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Abbreviations

ABCA1	ATP Binding Cassette Subfamily A Member 1
ADIPOQ	Adiponectin
AGE	Advanced Glycation Endproducts
APOA1	Apolipoprotein A1
APOB	Apolipoprotein B
BAX	BCL2 Associated X, Apoptosis Regulator
BCL2	BCL2 Apoptosis Regulator
CCL2	C-C Motif Chemokine Ligand 2
CEBPA	CCAAT Enhancer Binding Protein Alpha
CRP	C-Reactive Protein
CXCL8	C-X-C Motif Chemokine Ligand 8
EDN1	Endothelin 1
EGCG	Epigallocatechin gallate
FOXC1	Forkhead Box C1
GATA2	GATA Binding Protein 2
GDF	Growth Differentiation Factor
HIF	Hypoxia Inducible Factor
HMOX1	Heme Oxygenase 1
IBD	Inflammatory Bowel Disease
ICAM1	Intercellular Adhesion Molecule 1

74	IL2	Interleukin 2
75	IL4	Interleukin 4
76	IL6	Interleukin 6
77	IL10	Interleukin 10
78	ITGAM	Integrin Subunit Alpha M
79	ITGB1	Integrin Subunit Beta 1
80	JUN	Jun Proto-Oncogene: AP-1 Transcription Factor Subunit
81	KEGG	Kyoto Encyclopedia of Genes and Genomes
82	LDL	Low Density Lipoprotein
83	LDLR	Low Density Lipoprotein Receptor
84	LPL	Lipoprotein Lipase
85	LPS	Lipopolysaccharide
86	MAPK8	Mitogen-Activated Protein Kinase 8
87	miRNA	MicroRNA
88	MMP9	Matrix Metallopeptidase 9
89	MT-CO3	Mitochondrially Encoded Cytochrome C Oxidase III
90	NAFLD	Non-Alcoholic Fatty Liver Disease
91	NFKB1	Nuclear Factor Kappa B Subunit 1
92	NLRP3	NLR Family Pyrin Domain Containing 3
93	NOS2	Nitric Oxide Synthase 2
94	NOS3	Nitric Oxide Synthase 3

95	PBMC	Peripheral Blood Mononuclear Cell
96	PECAM1	Platelet and Endothelial Cell Adhesion Molecule 1
97	PPARA	Peroxisome Proliferator Activated Receptor Alpha
98	PPARG	Peroxisome Proliferator Activated Receptor Gamma
99	PPARs	Peroxisome Proliferator Activated Receptors
100	PPI	Protein-Protein Interaction
101	PTGS2	Prostaglandin-Endoperoxide Synthase 2
102	RAGE	Receptor for AGE
103	RETN	Resistin
104	ROCK1	Rho Associated Coiled-Coil Containing Protein Kinase 1
105	SELE	Selectin E
106	SERPINE1	Serpin Family E Member 1
107	SP1	Sp1 Transcription Factor
108	SREBF1	Sterol Regulatory Element Binding Transcription Factor 1
109	STAT1	Signal Transducer and Activator of Transcription 1
110	STAT3	Signal Transducer and Activator of Transcription 3
111	TGF-beta	Transforming Growth Factor Beta
112	TLDA	Taqman Low Density Array
113	TLR4	Toll Like Receptor 4
114	TNF	Tumor Necrosis Factor
115	TOLLIP	Toll Interacting Protein

116	VEGF	Vascular Endothelial Growth Factor
117	VCAM1	Vascular Cell Adhesion Molecule 1
118	YY1	Yin Yang 1 Transcription Factor

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Abstract: Flavanols intake positively influences several cardiometabolic risk factors in humans. However, the specific molecular mechanisms of action of flavanols, in terms of gene regulation, in the cell types relevant to cardiometabolic disease have never been systematically addressed. On this basis, we conducted a systematic literature review and a comprehensive bioinformatic analysis of genes which expression is affected by flavanols in cells defining the cardiometabolic health: hepatocytes, adipocytes, endothelial, smooth muscle and immune cells. A systematic literature search was performed using the following pre-defined criteria: treatment with pure compounds and metabolites (no extracts), at low concentrations that are close to their plasma concentrations. Differentially expressed genes were analyzed using bioinformatics tools to identify gene ontologies, networks, cellular pathways and interactions, as well as transcriptional and post-transcriptional regulators. The systematic literature search identified 54 differentially expressed genes at mRNA level in *in vitro* models of cardiometabolic disease exposed to flavanols and their metabolites. Global bioinformatic analysis revealed that these genes are predominantly involved in inflammation, leukocyte adhesion and transendothelial migration, and lipid metabolism. We observed that, although the investigated cells responded differentially to flavanol exposure, the involvement of anti-inflammatory responses is a common mechanism of flavanol action. We also identified potential transcriptional regulators of gene expression: transcriptional factors, such as GATA2, NFKB1, FOXC1 or PPARG, and post-transcriptional regulators: miRNAs, such as mir-335-5p, let-7b-5p, mir-26b-5p or mir-16-5p. In parallel, we analyzed the nutrigenomic effects of flavanols in intestinal cells and demonstrated their predominant involvement in the metabolism of circulating lipoproteins. In conclusion, the results of this systematic analysis of the nutrigenomic effects of flavanols provides a more comprehensive picture of their molecular mechanisms of action and will support the future setup of genetic studies to pave the way for individualized dietary recommendations.

Keywords: flavanols; cardiometabolic; gene expression; in vitro; bioinformatics; cell signaling

1. Introduction

Cardiometabolic disease is a cluster of metabolic dysfunctions including insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension and central adiposity that, over time, may translate in type 2 diabetes and cardiovascular disease [1]. Unhealthy eating habits leading to overweight and obesity have been recognized as key determinants in the development of cardiometabolic disease [2]. Since dietary factors interfere with cardiometabolic disease progression in connection to individual genetic setting [3], the understanding of the impact of nutrients and bioactives on the complex networking of human genes has been long envisaged as a recommended research goal [4]. Even though this research focus has produced novel results to date, the recent application of bioinformatics and molecular biology tools to nutritional science has produced a large body of new exciting evidence on how food and food bioactives may interact with the genome to control health and wellness [5].

Among plant food bioactives, the most impressive advancements have been achieved in the field of polyphenols [6]. Polyphenols are secondary plant metabolites, which are classified into flavonoids and non-flavonoid compounds. The main subclasses of flavonoids include flavanols (flavan-3-ols), flavonols, flavones, flavanones, isoflavonoids, and anthocyanins [7]. Flavanols, the focus of our study, are among the most abundant polyphenols in the human diet [8] with main dietary sources in green tea, cocoa, apples and grapes. From a chemical point of view, flavanols represent a complex subclass of flavonoids, which encompass a variety of monomeric, oligomeric and polymeric compounds. The main monomeric forms include: (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate. Proanthocyanidins (also known as condensed tannins) are oligomers or polymers of

202 flavanols, whereas polymers composed exclusively of catechin or epicatechin are called
203 procyanidins. In foods, flavanols exist predominantly as aglycones [9].
204
205 The metabolism of dietary flavanols in the human body includes series of biochemical
206 transformations that involve both host-microbiome interactions in the large intestine and
207 microbiome independent routes. Flavanol absorption largely depends on their
208 physicochemical properties; monomers can be absorbed in the small intestine but most of
209 ingested flavanols reach intact the large intestine [10,11]. In enterocytes, most of the absorbed
210 monomers are subjected to initial phase II metabolism, which include conjugation reactions
211 such as glucuronidation, sulfatation and methylation. Exception are (-)-epicatechin-3-O-
212 gallate and (-)-epigallocatechin-3-O-gallate [12], where 3-O-galloyl moiety is considered to
213 interfere the enzymes of phase II metabolism [9], and as such they reach the circulation as
214 parent compounds. Some of the phase II metabolites are transported back from the
215 enterocytes to the intestinal lumen, whereas the others are transported to the liver, where their
216 metabolism by phase II enzymes continues [13]. Since conjugation reactions facilitate the
217 excretion of flavanol derivatives, the plasma concentrations and half-life of flavanol phase II
218 metabolites result to be very low: their maximal plasma concentrations are usually found in
219 the range of nanomolar to low micromolar [14], which are reached approximately two hours
220 post-ingestion and followed by a rapid elimination [12]. A small number of dimeric
221 compounds are also absorbed in the small intestine. Most of the ingested flavanols reach the
222 large intestine where, together with the residual products of intestinal and liver phase II
223 metabolism, they are catabolized by the microbiome. Small phenolic and aromatic acids, such
224 as phenyl-γ-valerolactones, are generated through the biochemical transformations of
225 flavanols by gut microbiota. These metabolites can be absorbed and further subjected to
226 phase II metabolism before their elimination from the human body [9,15]. Therefore, besides
227 epicatechin-3-O-gallate and (-)-epigallocatechin-3-O-gallate that appear in the systemic

228 circulation as parent compounds, several flavanol glucuronidated, sulfatated and methylated
229 metabolites, and phenolic acids represent the most common forms traceable in the systemic
230 circulation and are those that likely mediate the beneficial health effects of their parent
231 compounds. These metabolites are chemically and, in many instances, functionally distinct
232 from the parent dietary forms, and such features determine their biological effectiveness [16].
233 In particular, conjugated forms of flavonoids were shown to have a significantly lower
234 capacity for donating hydrogens and scavenging free radicals compared to the parent
235 compounds [17].
236
237 Growing evidence from cohort studies and randomized trials indicate that higher dietary
238 intake of polyphenols reduces the risk of cardiovascular mortality [18] and positively
239 influences some of the key cardiometabolic risk factors, such as blood glucose, blood lipids,
240 blood pressure, endothelial dysfunction and arterial stiffness [19-21]. Despite the large body
241 of clinical and experimental data [22], evidence regarding the role of polyphenols in
242 cardiometabolic protection remains not entirely consistent. This inconsistency can be
243 explained by differences in study designs and polyphenols tested [23,24]. However, recent
244 findings are also pinpointing role of sex, age, gut microbiome, life-style but also genotype
245 and more recently epigenetic variations as potential factors contributing to heterogeneity in
246 the individual response to the consumption of polyphenols [25-27].
247
248 Although cardiovascular benefits of polyphenols have been in the past attributed to their
249 antioxidant properties (as free radical scavengers) [28], this view was not in agreement with
250 available knowledge about their bioavailability and *in vivo* metabolism [29]. Complementary
251 evidence suggests that their protective activities may mainly occur through genomic effects,
252 by interfering with the expression of genes [29]. Nutrigenomics can be defined as approach
253 to elucidate the diet-gene interaction by assessing gene or protein expression and gene

regulation [30,31]. The capacity of polyphenols to modulate gene expression has been identified in different cell types and for different families of polyphenols. For example, in endothelial cells, flavanone metabolites have been shown to affect the expression of a number of genes related to atherogenesis and especially those involved in cell adhesion, cytoskeleton organization, inflammation, and chemotaxis [32]. Similarly, the exposure of endothelial cells to curcumin before applying a pro-inflammatory stress, induced positive changes in the expression of genes involved in the control of cytoskeleton and endothelial junction dynamics, and in the pro-inflammatory redox-sensitive transcription factor NF-kappa B [33]. In a complementary fashion, the adoption of untargeted approaches has shown that plasma epicatechin metabolites affect the expression of more than two hundred of genes, some of them involved in endothelial permeability and interaction with immune cells, thus demonstrating a multi-targeted mode of action for flavanols [34]. Together with *in vitro* investigations, nutrigenomic modifications of polyphenols have also been demonstrated in several *in vivo* models of cardiometabolic disease. Curcumin [35] and naringin [36] modulate, in an anti-atherogenic manner, the gene expression profile in the aorta of mice model of atherosclerosis. Naringin is also able to modulate the expression of genes related to lipid metabolism, inflammation and insulin signaling in the liver of mice fed a high-fat diet [37]. Finally, in rats, quercetin was shown to affect the expression of genes involved in fatty acids metabolism in lung tissue [38]. In humans, several studies have confirmed the capacity of many of these food bioactives, including flavanols [39] and flavanones [40] to exert nutrigenomic regulation. However, most nutrigenomic findings with polyphenols are from *in vitro* studies focusing on expression of few target genes (targeted approaches), and using non-physiologically relevant conditions, that is high concentrations of non-circulating compounds for long period of time, conditions that do not take into account the bioavailability and metabolism of polyphenols following their intake. For these reasons we decided to work only on studies that were performed in physiologically relevant conditions,

that is use of circulating forms and right concentrations, studies that provided findings that are possible to happen *in vivo*. Furthermore, several studies reported opposite effects depending on concentrations used, for example significant effect at physiologically relevant concentrations on prevention of monocyte adhesion to endothelial cells, which is not observable at higher concentrations [22].

