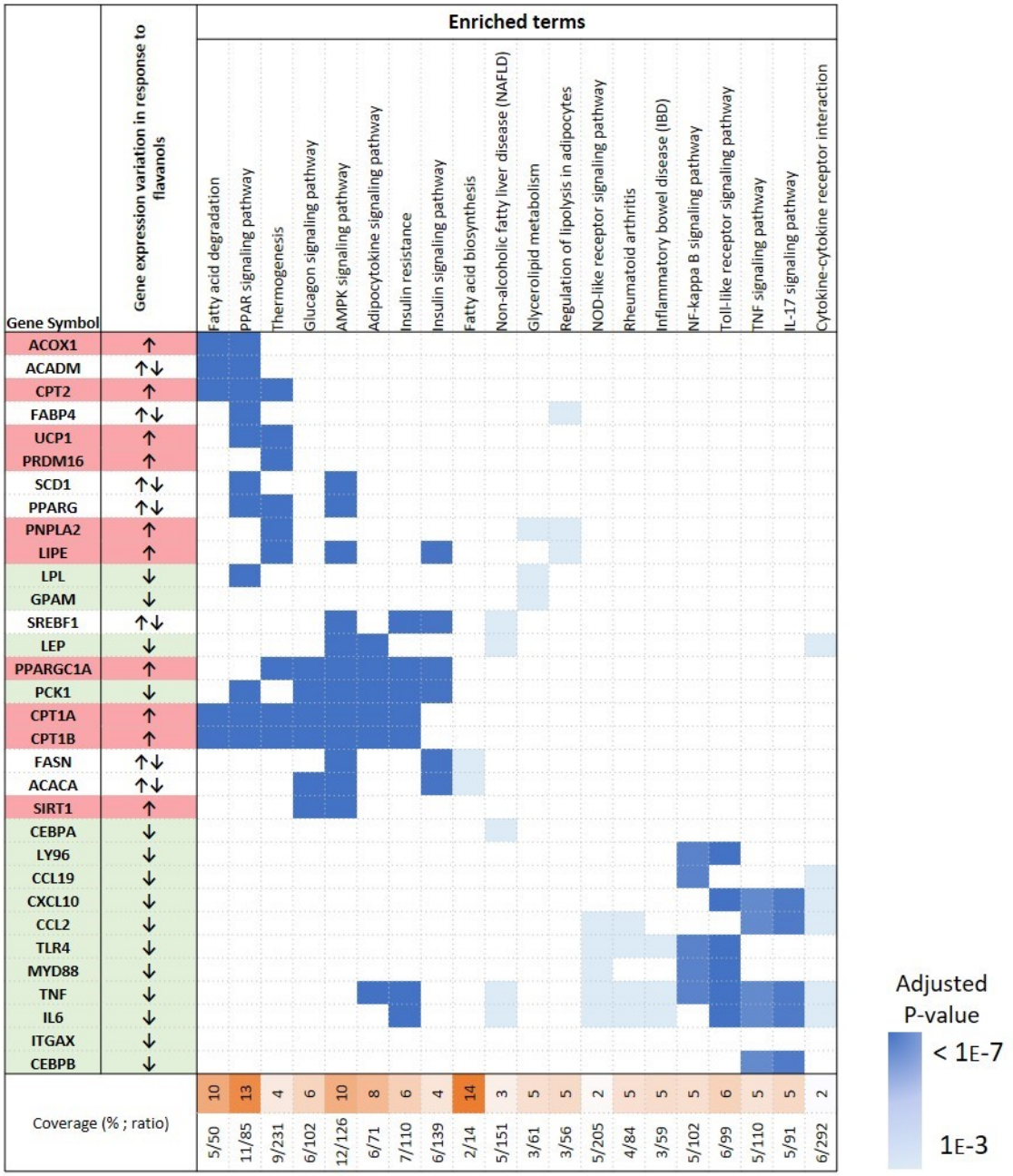
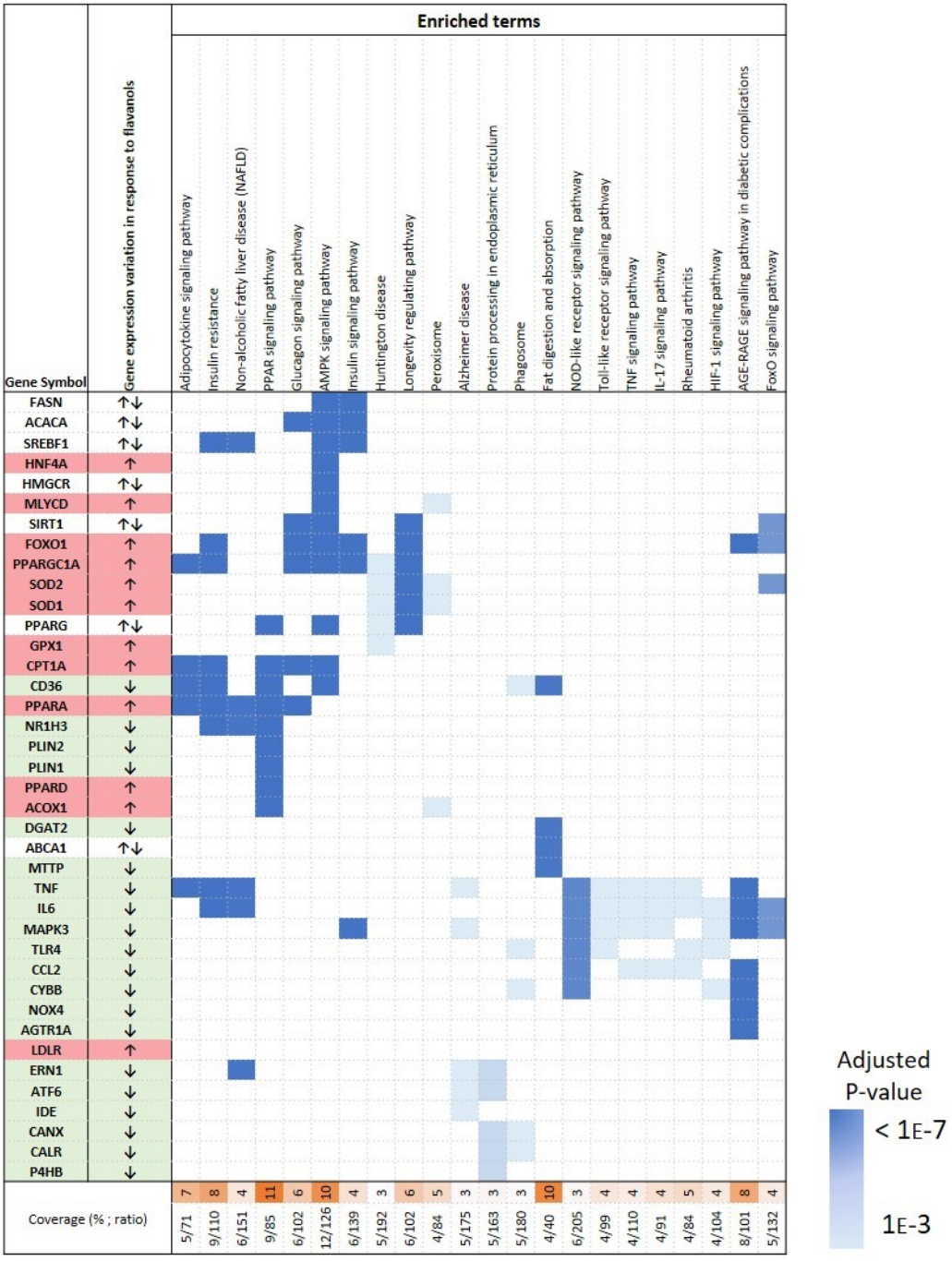