On this background, experts involved in the COST POSITIVe network (https://www6.inrae.fr/cost-positive) [41] aimed to identify the most significant target genes and cellular pathways of flavanols underlying their cardiometabolic health properties by performing systematic bioinformatic analyses of available nutrigenomic data. To this aim, we conducted a systematic literature search for gene expressions modulated by flavanols in cellular models of cardiometabolic disease. We included hepatocytes, adipocytes, endothelial, smooth muscle and immune cells, selecting only studies adopting research protocols testing monomeric or dimeric compounds or related metabolites at concentrations in the range of those fund in the plasma after flavanol intake. The identified differentially expressed genes were then subjected to a comprehensive and integrative bioinformatic analysis among the different cell models to decipher and characterize key target genes and mechanisms of action of flavanols within a new, more holistic perspective. In parallel, we also analyzed the nutrigenomic effects of flavanols in intestinal cells exposed to high concentrations of extracts or oligomeric compounds, as occurring after the ingestion of flavanols rich sources. The results of these analyses will pave the way for the identification of genes and pathways underlying the health effects of flavanols. This knowledge will allow us to identify potential genes which polymorphisms can be investigated in humans with the aim to better explain some aspects of the inter-individual variability in response to consumption of flavanols. It will also guide the setup of future nutrigenetic studies aiming to

identify flavanol responsive genotypes, whereby flavanol intake will be optimized to reduce the disease risk.

2. Methods

2.1. Data sources and search strategy

Literature searches were performed using two main scientific repositories, PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and Web of Science (https://www.webofknowledge.com). Both databases were searched for all relevant studies published until January 23, 2018. Search terms included, as "plant food bioactives", catechin OR epicatechin OR epigallocatechin OR procyanidin OR proanthocyanidin AND, as "cells", endothelial OR endothelial cells OR endothelium OR pancreatic OR pancreatic cells OR adipose OR adipose cells OR adipocyte OR intestinal OR intestinal cells OR intestinal enteroendocrine cells OR monocytoid OR monocytoid cells OR monocytes OR macrophagic OR macrophagic cells OR macrophage OR hepatic OR hepatic cells OR liver cell OR hepatocyte OR smooth muscle cell OR muscle cells OR caco-2 OR PBMC AND, as "gene/gene expression", gene expression OR miRNA OR transcript OR nutrigenomic OR TLDA OR microarray OR genomic OR mRNA.

2.2. Study selection and data extraction

To be eligible, the studies had to meet the following criteria: (1) published in English; (2) assess the effects of flavanols in *in vitro* cell models suitable to study cardiometabolic dysfunction, including endothelium, adipocytes, monocytes/macrophages, pancreatic, smooth muscle, hepatic and intestinal cells, as primary cells or cell lines; (3) show lack of toxicity at the tested concentrations; (4) evaluate data on gene expression in terms of mRNA

329 and miRNA modulation, but not proteins; (5) assess cardio-metabolic health outcomes. The
330 exclusion criteria were the following: (1) treatment of the cells with bioactive compounds at
331 concentrations higher than 10 μM (except for the intestinal cells); (2) studies performed using
332 extracts (again with the exception of the intestinal cells); (3) redundant publications; (4)
333 incomplete information; (5) insufficient or insignificant statistical analysis, (6) outcomes
334 unrelated to the study objectives; (7) lack of appropriate controls; (8) studies in animal
335 models, in humans and reviews. Also, we aimed to identify papers that showed an effect on
336 cellular function together with changes in the expression of genes to associate genomic
337 modifications with potential health impact. The initial lists of titles, as retrieved from
338 PubMed and Web of Science, were merged by using EndNote X6 reference manager
339 software, and duplicates were discarded. The resulting list of papers was screened twice, by
340 two different co-authors, to identify those that fulfilled the predefined criteria. Data were
341 extracted using a standardized template. The template was pilot-tested on a small subset of
342 studies to identify and reduce misinterpretations. Extracted data from the eligible studies
343 included: name of the first author, title, year of publication, accession number, cell type with
344 detailed description, type of challenge, associated disease, cell function evaluated, bioactive
345 compounds (if single or mixed; if pure or extract) and their concentrations, number of genes
346 studied, number of differentially expressed genes, modulation (up/down), official gene
347 symbols and full names of the differentially expressed genes, and species. Data were
348 extracted only for those genes that were identified as modulated by flavanols exposure with
349 a p-value <0.05. Extracted data were then further crosschecked by two co-authors; in case of
350 doubts and/or disagreement, a third co-author was consulted.
351

352 *2.3. Bioinformatic analysis*

353 To identify gene ontologies of the differentially expressed genes extracted from *in vitro*
354 studies, David database has been used (https://david.ncifcrf.gov) [42,43], and the identified
355 gene ontologies were plotted in treemap plot using Revigo tool (http://revigo.irb.hr/) [44].
356 Gene network analyses were searched using a text-mining algorithm of MetaCore software
357 from Clarivate Analytics (https://portal.genego.com). To identify pathways that are
358 significantly associated with the genes, we used the web tool GeneTrail2
359 (https://genetrail2.bioinf.uni-sb.de/) [45], version 1.6, as a platform to access Kyoto
360 Encyclopedia of Genes and Genomes (KEGG) and BioCarta databases, using the following
361 settings: over-representation analysis; null hypothesis (for p-value computation) – two-sided;
362 method to adjust p-values – Benjamini-Yekutieli; significance level – 0.05. Interactions
363 between functional groups of genes were searched using the online tool Metascape
364 (http://metascape.org), using the option "Multiple Gene List" [46]. The network obtained was
365 further visualized using Cytoscape platform for molecular interaction networks visualization
366 (https://cytoscape.org/) [47]. Bioinformatic analysis on protein-protein interaction (PPI)
367 between the proteins that are coded by the differentially expressed genes, including their
368 neighboring proteins, was conducted using the database STRING, version 10.5
369 (https://string-db.org/) [48]. For protein-protein interaction in adipocytes, hepatocytes,
370 immune, smooth muscle and endothelial cells we applied the following settings: confidence;
371 text-mining, experiments, databases, co-expression; high confidence – 0.700; no more than
372 20 interactions in the first shell and no more than 20 interactions in the second shell, without
373 clustering. STRING settings for the intestinal cells were the following: confidence; text-
374 mining, experiments, databases, co-expression; high confidence – 0.700; no more than 15
375 interactions in the first shell and no more than 15 interactions in the second shell. The
376 resulting protein network was organized in two clusters. For integrated functional analyses
377 of identified genes and their associated transcription factors and potential miRNAs involved
378 in their post-transcriptional regulation, we used OmicsNet online tool from MetaboAnalyst

(https://www.omicsnet.ca/faces/home.xhtml) [49,50]. miRNet 2.0 was used for identification of potential miRNAs (https://www.mirnet.ca). For identification of official names and symbols of flavanol modulated genes, we used GeneCards (https://www.genecards.org/) [51] and NCBI (https://www.ncbi.nlm.nih.gov/) databases.

3. Results

3.1. Literature search and characteristics of papers selected for bioinformatic analysis

The initial systematic search in PubMed and Web of Science using the pre-defined words identified more than 1500 publications. Publications that were out of scope or in duplicate were removed. The remaining 658 papers were distributed among the co-authors for screening. The screening based on title and abstract retrieved 79 papers as eligible for data extraction. Following a detailed analysis of the full text, 41 papers were considered for bioinformatic analysis (Table 1 and supplemental Table S1), that is *in vitro* studies in which cells have been exposed to flavanols (from tea, cocoa, apple or grape seed), at concentrations lower than 10 μM (intestinal cells were an exception), and for which expression of genes at mRNA level had been analyzed. The flow diagram of the literature search and data extraction is summarized in Figure 1.

The majority of the studies, 26 out of 41 (63.4%), were conducted on cells of human origin, and 15 (36.6%) of studies were conducted on rodent cells, 10 from mouse and 5 from rat. Out of 41 studies, 37 reported results from *in vitro* studies using different cell models related to cardiometabolic disease: adipocytes, hepatocytes, immune, smooth muscle, and endothelial cells, and 5 used intestinal cells (in one paper both hepatocytes and intestinal cells were used [52]). Although pancreatic cells were included in the search criteria, we were not able to identify any eligible study conducted on this type of cells. As shown in Table 1, within

404 the 37 papers, the majority of experiments were conducted on cells that were challenged with
405 dysmetabolic or pro-inflammatory stimuli, while the others examined the effects of flavanols
406 under resting (basal) conditions. Most of these studies were carried out on endothelial cells
407 (37.8%), followed by immune cells (27%), adipocytes (13.5%), smooth muscle cells (13.5%),
408 and finally hepatocytes (8.1%). About half of the studies were conducted using primary cells,
409 while the others used cell lines. Flavanols that were used for treatment of the cells include
410 monomers, such as catechin, epicatechin, epicatechin gallate, epigallocatechin and
411 epigallocatechin gallate (EGCG), the dimer procyanidin B2, and various flavanol
412 metabolites. As shown in Table 1, flavanol metabolites were analyzed only in a small number
413 of studies. Concentrations of flavanols and their metabolites varied from 0.1 to 10 µmol/L,
414 in average 5 µmol/L, and the cells were treated for a period from 3 hours to over 24 hours.
415
416 In experiments conducted on intestinal cells, Caco-2 cells were used as an exclusive cell
417 model. In these experiments, cells were exposed to grape seed extract or oligomeric
418 compounds, most often at high concentrations (Table 1), which is out of our pre-established
419 inclusion criteria for the other cell types. However, because these experimental conditions
420 resemble physiological conditions for the intestinal cells, these papers were included in our
421 study, but the differentially expressed genes were analyzed separately.
422
423 *3.2. Identification of differentially expressed genes in cell models of cardiometabolic*
424 *disease*
425 Most of the retrieved studies adopted a targeted approach, analyzing the expression of a
426 selection of targeted genes at the mRNA level. Only two studies adopted an untargeted
427 (holistic) approach, using microarray methods [22,53]. However, for these studies, only RT-
428 PCR data, used to validate microarray data, have been extracted and used for global
429 systematic analysis.

Detailed analysis of human and rodent cell models of cardiometabolic disease (adipocytes, hepatocytes, immune, smooth muscle, and endothelial cells) exposed to flavanols (monomers, dimers, or their metabolites) identified 92 differentially expressed genes at the mRNA level. An overview of data extracted from the papers reporting experiments on human and rodent cell models of cardiometabolic disease is given in Table 1, while more detailed information can be found in the supplemental Table S1. We observed that some genes had been studied more frequently than others, which results in their more frequent identification as differentially expressed. For example, *CCL2* has been identified as differentially expressed by flavanols in seven different studies, *APOA1* in five experiments, *TNF* in four different studies, whereas *MMP9, IL6, LDLR, APOB, ABCA1, PPARG* and *CRP* were identified as differentially expressed three times each (Figure 2A). After removal of the duplicates, a total number of genes whose expression was modulated by flavanols was 54, which were subjected to bioinformatic processing. Of these 54 genes, 42 genes were identified as having expression modulated by flavanols using human cells, 14 in mouse cell models, and 3 in cells of rat origin (Figure 2B). The analysis of papers examining the effects of flavanols in intestinal cells identified 15 differentially expressed genes (Table 1 and supplemental Table S1), i.e., 14 genes after removal of one duplicate, which were analyzed through a separate bioinformatic analysis.

3.3. Global gene enrichment and functional annotation analysis of differentially expressed genes

In order to identify biological functions of the genes differentially expressed by flavanols in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells, we first performed a global gene ontology analysis. As shown in Figure 3, the analysis suggests that these genes

455 are involved in the regulation of different biological processes, including cell signal
456 transduction, biosynthesis, immune response, cell adhesion, and cell proliferation/death.
457
458 Aiming to deepen the identification of biological processes in which these genes are
459 involved in, we performed gene network analysis using a text-mining approach. We used the
460 list of differentially expressed genes identified in different studies to construct gene-gene
461 networks. The networks were grouped in clusters representing specific biological processes,
462 which are presented in the pie slice (Figure 4). As shown in Figure 4, flavanol modulated
463 genes are involved in processes regulating inflammation, immune response, cell adhesion,
464 apoptosis and cell signaling. Within the inflammation network cluster are pathways that
465 include IL-2, 4, 6 signaling, chemotaxis, or IL-10 anti-inflammatory response. Within the
466 signal transduction network cluster are pathways involved in insulin signaling, nitric oxide
467 signaling or TGF-beta, GDF and activin signaling. The cell adhesion network cluster includes
468 processes regulating cell junctions, integrin-mediated cell-matrix adhesion, leucocyte
469 chemotaxis, or platelet-endothelium-leucocyte interactions. Overall, this analysis suggests
470 that flavanols can modulate the expression of genes identified from different cell models of
471 cardiometabolic disease that are collectively implicated in the regulation of inflammation,
472 cell adhesion and metabolic processes.
473
474 To further investigate the functional role of flavanol modulated genes, we aimed to
475 search for cellular pathways in which these genes are involved using the online platform
476 GeneTrail2, which allows accesses to KEGG and BioCarta databases. Of 54 genes that were
477 found differentially expressed at mRNA level in adipocytes, hepatocytes, immune, smooth
478 muscle and endothelial cells, 53 genes were mapped in GeneTrail2, whereas *MT-CO3* failed
479 the identification. The enquiring of KEGG database revealed that the differentially expressed
480 genes are placed within pathways related to both cellular processes and human diseases.

Among the top pathways related to cellular processes are those involved in cell signaling and endothelial cell permeability, including cell adhesion, regulation of cytoskeleton organization, or focal adhesion (Figure 5). The top five KEGG pathways related to cellular processes are all involved in cell signaling and include "TNF signaling pathway", which encompasses eleven differentially expressed genes (*CCL2, EDN1, ICAM1, IL6, JUN, MMP9, NFKB1, PTGS2, SELE, TNF* and *VCAM1*), "NF-kappa B signaling pathway" encompassing eight genes (*BCL2, CXCL8, ICAM1, NFKB1, PTGS2, TLR4, TNF* and *VCAM1*), "HIF-1 signaling pathway", also with eight genes (*BCL2, EDN1, HMOX1, IL6, NFKB1, NOS2, SERPINE1* and *TLR4*), "Toll-like receptor signaling pathway" with seven genes (*CXCL8, IL6, JUN, NFKB1, TLR4, TNF* and *TOLLIP*) and "NOD-like receptor signaling pathway" with six genes (*CCL2, CXCL8, IL6, NFKB1, NLRP3* and *TNF*). Among pathways related to regulation of the endothelial cell permeability, the highest number of encompassed genes modulated by flavanols have been found in "leukocyte transendothelial migration" (six genes: *ICAM1, ITGAM, ITGB1, MMP9, ROCK1* and *VCAM1*) and "cell adhesion molecules" (five genes: *ICAM1, ITGAM, ITGB1, SELE* and *VCAM1*). Among top KEGG pathways related to human diseases, infectious diseases were predominant, but non-alcoholic fatty liver disease (NAFLD), which is a consequence of complex metabolic dysfunctions, was also present encompassing nine genes (*ADIPOQ, BAX, CEBPA, CXCL8, IL6, JUN, NFKB1, SREBF1* and *TNF*).

Accordingly, the enquiring of BioCarta database returned pathways involved in inflammation, lipid metabolism and cell signaling (Figure 5). Top BioCarta pathways related to inflammation include "cells and molecules involved in local acute inflammatory response", which encompasses six differentially expressed genes (*CXCL8, ICAM1, IL6, ITGB1, TNF* and *VCAM1*), "monocyte and its surface molecules", encompassing four genes (*ICAM1, ITGAM, ITGB1* and *SELE*), "adhesion and diapedesis of granulocytes" (*CXCL8,*

ICAM1, ITGAM and *TNF*), and "adhesion and diapedesis of lymphocytes" (*CXCL8, ICAM1, ITGB1* and *VCAM1*), also encompassing four genes each. Top BioCarta pathways related to lipid metabolism are the following: "visceral fat deposits and the metabolic syndrome", encompassing five genes (*ADIPOQ, LPL, PPARG, RETN* and *TNF*), "mechanism of gene regulation by PPARA", encompassing six genes (*APOA1, JUN, LPL, NOS2, PTGS2* and *TNF*) and "LDL pathway during atherogenesis", with four genes (*CCL2, IL6, LDLR* and *LPL*).

Together with the identification of cellular pathways in which the genes are involved in, to facilitate their biological interpretation, we also performed network meta-analysis of interactions between functional groups of genes using text-mining approach implemented in the Metascape online tool. This analysis reveals not only the list of functions of the genes but also functional interaction between them in different cellular processes. This analysis has been performed using the option "Multiple Gene List", that is lists of genes identified as modulated by flavanols in different cell types: adipocytes, smooth muscle cells, immune cells, endothelial cells and hepatocytes, allowing us to identify which functions are specific to which cell types. Global analysis has shown that flavanol modulated genes are involved in processes regulating lipid metabolism, inflammatory response, cellular response to TNF, AGE-RAGE pathway in diabetes, or regulation of binding. Some of the functions are common to all cell types studied, such as inflammatory response and cellular response to TNF. Functions such as steroid metabolic response are more specific to hepatocytes, or HIF-1 signaling to endothelial cells (Figure 6). These analyses showed that exposure of cells to flavanols could modulate different cellular processes that are interacted at the cellular level.

For analysis of functional links between proteins coded by the differentially expressed genes extracted from the literature and their neighboring proteins, we used the database STRING. All 54 differentially expressed genes were identified as valid by STRING software.

The network obtained consists of 94 nodes (proteins) having 515 edges (interactions) with PPI enrichment value <1.0e-16 (Figure 7). Notably, some of the proteins have more interactions with other proteins within the network than others, indicating their key role in the cellular response to flavanols. For example, TNF, IL6, JUN, TLR, NFKB1, and MAPK8 are on the top of the list with ≥ 30 interactions (Table 2).

3.4. Transcriptional and post-transcriptional regulation of gene expression by flavanols

Our next step of analyses aimed to identify potential transcriptional and post-transcriptional regulators involved in the observed modulation of gene expression by flavanols. Expression of genes can be regulated at the transcriptional level by the activity of transcription factors or post-transcriptionally by non-coding RNAs such as miRNAs. Using the bioinformatics tool OmicsNet, we first searched for protein-protein interactions followed by the evaluation of potential transcription factors and then potential miRNAs that could bind to mRNA of the identified protein-protein network to exert post-transcriptional regulations. Top 20 transcription factors and miRNAs, with the highest number of interactions in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells are presented in Table 3**.** Among the most significant transcription factors identified are GATA2, NFKB1, FOXC1, or PPARG. The miRNAs identified to interact with flavanol modulated genes identified are mir-335-5p, let-7b-5p, mir-26b-5p or mir-16-5p. Visualization of the interaction between the proteins of protein-protein interaction network with miRNAs and transcription factors is presented in a 3-layer 3D mode in Figure 8. These analyses showed a “dense” interaction between proteins and the regulatory elements, with each miRNA being able to regulate several proteins and one protein being potentially regulated by several miRNAs. The same is observed for transcription factors. This analysis revealed potential regulators of gene expression whose activity or level might be affected by flavanols, which determines the observed nutrigenomic modifications.

559

560 *3.5. Nutrigenomic effects of flavanols in intestinal cells*

561 Fifteen differentially expressed genes have been identified in the intestinal cells, i.e., 14
562 different genes, after removal of one duplicate (Table S1; Table 1). Bioinformatic analysis
563 demonstrated that these genes are most significantly associated with "PPAR signaling
564 pathway", which encompasses seven of 14 differentially expressed genes, and the
565 "adipocytokine signaling pathway", encompassing four of 14 genes. Other KEGG pathways
566 that are significantly related to the differentially expressed genes in the intestinal cells include
567 "fat digestion and absorption", "fatty acid degradation", "fatty acid metabolism", "bile
568 secretion" and "peroxisome", all of them encompassing 3 different genes, as well as "vitamin
569 digestion and absorption", encompassing two genes. The enquiring of BioCarta database
570 revealed only "mechanism of gene regulation by peroxisome proliferators via PPARA"
571 (Figure 9A). By analyzing the protein-protein interactions using the STRING database, two
572 protein clusters were identified for the intestinal cells. One of them includes proteins that are
573 mostly involved in the metabolism of circulating lipoproteins. Proteins that belong to this
574 cluster are shown in red. The second cluster is connected to the previous one through NOS2
575 and NOS3 and covers mainly proteins that are involved in calcium signaling. Proteins that
576 belong to this cluster are shown in green (Figure 9B). Proteins that have the highest number
577 of interactions within the clusters are lipoprotein lipase, apolipoproteins, and calmodulins
578 (Table S2).