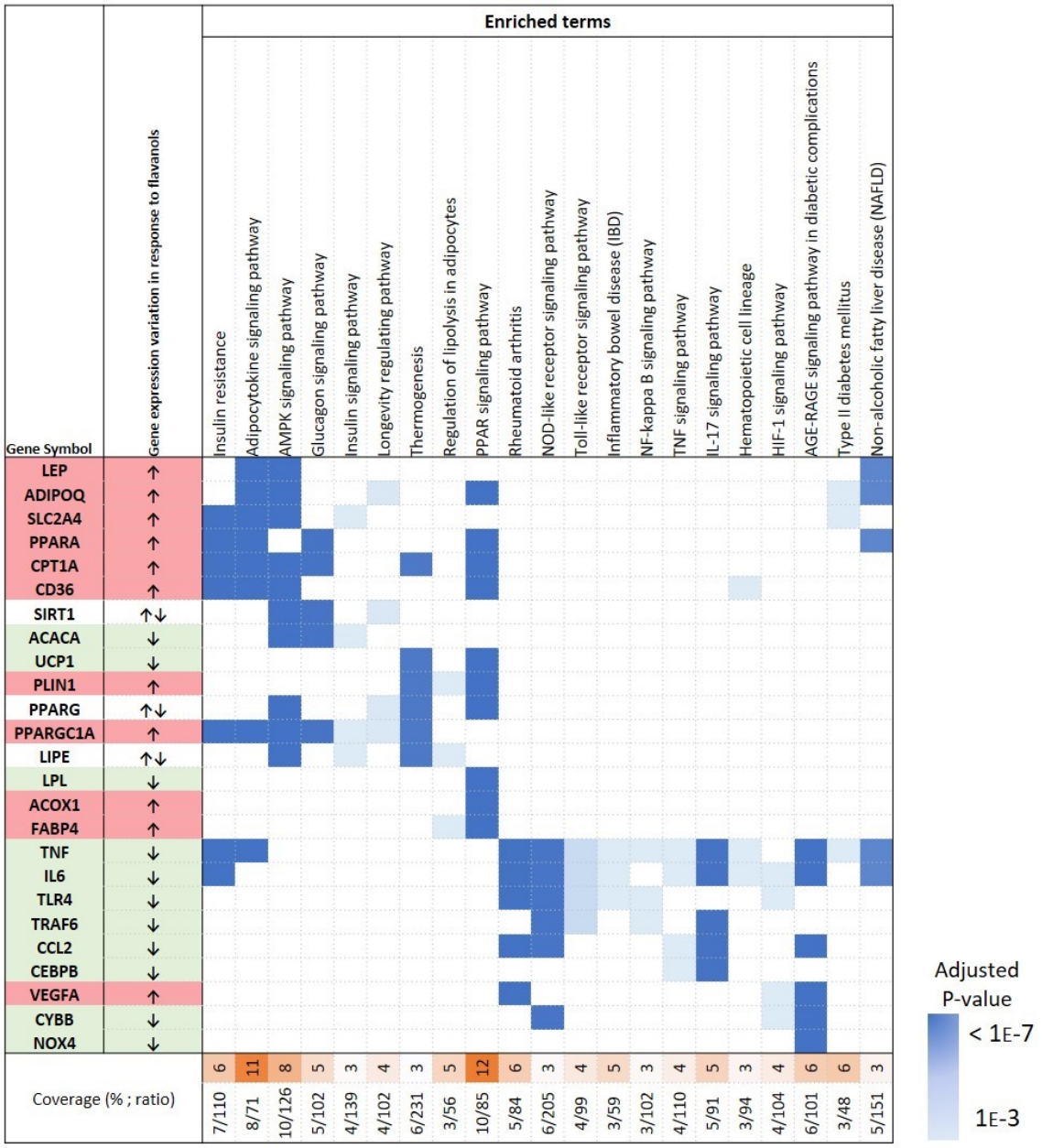