579

580 Transcriptional and post-transcriptional regulation of flavanol modulated genes in the
581 intestinal cells was also analyzed using the bioinformatics tool OmicsNet. This analysis
582 revealed that master regulators of proteins that belong to the protein-protein interaction
583 network emerging from the differentially expressed genes extracted from the literature

584 include SP1, NFKB1, STAT3, PPARG or STAT1 among the transcription factors, and mir-
585 335-5p, mir-26b-5p, mir-16-5p, mir-124-3p or mir-92a-3p among the miRNAs. A 3-Layer
586 3D presentation of this regulatory network is given in Figure 9C.

587

588 **4. Discussion**

589 Facing an unprecedented increase of cardiometabolic, neurodegenerative and other non-
590 communicable diseases, contemporary science strives to find effective strategies for their
591 prevention and treatment. In this context, there is a growing body of scientific evidence about
592 the role of diet in general, as well as of various food constituents, including bioactives, as
593 important modulators of the cardiometabolic risk. In this review, we have systematically
594 examined the effects of flavanols in terms of modulation of gene expressions relevant to the
595 pathogenesis of cardiometabolic disease and identified potential pleiotropic pathways and
596 cellular and molecular mechanisms underlying their protective actions.

597

598 Living in the era of personalized medicine, we are witnessing an enhanced awareness of
599 the need for a personalized approach to dietary recommendations. This applies to the general
600 population in terms of good health preservation, and secondary prevention in patients with
601 various non-communicable diseases. As recently reviewed, variability in the cardiometabolic
602 response to consumption of plant food bioactives, including polyphenols, is considered as
603 one major cause of inconsistency in the results of human intervention studies [26]. This
604 variability is determined by a number of factors, among which a central role is ascribed to
605 the genetic variability beside to gut microbiome composition and functionality, sex, age,
606 lifestyle and various comorbidities (overweight and obesity, diabetes, hypertension,
607 dyslipidemia, etc.). Aiming to take the pioneering step towards the ultimate goal - identify
608 genetic variants in the human population underlying the individual metabolic response to the

609 consumption of dietary flavanols - we conducted this systematic literature search to identify
610 target genes involved in the protective effect of these compounds and which polymorphism
611 expressions may explain the inter-individual variability in response to flavanols
612 consumption. This is the first-ever systematic analysis of nutrigenomic data about the effects
613 of flavanols in cell models relevant for cardiometabolic health. In order to provide
614 physiologically relevant data, we applied rigorous criteria for inclusion/exclusion of the
615 studies, resulting in the retrieval of relatively small number of relevant papers and
616 differentially expressed genes.
617
618 The complex pathogenesis of cardiometabolic disease development, in terms of many
619 different cell types and cellular processes involved, makes the choice of relevant *in vitro*
620 models to be assessed rather challenging. Indeed, one single cell model would not be able to
621 replicate the entire pathogenesis of the disease and/or may not be sufficient to intercept the
622 therapeutic potential of a given product. Rather, taking into account different cell models,
623 evaluated together, was needed to cover the wide spectrum of different cellular processes.
624 Thus, to obtain comprehensive assessment of the genomic effects of flavanols, we extracted
625 gene expression data from intestinal cells besides to five cell types known for their major
626 contribution in cardiometabolic dysfunction, such as adipocytes, hepatocytes, endothelial
627 cells, immune cells and smooth muscle cells. We examined results from cells exposed to
628 flavanols in the presence or absence of dysmetabolic and/or pro-inflammatory stimuli (such
629 as lipopolysaccharide (LPS), glucose or cytokines), classically used to better simulate the *in*
630 *vivo* dysmetabolic conditions, and processed the gene dataset retrieved by integrated
631 functional analysis tools. The assessment of the flavanol effects in these cell models of
632 cardiometabolic disease allow circumventing several important confounding factors inherent
633 to *in vivo* studies, such as age, diet, use of drugs, and chronobiological variations. For this
634 reason, cell models are useful to unveil all those metabolic alterations induced by a treatment

635 with flavanol that might not be revealed in studies using animal models or human subjects,
636 due to biological sample complexity. Notwithstanding, these *in vitro* models present some
637 limitations, particularly the fact that cultured cells fail to reproduce the complex cell-cell and
638 cell-matrix interactions recognized as a key determinant in the definition of the final cell
639 homeostasis. In the attempt to interpret the data extracted in a more complex cell networking
640 and circumvent the use of monotype cell models, data were also subjected to an integrated
641 bioinformatic analysis among different cell models. Nevertheless, the findings obtained from
642 these *in vitro* studies need confirmation and validation in animal models and human studies.
643
644 To understand the biological role of the differentially expressed genes extracted from the
645 literature, they were subjected to a global bioinformatic analysis. By integrating the relatively
646 small amount of data scattered across different cell models on the one hand, and applying the
647 powerful bioinformatics tools driven by a large amount of information on the other, we have
648 been able to obtain a broader and more complex insight into the molecular effects of flavanols
649 on the cardiometabolic health. This strategy allowed us to overcome the limitation of the
650 targeted-approach (i.e., analysis of a selected, limited and predefined target genes) featuring
651 most of the studies selected. The global analysis using the bioinformatics tools allowed us to
652 identify, quantify and describe their role in the cellular functions. Furthermore, by integrating
653 data from different cell types, the derived model could mimic, to some extent, the whole
654 organism, which is particularly important for the cardiometabolic disease where several
655 organs and tissues are implicated, connected with complex causal links.
656
657 This systematic review has identified 37 *in vitro* studies with 54 different genes up- or
658 down-regulated by flavanol exposure in adipocytes, hepatocytes, immune, smooth muscle,
659 and endothelial cells. Global bioinformatic analysis of differentially expressed genes
660 extracted from literature has demonstrated that flavanols primarily modulate different

pathogenic aspects of cardiometabolic disease particularly processes of inflammation, cell adhesion and transendothelial migration, or lipid metabolism (Figure 10).

Low-grade inflammation is a risk factor that induces endothelial dysfunction in medium- and large-sized arterial blood vessels [54]. Dysfunctional endothelium is characterized by an increased permeability to atherogenic lipoproteins [54] and circulating immune cells [55]. Under such conditions, endothelial cells increase the expression of leukocyte adhesion molecules on their surface [55]. In particular, ICAM1 and VCAM1, along with a plethora of adhesion molecules and ligands, play major roles in the process of adhesion and transendothelial migration of circulating monocytes, which includes a series of complex sequential events, such as capture, slow rolling, firm adhesion, adhesion strengthening, intraluminal crawling and finally, the transendothelial migration [55]. Flavanols have been shown to decrease the expression of leukocyte adhesion biomarkers in humans [56], as well as the leukocyte rolling over endothelium in an animal model of inflammation [34]. However, a more in-depth analysis of molecular mechanisms underlying the protective effects of flavanols on the arterial endothelium has been made only recently, demonstrating a high level of modulation of pathways defining cell adhesion and transendothelial migration [34]. Concordantly, we also identified several regulators of cell adhesion, such as the "platelet-endothelium-leucocyte interaction" and "cell adhesion molecules", including *ICAM1, ITGAM, ITGB1, SELE* and *VCAM1* genes as primarily affected by flavanols. The interaction between immune and endothelial cells requires the attraction of immune cells to endothelium. This process is regulated by several chemokines, which are involved in "leucocyte chemotaxis" and "chemokine signaling" pathways. In line with previous results, these pathways have also been recognized to be affected by flavanols. Upon adhesion to endothelium, immune cells migrate in sub-endothelial space, predominantly following paracellular routes [55]. Paracellular transendothelial migration requires the reorganization

687 of endothelial cytoskeleton, which is mediated by several genes, including *ROCK1* [57].
688 Interestingly, our bioinformatic analyses identified pathways and gene networks regulating
689 the monocyte transmigration, such as "leukocyte transendothelial migration pathway",
690 "regulation of actin cytoskeleton", "focal adhesion" or "cell junctions". "Leukocyte
691 transendothelial migration pathway" exhibited the highest statistical significance among the
692 pathways defining the endothelial cell function and include the following genes extracted
693 from *in vitro* studies: *ICAM1, ITGAM, ITGB1, MMP9, ROCK1* and *VCAM1*. Concordantly,
694 bioinformatic analysis of protein-protein interactions of extracted genes that are placed in the
695 modulated cellular pathways responsible for endothelial cell function, demonstrated that
696 TNF, MAPK8 and NFKB1 are central to the network of protein-protein interactions, also
697 revealing the role of inflammation as a common underlying mechanism of cardiometabolic
698 disease. Taken together, these systematic bioinformatic analyses showed that regulation of
699 endothelium by flavanols is one of the key molecular mechanisms of these bioactives
700 underlying their health properties. Genes regulating this function present potential candidates
701 for further analyses of their importance for the inter-individual variability in response to
702 consumption of dietary flavanols.
703
704 The enquiring of BioCarta database identified pathways linked to lipid metabolism
705 including "visceral fat deposits and the metabolic syndrome", "mechanism of gene regulation
706 by peroxisome proliferators via PPARA" and "LDL pathway during atherogenesis". It is well
707 known that adipose tissue exerts immune-metabolic functions. Besides functioning as an
708 energy storage tissue (storing energy in the form of lipid) and controlling the lipid
709 mobilization and distribution in the body, it acts as an active endocrine organ by releasing a
710 cluster of active molecules, named adipokines with autocrine and paracrine functions and
711 modulating a range of metabolic pathways [58]. It is now widely recognized that adipose
712 tissue dysfunction, as in terms of adipose hypertrophy and deregulated release of adipokines,

713 plays a prominent role in the development of obesity and its related disorders such as insulin
714 resistance or cardiovascular disease [59]. Visceral fat accumulation, linked with levels of
715 some adipokines, induces chronic inflammation and metabolic disorders, including glucose
716 intolerance, hyperlipidemia, and arterial hypertension. Together, these conditions contribute
717 to a diagnosis of metabolic syndrome, directly associated with the onset of cardiovascular
718 disease [60]. Our data suggest that flavanols significantly interfere with the pathway related
719 to "visceral fat deposits and the metabolic syndrome" regulating the expression of five
720 interesting genes within this pathway: *PPARG, LPL, TNF, RETN* and *ADIPOQ*. Several
721 epidemiological and experimental studies have shown robust hypolipidemic and anti-
722 obesogenic effects by flavanols [61,62]. Regulation of peroxisome proliferator-activated
723 receptors (PPARs) activity and expression by these compounds has been largely suggested
724 as the primary mechanism of hypolipidemic and anti-obesogenic effects exerted by most
725 flavanols [63]. PPARs are nuclear hormone receptors that function as transcription factors
726 [64]. Up to now, three PPARs have been identified, PPARA, D/B, and G with different tissue
727 distribution and pharmacological ligand activation profile [64]. Among them, PPARG is
728 abundantly expressed in adipose tissue and muscle cells whereas it mediates the expression
729 of genes associated with adipogenesis and insulin sensitivity [65], thus making it a molecular
730 target of choice for the development of therapeutic treatments of both synthetic and natural
731 origin.
732
733 Bioinformatic analyses of the extracted nutrigenomic data were not focused only to gene
734 ontology analysis and identification of cellular pathways significantly associated to
735 differentially expressed genes, but also to the gene network analyses, analysis of interactions
736 between functional groups of genes and protein-protein interactions. Furthermore, we have
737 also taken a step forward by analyzing the transcriptional (transcription factors) and post-
738 transcriptional (miRNAs) regulation of differentially expressed genes. Among the most

739 significant transcription factors identified, we recognized PPARG and GATA2. Previous
740 studies demonstrated that in addition to its role in hematopoietic stem cell development [66],
741 GATA2 also has an important role in mediating cardiovascular disease development [67]. It
742 is abundantly expressed in vascular endothelial cells and regulates endothelial-specific genes,
743 such as *VCAM1, P-selectin* and *PECAM1*, involved in endothelial activation and dysfunction
744 that can lead to development of atherosclerosis and cardiovascular disease [67]. It has also
745 been observed that inactivation of GATA2 decreases the expression of cell adhesion
746 molecules, and that it plays an essential role in endothelial cell activation by acting together
747 with NF-kappa B, which is a critical factor in the molecular pathogenesis of atherosclerosis
748 [67]. Our results, suggesting a role for flavanols in modulating *GATA2*, reveal a new potential
749 regulatory site for flavanol effects. The PPARs modulate several biological processes that
750 are perturbed in obesity, including inflammation, lipid and glucose metabolism and overall
751 energy homeostasis. PPARs agonists have some efficacy in reducing cardiovascular risk in
752 patients with type 2 diabetes who also have pro-atherogenic dyslipidemia [68]. Use of PPARs
753 agonists, such as aleglitazar, was shown to improve insulin sensitivity, glucose control and
754 lipid levels in people with type 2 diabetes [69]. Interestingly, two studies have suggested that
755 polyphenols could act as PPARs agonists and prevent risk factors for obesity-related
756 metabolic disorders and cardiovascular disease, such as polyphenols from plum [70] or grape
757 seeds [71]. Together with these 2 transcription factors, our systematic bioinformatic analyses
758 also identified other ones that present key players in the genomic response to flavanol intake,
759 like YY1, FOXC1 or NFKB1.
760
761 Along with the identification of transcriptional regulators, we also searched for potential
762 post-transcriptional regulators, particularly miRNAs. miRNAs are endogenous small non-
763 coding RNAs that can interact with mRNAs, in this way exerting post-transcriptional
764 regulation activities [72]. It has been shown that they play an important role in the regulation

765 of lipid metabolism, endothelial function, and consequently, in the development of chronic
766 diseases such as cardiometabolic disorders [72] or cancer. Our bioinformatic analysis
767 identified the mir-335-5p as the most significant miRNAs affected by flavanols. It has been
768 shown that mir-335-5p plays a role in regulating endothelial function [73], insulin secretion
769 and diabetes development [74], and in suppressing lower extremity deep venous thrombosis
770 [75]. Concordantly to our results, in mouse models of atherosclerosis catechins, hesperidin,
771 quercetin, curcumin, or anthocyanins were shown to modulate the expression of this miRNA
772 [76]. Among the other miRNAs identified by our bioinformatic analysis, there is the mir-16-
773 5p. mir-16-5p has been interestingly suggested to be associated with insulin sensitivity and
774 cardiometabolic risk factors in humans [77]. Capacity of polyphenols to regulate the
775 expression of this miRNA has been described in a few studies, such as with epigallocatechin
776 gallate and quercetin [78,79]. For let-7b-5p or mir-193b-3p, no role has been reported before
777 in regulation of cardiometabolic disorders, whereas mir-26b-5p is involved in the regulation
778 of inflammation in myocardial infarction [80]. Taken together, this systematic analysis of
779 genomic data of flavanols related to cardiometabolic effects revealed potential transcriptional
780 and post-transcriptional regulators involved in the genomic modifications of flavanols and
781 therefore novel mechanisms of action and key players in the observed effects.
782
783 Conducting this systematic bioinformatic analysis of published nutrigenomic data about
784 the effects of flavanols in cellular models of relevance for cardiometabolic health, such as
785 adipocytes, hepatocytes, immune, smooth muscle and endothelial cells, we demonstrated that
786 only in a small number of studies that were identified as eligible for inclusion in our analysis,
787 the cells were treated with flavanol metabolites (Table 1). Given the growing scientific
788 evidence that flavanol phase II and gut microbiota metabolites represent the main circulating
789 forms of the majority of these bioactives and mediate the effects of their parent compounds
790 at cellular level [9], this finding identifies a major gap in the literature limiting the power of

791 the available *in vitro* studies to demonstrate the true molecular effects of flavanols. This gap
792 in the literature should be addressed in future.
793
794 Intestinal cells are not only mediators of macro- and micronutrients absorption, but they
795 also exhibit various functions that may affect the cardiometabolic health. By synthesizing
796 triglycerides [129] and apolipoproteins [52], intestinal cells actively contribute to the
797 regulation of plasma lipoprotein pools. Noteworthy, an increased atherogenic risk features
798 patients with inflammatory bowel disease (IBD) [81]. A recent literature review has indeed
799 suggested that patients with IBD may be at an increased risk of cardiovascular diseases
800 [82,83]. Several studies have shown that chronic systemic inflammation in IBD can lead to
801 endothelial dysfunction and increased platelet activation, conditions preceding the
802 development of atherosclerotic vascular disease [84] or favoring its clinical manifestations.
803 High levels of tumor necrosis factor (TNF), C-reactive protein (CRP) and vascular
804 endothelial growth factor (VEGF) are characteristic of IBD and may therefore contribute to
805 endothelial dysfunction and atherogenesis [85]. Furthermore, in both cardiovascular disease
806 and IBD pro-inflammatory angiogenesis is recognized as a common trait sustaining both
807 atherosclerotic plaque growth and intestinal inflammation [86-88]. Finally, during IBD
808 flares, the adhesion of circulating monocytes to the intestinal microvascular endothelial cells,
809 as well as their infiltration and transformation into macrophages occurs, in tight analogy with
810 what happens in the early phases of arterial atherosclerosis [89]. Results of our bioinformatic
811 analysis suggest that flavanols may reduce cardiovascular risk also affecting the intestinal
812 homeostasis. For example, our data suggest that flavanols affect the expression of genes
813 involved in PPAR signaling pathway. Beside to adipose tissue and muscle cells, PPARG is
814 also abundantly expressed in colonic epithelial cells whereas it seems to play important anti-
815 inflammatory and anti-carcinogenic effects [90]. In experimental animal model of IBD, the
816 activation of PPARG by synthetic agonist rosiglitazone was shown to reduce the expression

817 of inflammatory genes by interfering with the activation of NF-kappa B transcription factor
818 [91]. Several experimental evidences suggest that dietary polyphenols possess both
819 protective and therapeutic effects in the management of IBD [92]. However, further
820 preclinical and clinical studies are needed in order to understand the efficacy of dietary
821 polyphenols in IBD patients.
822
823 Although cellular models do not reflect the variability across individuals within
824 population, in this work, by integrating the mechanistic *in vitro* data, we gain insights on
825 which genes or proteins are of major importance in mediating the anti-inflammatory and
826 vasoprotective effects of flavanols. Our integrative bioinformatic meta-analyses of the
827 existing genomic data from the literature allow us to better identify molecular mechanisms
828 underlying cardiometabolic health properties of flavanols and identify major molecular
829 pathways and target genes involved. Nevertheless, from the data here presented, as well as
830 from the data in the literature, there is no doubt that *TNF* and *IL6* are among the key gene
831 players in mediating flavanol anti-inflammatory activity, since their polymorphisms have
832 already been associated with lifestyle dependent cardiometabolic risk factors [93]. Our data
833 confirm and suggest the need to systematically investigate flavanol effects in relation to *TNF*
834 and *IL6* polymorphic expressions. Deeper analyses of our data and the data from the literature
835 may also identify other potential key target genes and polymorphisms that are worth further
836 studying in the context of inter-individual variability of the effects of flavanols on
837 cardiometabolic health. In conclusion, integrative biology approaches allow to identify
838 potential key players of flavanols involved in cardiometabolic disease prevention associated
839 to gene-protein-miRNA networks, which can be exploited for personalized nutritional
840 recommendations in cardiometabolic disease prevention.
841
842

Figure legends

Figure 1. Data collection flowchart. For search criteria, see Methods section.

Figure 2. A) Number of genes repeated in studies conducted on adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. B) Number of differentially expressed genes extracted from the studies on adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

Figure 3. Gene ontology for adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. Each rectangle is a single cluster representative, and they are joined into 'superclusters' of related terms, represented with different colors. Size of the rectangles reflects the p-value of the GO.

Figure 4. Gene network pie chart for adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

Figure 5. BioCarta and KEGG pathways related to cellular processes in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. *: KEGG; **: BioCarta.

Figure 6. Functional enrichment and interactome meta-analysis based on gene lists for different cell types exposed to flavanols. Enrichment network visualization of the results

from the lists of genes identified for adipocytes, smooth muscle cells, immune cells, endothelial cells and hepatocytes. Nodes are functional groups represented by pie charts indicating their associations with each cell type. Cluster labels were added manually. Color code represents the identities of gene lists (adipocytes: red, endothelial cells: blue, hepatocytes: green, immune cells: violet) and size of each color is proportional to the percentage of the genes from different types of cells.

Figure 7. Protein-protein interactions in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. Colored nodes: query proteins and first shell of interactors; white nodes: second shell of interactors; filled nodes: some 3D structure is known or predicted; empty nodes: proteins of unknown 3D structure.

Figure 8. Regulation of protein-protein interaction network by transcription factors and miRNAs in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

Figure 9. A) KEGG and BioCarta (marked with *) pathways for the intestinal cells exposed to flavanols. B) Protein-protein interactions for the intestinal cells exposed to flavanols. Protein network is organized in two clusters: in red – proteins that are mostly involved in the metabolism of circulating lipoproteins; in green – proteins that are mainly involved in calcium signaling. C) Regulation of protein-protein interaction network by transcription factors and miRNAs in the intestinal cells exposed to flavanols.

Figure 10. Summary of identified differentially expressed genes modulated by flavanol and related to cardiometabolic health.

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Conflict of interest

The authors do not have conflict of interest

Contribution of authors

All authors contributed to conceptualization, methodology, data extraction and validation of the last version of the manuscript. TR, MM, DM contributed to writing, reviewing and editing of the manuscript and preparation, creation and/or presentation of the published work.

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Table 1. Overview of data extraction for cell models exposed to different flavanols or flavanol metabolites at physiological concentrations.