Figure 3: Functional enrichment analysis of the modulated genes in response to flavanol intake in liver (A, C), adipose tissues (B, D), muscles and aortas (E) of rodents (A-B-E: mouse; C-D-E: rat). Clustergrams were built on enriched pathways retrieved from KEGG using Enrichr. Enriched pathways were ranked based on their enrichment score and gene hierarchically clustered based on their association with the top enriched terms. Heatmap is proportional to p-value of the enriched terms. Gene modulation was *a posteriori* added onto the clustergram as well as gene coverage (expressed in percentage and ratio).









GSEA in Muscles	Mouse		Rat	
Enriched Terms	Overlap	*Adjusted P-value*	Overlap	*Adjusted P-value*
Adipocytokine signaling pathway			8,5	*7,17E-10*
AMPK signaling pathway			2,4	*1,02E-03*
Insulin resistance			7,3	*5,72E-13*
Fatty acid degradation	4,0	*2,01E-02*		
PPAR signaling pathway	3,5	*1,60E-03*	5,9	*2,41E-07*
Non-alcoholic fatty liver disease (NAFLD)	2,0	*6,69E-03*	3,3	*2,64E-06*
cAMP signaling pathway	1,4	*1,44E-02*		
Thermogenesis	1,7	*1,21E-03*		
Cardiac muscle contraction	2,6	*4,18E-02*		
C-type lectin receptor signaling pathway			2,7	*7,51E-04*
Fluid shear stress and atherosclerosis			2,1	*1,33E-03*
Hypertrophic cardiomyopathy (HCM)			3,5	*4,81E-04*
Hematopoietic cell lineage			3,2	*5,62E-04*
Osteoclast differentiation			2,3	*1,03E-03*
Huntington disease	2,1	*1,16E-03*		
Inflammatory bowel disease (IBD)			6,8	*3,38E-06*
Rheumatoid arthritis			3,6	*4,76E-04*
TNF signaling pathway			2,7	*7,43E-04*
Toll-like receptor signaling pathway			3,0	*6,24E-04*
Th17 cell differentiation			2,9	*6,49E-04*
IL-17 signaling pathway			3,3	*5,38E-04*
AGE-RAGE signaling pathway in diabetic complications			4,0	*2,55E-05*
FoxO signaling pathway			2,3	*1,09E-03*

GSEA in Aorta	Mouse		Rat	
Enriched Terms	Overlap	*Adjusted P-value*	Overlap	*Adjusted P-value*
Arginine biosynthesis	10,5	*2,57E-03*		
Hematopoietic cell lineage	3,2	*2,09E-03*		
HIF-1 signaling pathway	4,8	*3,21E-06*		
VEGF signaling pathway			3,4	*3,30E-03*
Fluid shear stress and atherosclerosis	7,0	*2,31E-14*	4,2	*3,01E-09*
Leukocyte transendothelial migration	4,3	*4,72E-06*	2,6	*3,06E-04*
Cell adhesion molecules (CAMs)	2,4	*6,19E-04*		
C-type lectin receptor signaling pathway			2,7	*3,03E-04*
Cytokine-cytokine receptor interaction			1,0	*2,94E-03*
MAPK signaling pathway			1,0	*2,88E-03*
MicroRNAs in cancer	1,8	*2,48E-04*		
Graft-versus-host disease			3,1	*3,74E-03*
NOD-like receptor signaling pathway	2,4	*6,78E-05*	1,5	*1,17E-03*
Insulin resistance	2,7	*2,80E-03*		
Non-alcoholic fatty liver disease (NAFLD)			2,0	*6,11E-04*
Type I diabetes mellitus			2,9	*4,20E-03*
Osteoclast differentiation			3,1	*6,62E-06*
Proteoglycans in cancer			1,5	*1,19E-03*
Relaxin signaling pathway			2,3	*4,24E-04*
Inflammatory bowel disease (IBD)			5,1	*5,57E-05*
Rheumatoid arthritis	4,8	*6,06E-05*	4,8	*1,81E-06*
IL-17 signaling pathway	5,5	*1,87E-06*	4,4	*2,15E-06*
TNF signaling pathway	7,3	*1,47E-11*	4,5	*4,31E-08*
NF-kappa B signaling pathway	3,9	*1,10E-04*	3,9	*2,98E-06*
AGE-RAGE signaling pathway in diabetic complications	9,9	*1,27E-15*	4,9	*3,72E-08*

Figure 4: Global Enrichment analysis in response to flavanols (A: mice; B: Rats). Functionally organized network connects the input genes modulated in response to flavanols and the top enriched KEGG pathways that predict the main functions modulated by flavanols.