Flavanol tested	Concentration	Challenge	Differentially expressed genes; p<0.05	Reference
		Adipocytes		
EGCG	10 μM	adipogenic cocktail	*CEBPA, PPARG*	[94]
Epicatechin	0.5 – 10 μM	TNF	*IL6, CCL2, RETN, TNF*	[95]
EGCG	1 – 5 μM	dexamethasone	*ADIPOQ, RETN*	[96]
Catechin	10 μM	adipogenic cocktail	*ADIPOQ, FABP4, LPL, PPARG*	[97]
EGCG	1 μM	adipogenic cocktail	*CFD*	[98]
		Endothelial cells		
EGCG	10 μM	phorbol-12-myristate-13-acetate	*CCL2*	[99]
Catechin	0.1 – 10 μM	no challenge	*SERPINE1*	[100]
Catechin	10 μM	homocysteine	*NRF1, TFAM, MT-CO3*	[101]
EGCG	2.5 – 10 μM	no challenge	*EDN1, HMOX1*	[102]
EGCG	10 μM	no challenge	*EDN1*	[103]
EGCG	0.5 – 10 μM	vascular endothelial growth factor	*CXCL8*	[104]
EGCG	10 μM	TNF	*CCL2*	[105]
EGCG	10 μM	no challenge	*ICAM1, CCL2*	[106]
EGCG	10 μM	TNF	*ICAM1, VCAM1, CCL2, BCL2, BAX, CASP9*	[107]
Procyanidin B2	1 – 2 μM	LPS and ATP	*NLRP3*	[108]
EGCG	10 μM	glucose	*VCAM1*	[109]
EGCG	10 μM	no challenge	*PIM1*	[110]
Epicatechin, Flavanol metabolites	1 – 10 μM 0.4 – 7.8 μM	no challenge	*ARG2*	[111]

Flavanol metabolites	1 μM	TNF	*CALD1, TJP1, ARHGEF7, CASK, NFKB1, SELE, CCL2, ITGB1, ROCK1*	[22]
		Hepatocytes		
Epicatechin, Catechin, Procyanidin B2	0.1 – 10 μM	no challenge	*APOA1, APOB, LDLR, ABCA1, SREBF1, SCARB1, SCAP*	[52]
Epicatechin, Flavanol metabolites	10 μM	no challenge	*APOA1, FOXA2*	[112]
EGCG	1 – 10 μM	angiotensin II	*AGTR1, PPARG*	[113]
		Immune cells		
EGCG	3 – 10 μM	phorbol-12-myristate-13-acetate	*S1PR2*	[114]
EGCG	3 μM	phorbol-12-myristate-13-acetate	*MMP9, PTGS2*	[115]
Epicatechin	2 μg/mL	LPS	*NOS2, PTGS2*	[116]
Epicatechin gallate	3 μM	no challenge	*ITGAM*	[117]
Catechin	10 μM	LPS	*IL6, TNF*	[118]
EGCG	10 μM	phorbol-12-myristate-13-acetate	*MMP9, BSG*	[119]
EGCG	2.5 μM	no challenge	*TOLLIP*	[120]
EGCG, (-)-epigallocatechin-3-O-(3-O-methyl)-gallate	5 μM 1 μM	no challenge; palmitic acid	*RNF216,* *TNF*	[121]
EGCG	1 μM	LPS	*MMP9, CCL2*	[122]
EGCG	1 μM	LPS; no challenge	*TNF, IL6,* *TLR4, TOLLIP*	[53]
		Smooth muscle cells		
EGCG	0.1 – 10 μM	no challenge	*TIMP2*	[123]

EGCG	10 μM	basic fibroblast growth factor	*JUN*	[124]
EGCG	3 – 10 μM 1 – 10 μM	IL-6; angiotensin II	*CRP*	[125]
EGCG	3 – 10 μM	endothelin 1	*CRP*	[126]
Epigallocatechin	10 μM	serum	*JUN*	[127]
		Intestinal cells		
Hexameric procyanidins	20 μM	TNF	*NOS2*	[128]
Grape seed extract	100 mg/L 25 – 100 mg/L	fasted state medium; postprandial state medium	*ACSL5, ACSL3, FABP2, PPARA, CPT1A*	[129]
Cinnamtannin A2	1 – 10 μM	no challenge	*APOA1, APOB*	[52]
Grape seed extract	100 mg/L	no challenge	*DPP4*	[130]
Grape seed extract	20 – 100 mg/L	chenodeoxycholic acid	*SLC10A2, FABP6, FGF19, SLC51A, SLC51B*	[131]

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1441 **Table 2.** Proteins with the highest number of interactions within the network (≥
1442 15).

Symbol	Name	Number of interactions
TNF	Tumor necrosis factor	40
IL6	Interleukin-6	39
JUN	Transcription factor AP-1	37
TLR4	Toll-like receptor 4	30
NFKB1	Nuclear factor NF-kappa-B p105 subunit	30
MAPK8	Mitogen-activated protein kinase 8	30
IL8	Interleukin-8	26
CCL2	C-C motif chemokine 2	24
MMP9	Matrix metalloproteinase-9	23
PPARG	Peroxisome proliferator-activated receptor gamma	22
BCL2	Apoptosis regulator Bcl-2	22
MMP2	72 kDa type IV collagenase	21
CYCS	Cytochrome c	21
FOS	Proto-oncogene c-Fos	21
ICAM1	Intercellular adhesion molecule 1	20
CRP	C-reactive protein	19
PTGS2	Prostaglandin G/H synthase 2	19
ADIPOQ	Adiponectin	19
CASP3	Caspase-3	18
NOS3	Nitric oxide synthase, endothelial	17
BCL2L1	Bcl-2-like protein 1	17
MYD88	Myeloid differentiation primary response protein MyD88	16
XIAP	E3 ubiquitin-protein ligase XIAP	16
VCAM1	Vascular cell adhesion protein 1	16
BAX	Apoptosis regulator BAX	15
EDN1	Endothelin-1	15
ITGAM	Integrin alpha-M	15

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1449 Table 3: Top 20 transcription factors and miRNAs that regulate the protein-protein
1450 interaction network in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells
1451 exposed to flavanols.

Symbol	Name	Number of hits
	Transcription factor	
FOXC1	Forkhead box protein C1	362
GATA2	Endothelial transcription factor GATA-2	266
YY1	Transcriptional repressor protein YY1	186
E2F1	Transcription factor E2F1	160
FOXL1	Forkhead box protein L1	149
USF2	Upstream stimulatory factor 2	141
RELA	Transcription factor p65	138
PPARG	Peroxisome proliferator-activated receptor gamma	137
NFKB1	Nuclear factor NF-kappa-B p105 subunit	136
CREB1	Cyclic AMP-responsive element-binding protein 1	134
TFAP2A	Transcription factor AP-2-alpha	131
TP53	Cellular tumor antigen p53	127
NFIC	Nuclear factor 1 C-type	123
POU2F2	POU domain, class 2, transcription factor 2	115
SRF	Serum response factor	115
HINFP	Histone H4 transcription factor	114
JUN	Transcription factor AP-1	113
SREBF1	Sterol regulatory element-binding protein 1	106
STAT3	Signal transducer and activator of transcription 3	106
MEF2A	Myocyte-specific enhancer factor 2A	92
	micro RNA	
mir-335-5p	microRNA-335-5p	105
mir-16-5p	microRNA-16-5p	83
mir-124-3p	microRNA-124-3p	80
mir-26b-5p	microRNA-26b-5p	79
mir-17-5p	microRNA-17-5p	77
let-7b-5p	let-7b-5p	74
mir-155-5p	microRNA-155-5p	70
mir-92a-3p	microRNA-92a-3p	70
mir-93-5p	microRNA-93-5p	66
mir-20a-5p	microRNA-20a-5p	64
mir-106b-5p	microRNA-106b-5p	61
mir-1-3p	microRNA-1-3p	53
let-7c-5p	let-7c-5p	52

mir-193b-3p	microRNA-193b-3p	51
mir-20b-5p	microRNA-20b-5p	51
mir-34a-5p	microRNA-34a-5p	51
mir-615-3p	microRNA-615-3p	50
mir-218-5p	microRNA-218-5p	49
mir-519d-3p	microRNA-519d-3p	49
mir-21-5p	microRNA-21-5p	48

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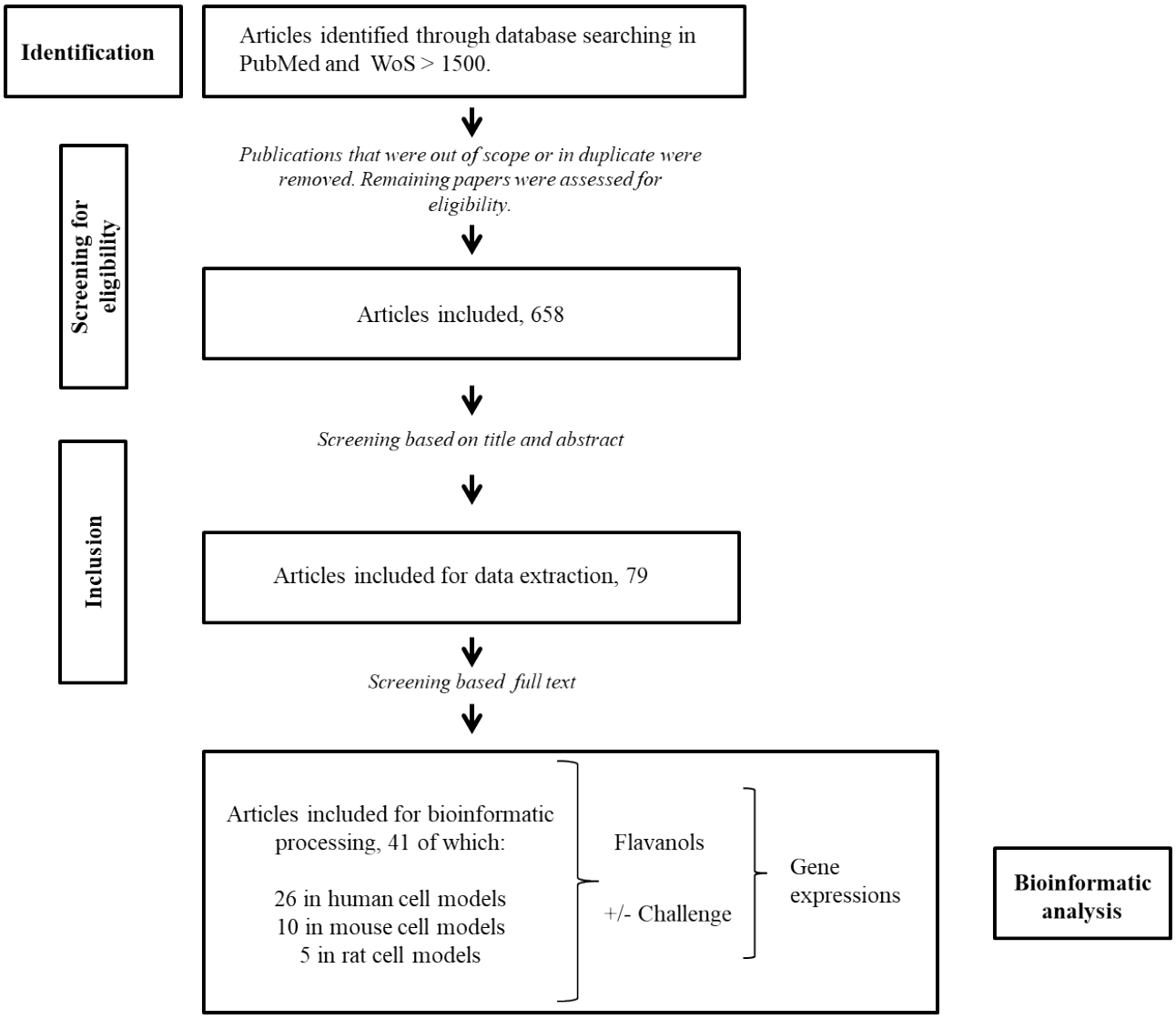

Figure 1. Data collection flowchart. For search criteria, see Methods section.