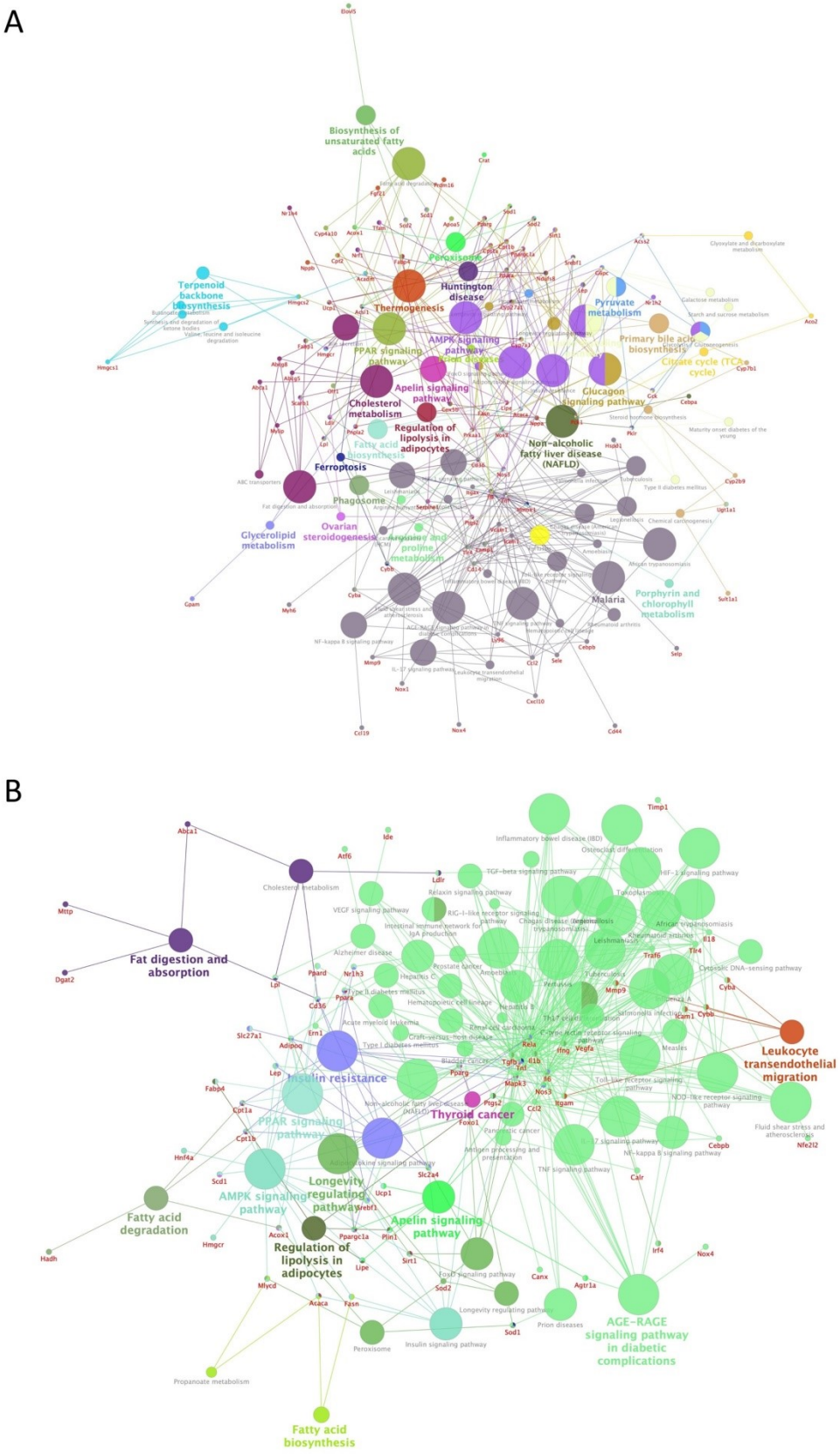
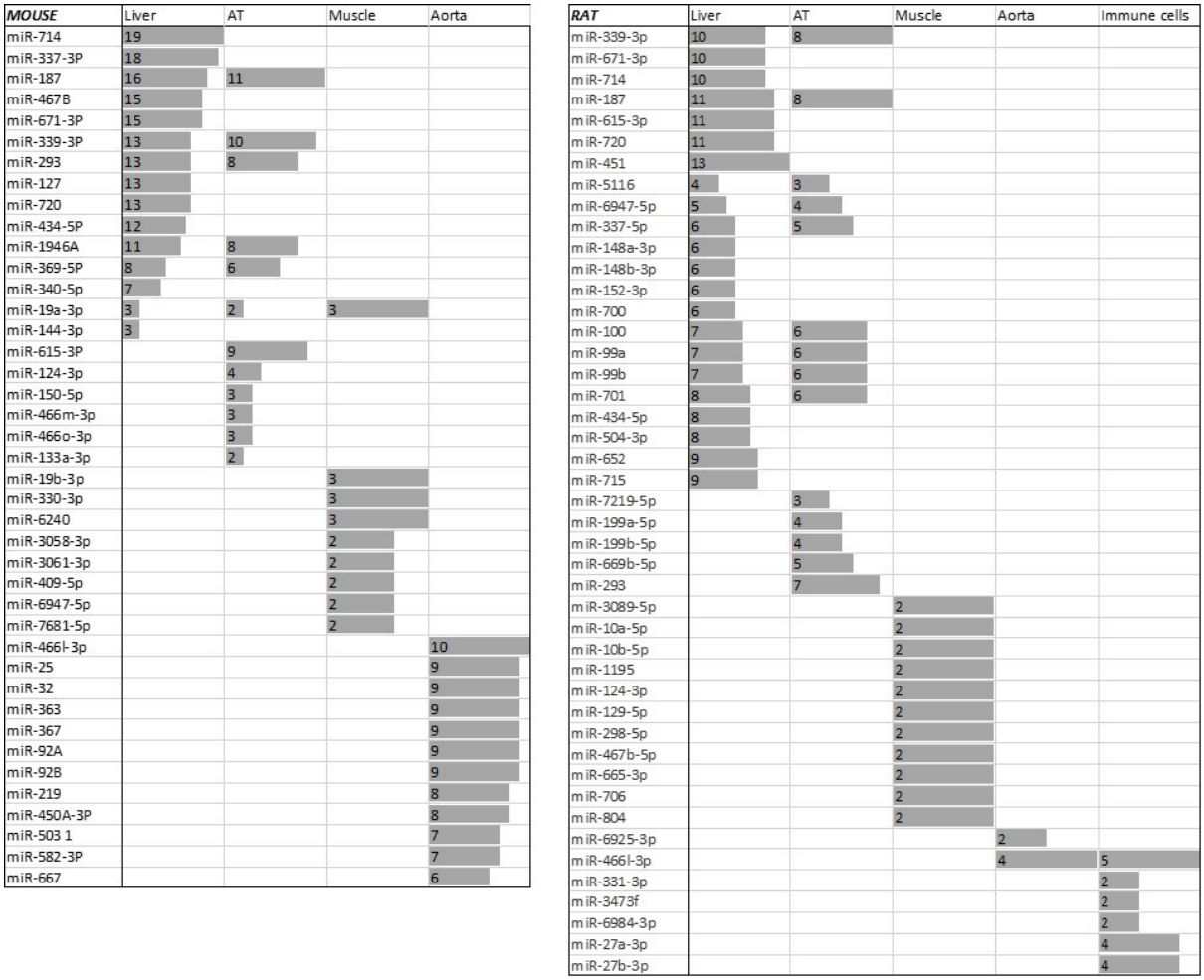


Figure 5: Predicted regulators responsible to the observed gene modulation. Transcription factors (A) and miRNA (B) were predicted submitting gene dataset to HOMER and miRwalk respectively. For a tissue, scales are proportional to hits from each gene inputs.

MOUSE	LIVER	Adipose Tissues	Aorta	Muscles
RXR	14			
PPARE	13			
MafA	10	8		
AP-1	9		8	
USF1	9		4	
FOXM1	8			
PU.1:IRF8	6	4		
E-box	5	4	3	
Srebp1a	5			3
IRF2	4	4		
IRF1	4	4		
LXRE	3	2		
Tbx5		15		
Tgif1		13		
Rfx6		11		
PRDM1		7		
CRE		6		
Jun-AP1		4	5	
ISRE		3		
Nrf2		2		
Atf3			7	
BATF			7	
STAT1			6	
Fra2			6	
Fra1			6	
RUNX-AML			6	
Stat3			6	
RUNX2			6	
Fosl2			5	
STAT5			5	
Ascl1			5	
Bach2			4	
Usf2			4	
bHLHE40			4	
Max			4	
CLOCK			4	
n-Myc			4	
c-Myc			4	
MITF			4	
NPAS2			4	
TFE3			3	
NF-E2			2	
Tcf3				2
Tcf4				2

RAT	Liver	Adipose Tissues	Aorta	Muscles	Immune Cells
Atf3	7			4	
AP-1	7			4	
Srebp1a	4				
PAX3:FKHR	3	3			
Bach1	2	2			2
ZNF528	1				
RUNX1		7			
HOXD13		6			
ZNF528		1			
Sp5			8		
Maz			8		
TATA-Box			7		
Fra1				3	
JunB				3	
BATF				3	
ZSCAN22					2



MOUSE	Liver	AT	Muscle	Aorta
miR-714	19			
miR-337-3P	18			
miR-187	16	11		
miR-467B	15			
miR-671-3P	15			
miR-339-3P	13	10		
miR-293	13	8		
miR-127	13			
miR-720	13			
miR-434-5P	12			
miR-1946A	11	8		
miR-369-5P	8	6		
miR-340-5p	7			
miR-19a-3p	3	2	3	
miR-144-3p	3			
miR-615-3P		9		
miR-124-3p		4		
miR-150-5p		3		
miR-466m-3p		3		
miR-466o-3p		3		
miR-133a-3p		2		
miR-19b-3p			3	
miR-330-3p			3	
miR-6240			3	
miR-3058-3p			2	
miR-3061-3p			2	
miR-409-5p			2	
miR-6947-5p			2	
miR-7681-5p			2	
miR-466l-3p				10
miR-25				9
miR-32				9
miR-363				9
miR-367				9
miR-92A				9
miR-92B				9
miR-219				8
miR-450A-3P				8
miR-503 1				7
miR-582-3P				7
miR-667				6

RAT	Liver	AT	Muscle	Aorta	Immune cells
miR-339-3p	10	8			
miR-671-3p	10				
miR-714	10				
miR-187	11	8			
miR-615-3p	11				
miR-720	11				
miR-451	13				
miR-5116	4	3			
miR-6947-5p	5	4			
miR-337-5p	6	5			
miR-148a-3p	6				
miR-148b-3p	6				
miR-152-3p	6				
miR-700	6				
miR-100	7	6			
miR-99a	7	6			
miR-99b	7	6			
miR-701	8	6			
miR-434-5p	8				
miR-504-3p	8				
miR-652	9				
miR-715	9				
miR-7219-5p		3			
miR-199a-5p		4			
miR-199b-5p		4			
miR-669b-5p		5			
miR-293		7			
miR-3089-5p			2		
miR-10a-5p			2		
miR-10b-5p			2		
miR-1195			2		
miR-124-3p			2		
miR-129-5p			2		
miR-298-5p			2		
miR-467b-5p			2		
miR-665-3p			2		
miR-706			2		
miR-804			2		
miR-6925-3p				2	
miR-466l-3p				4	5
miR-331-3p					2
miR-3473f					2
miR-6984-3p					2
miR-27a-3p					4
miR-27b-3p					4