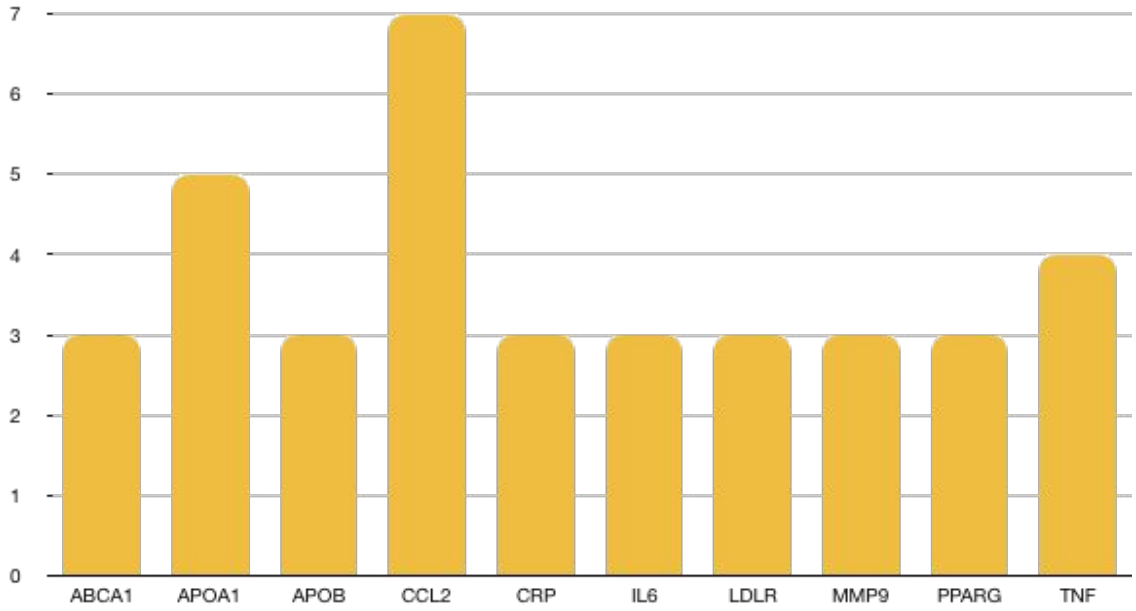

Figure 2A. Number of genes repeated in studies conducted on adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

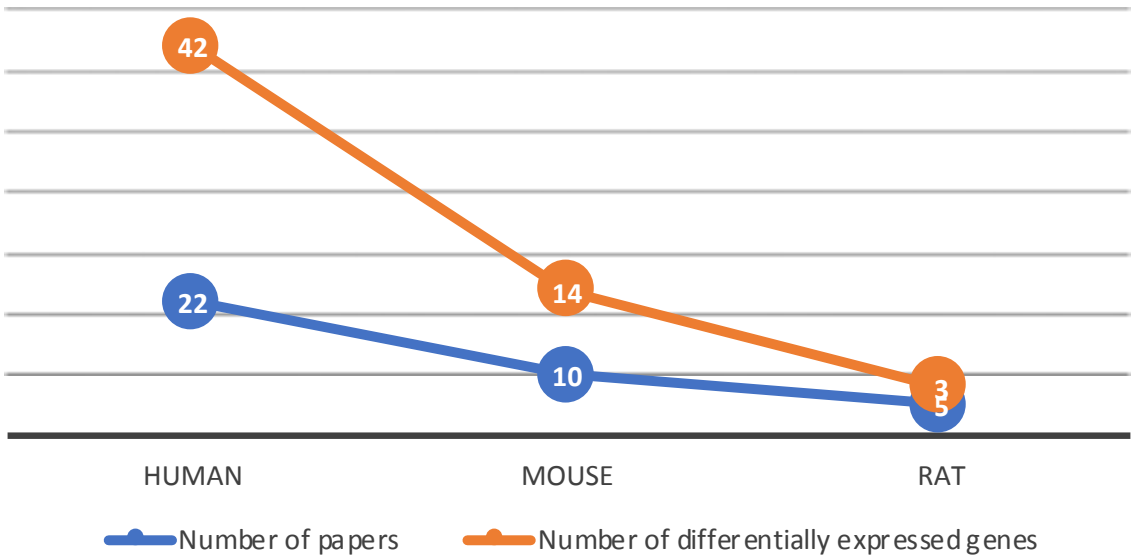

Figure 2B. Number of differentially expressed genes extracted from the studies on adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

Figure 3. Gene ontology for adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. Each rectangle is a single cluster representative, and they are joined into 'superclusters' of related terms, represented with different colors. Size of the rectangles reflects the p-value of the GO.

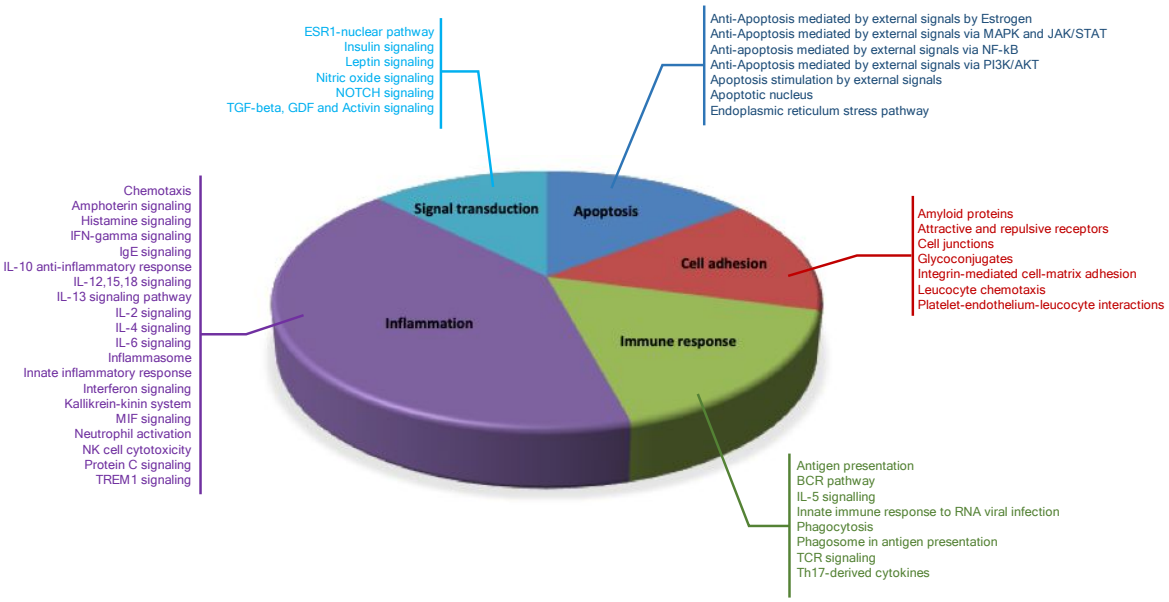

Figure 4. Gene network pie chart for adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

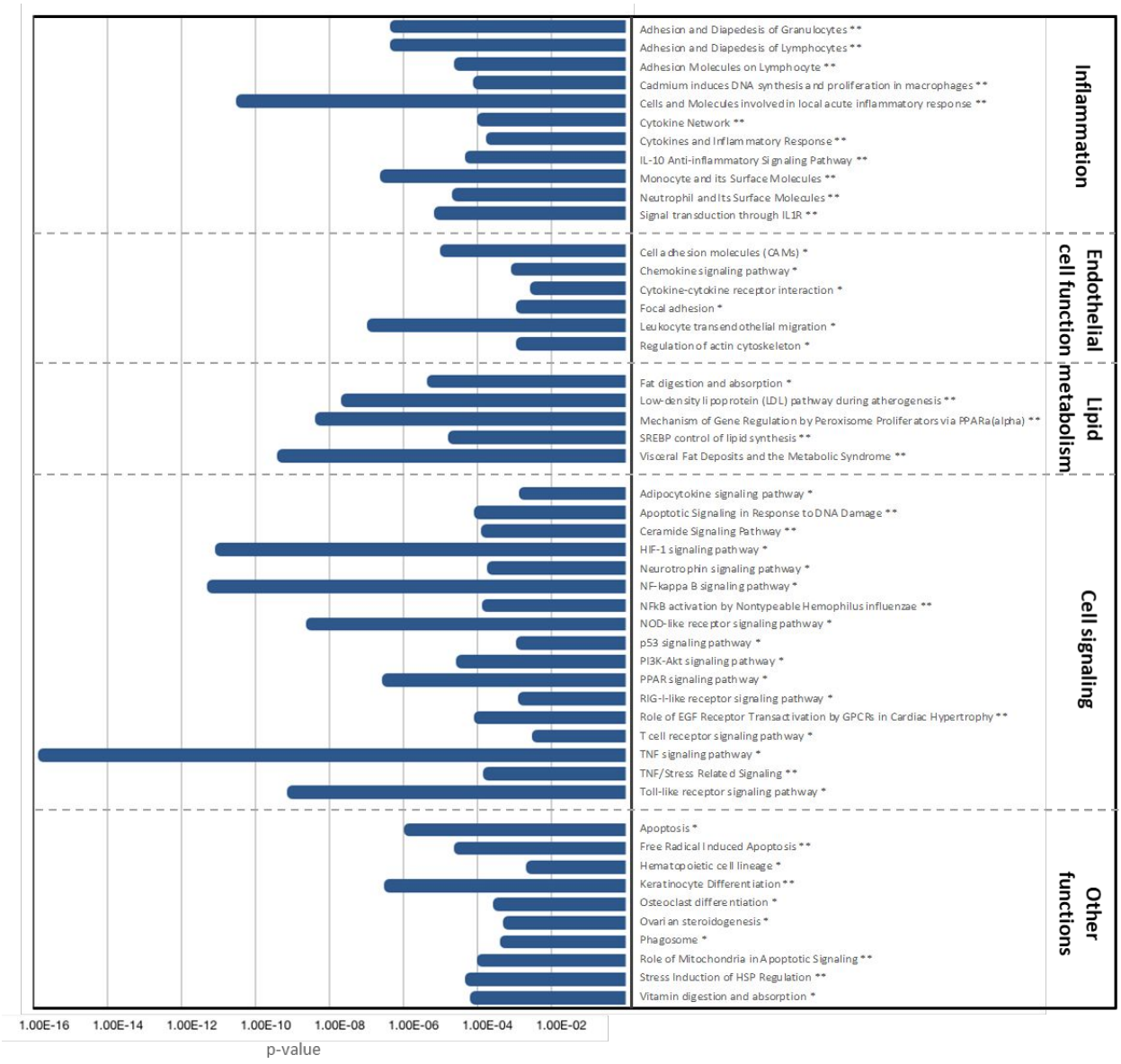

Figure 5. BioCarta and KEGG pathways related to cellular processes in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. *: KEGG; **: BioCarta.