Figure 6: Global network of interactions between genes identified as modulated by flavanols with potential transcriptional regulators (i.e. transcription factors) and post-transcriptional regulators (i.e. miRNAs). The total interactions between mRNAs (yellow nodes labelled with the protein coding gene name), transcription factors (red nodes) and miRNAs (blue nodes) in mouse (A) and in rat (B) were built using the cytoscape software.

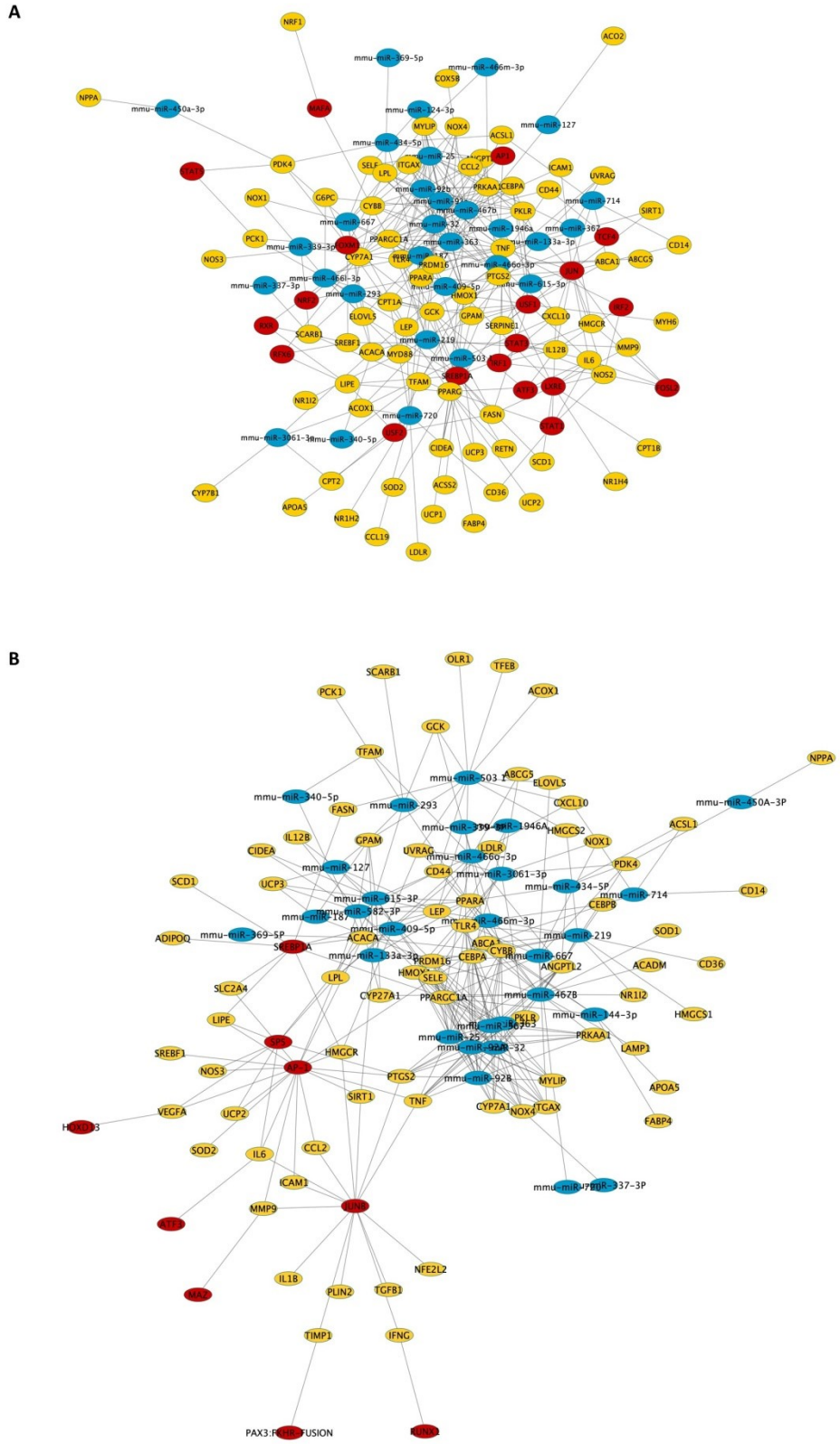
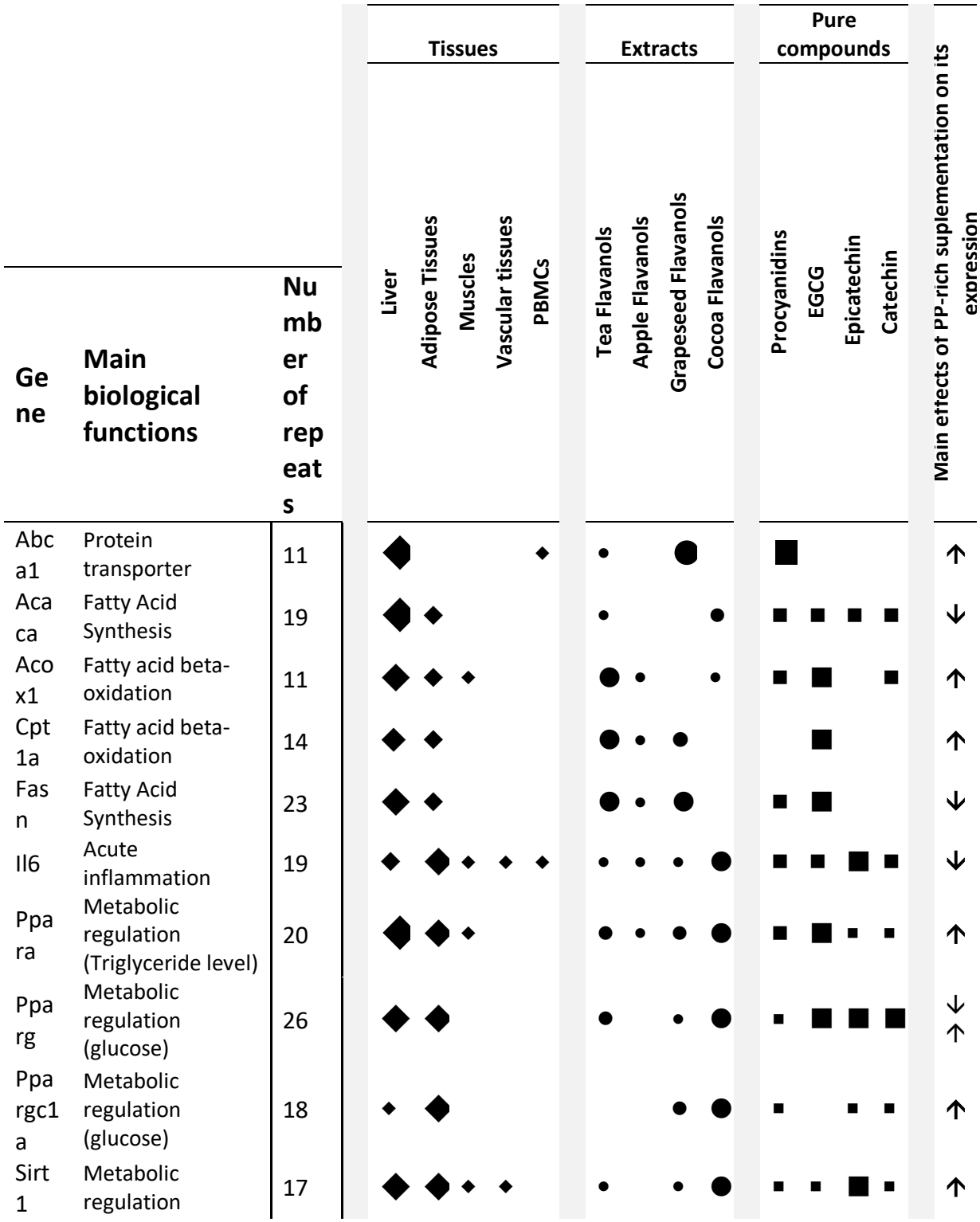


Table 1: Genes mostly investigated in rodent studies using targeted approach for assessing gene expression. The symbol size (▪,●,♦) is proportional to the number of repeats in respective categories. Reported main effect of flavanols on the gene expression corresponds to the effect observed in >80% of the studies.



Srebf1	Lipid and cholesterol synthesis	20	◆ ◆	● •	▪ ■	↓
Tnf	Acute inflammation	28	◆ ◆ ◆ ◆	● ● • ●	▪ ▪ ■ ▪	↓
Ucp1	Energy expenditure	15	◆	• ●	▪ ▪ ▪ ▪	↑

Nutrigenomic reveals the complex and multilevel action of dietary flavanols contributing to their strong potential to preserve cardiometabolic health.