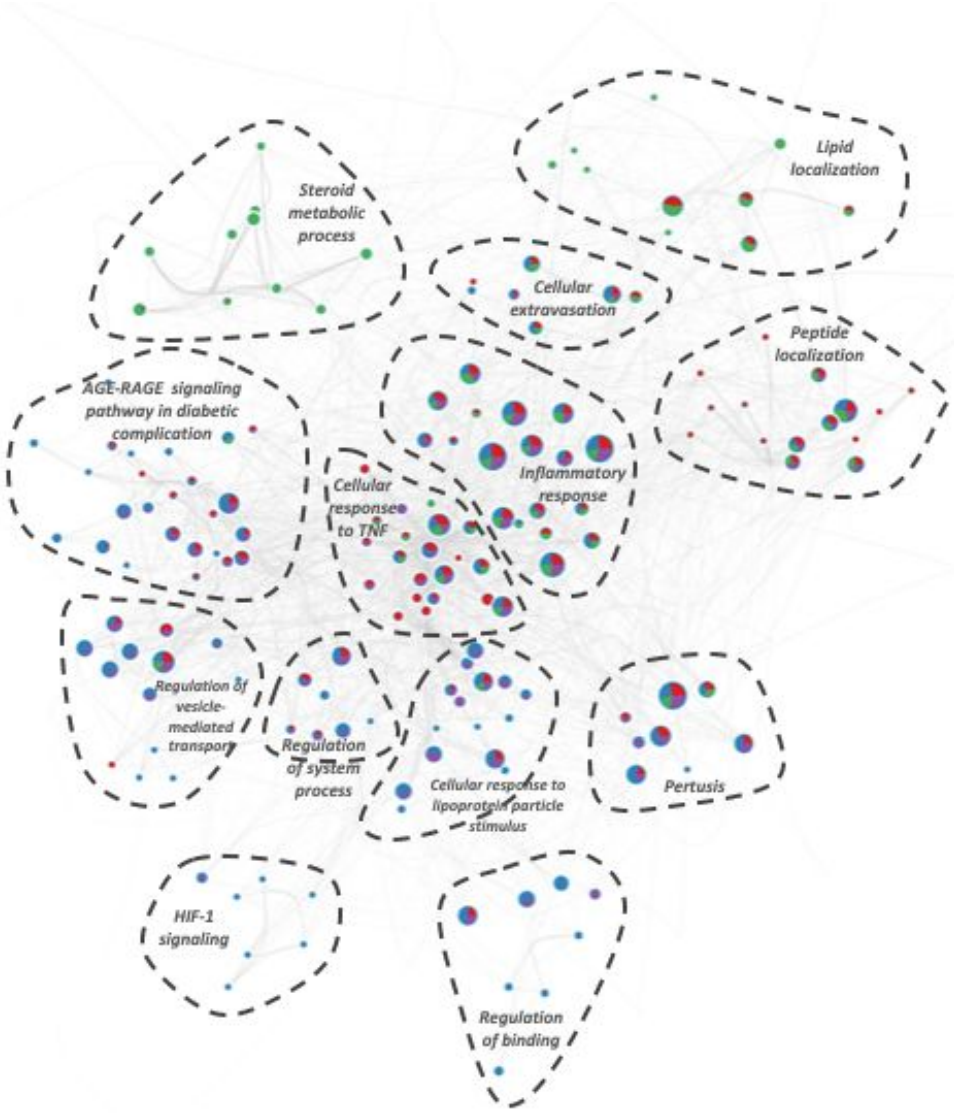

Figure 6. Functional enrichment and interactome meta-analysis based on gene lists for different cell types exposed to flavanols. Enrichment network visualization of the results from the lists of genes identified for adipocytes, smooth muscle cells, immune cells, endothelial cells and hepatocytes. Nodes are functional groups represented by pie charts indicating their associations with each cell type. Cluster labels were added manually. Color code represents the identities of gene lists (adipocytes: red, endothelial cells: blue, hepatocytes: green, immune cells: violet) and size of each color is proportional to the percentage of the genes from different types of cells.

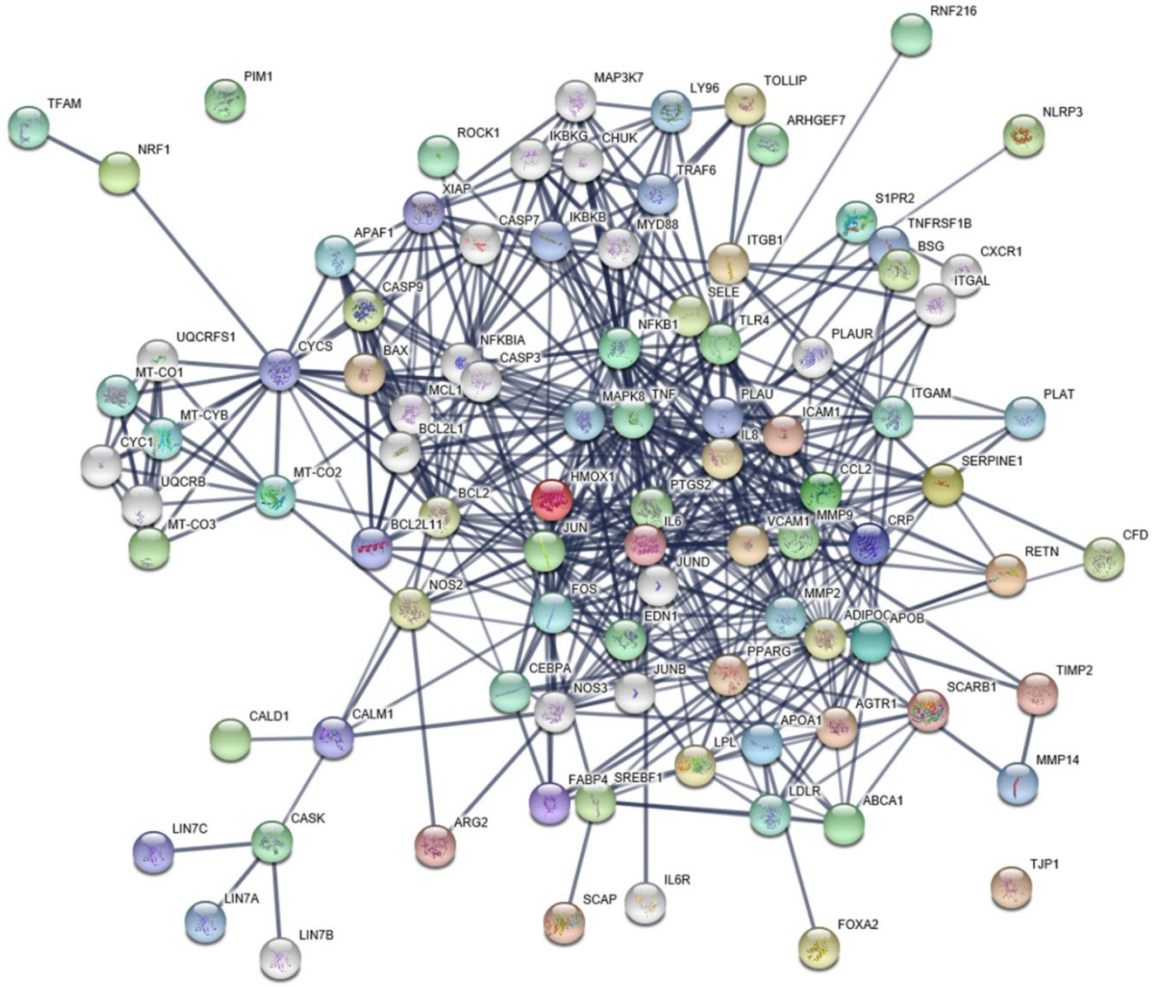

Figure 7. Protein-protein interactions in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols. Colored nodes: query proteins and first shell of interactors; white nodes: second shell of interactors; filled nodes: some 3D structure is known or predicted; empty nodes: proteins of unknown 3D structure.

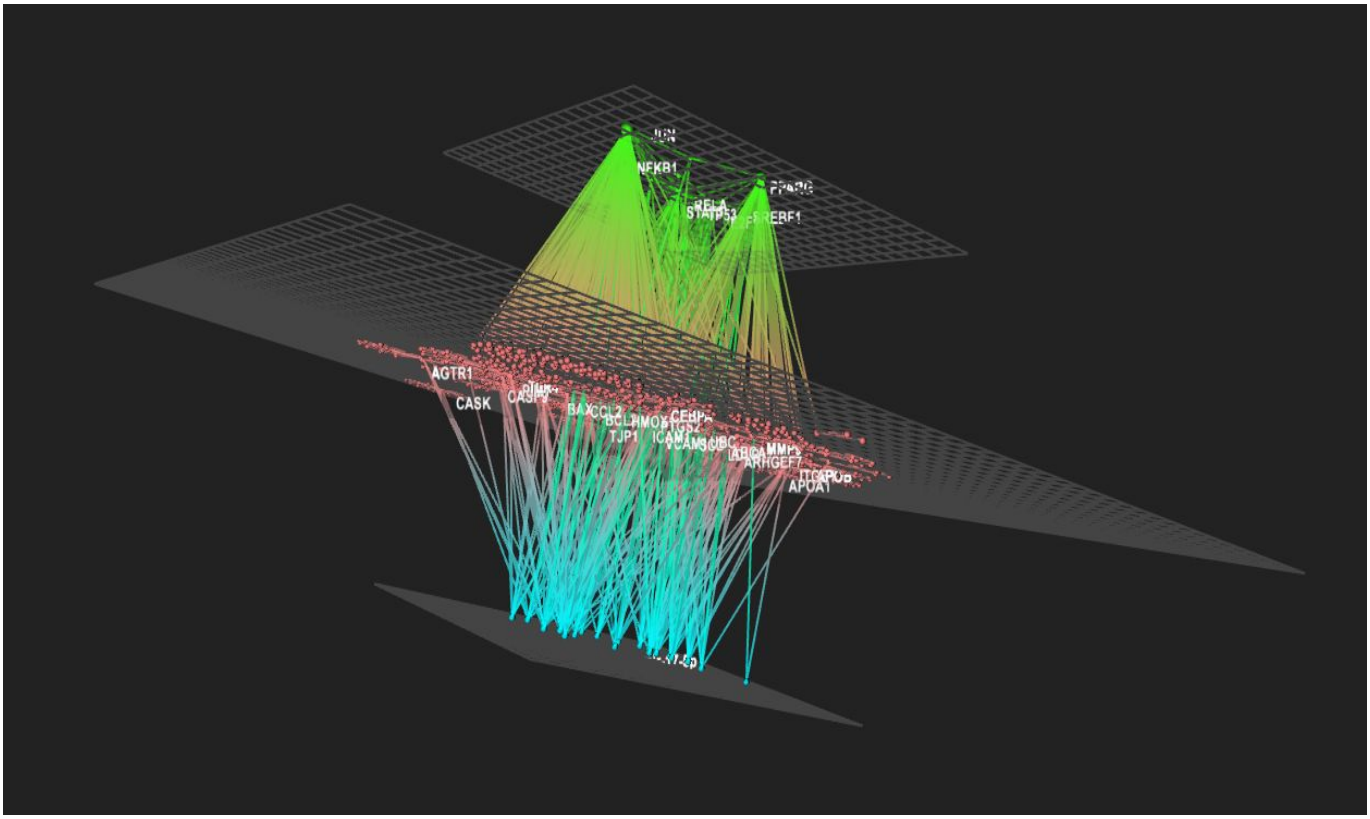

Figure 8. Regulation of protein-protein interaction network by transcription factors and miRNAs in adipocytes, hepatocytes, immune, smooth muscle and endothelial cells exposed to flavanols.

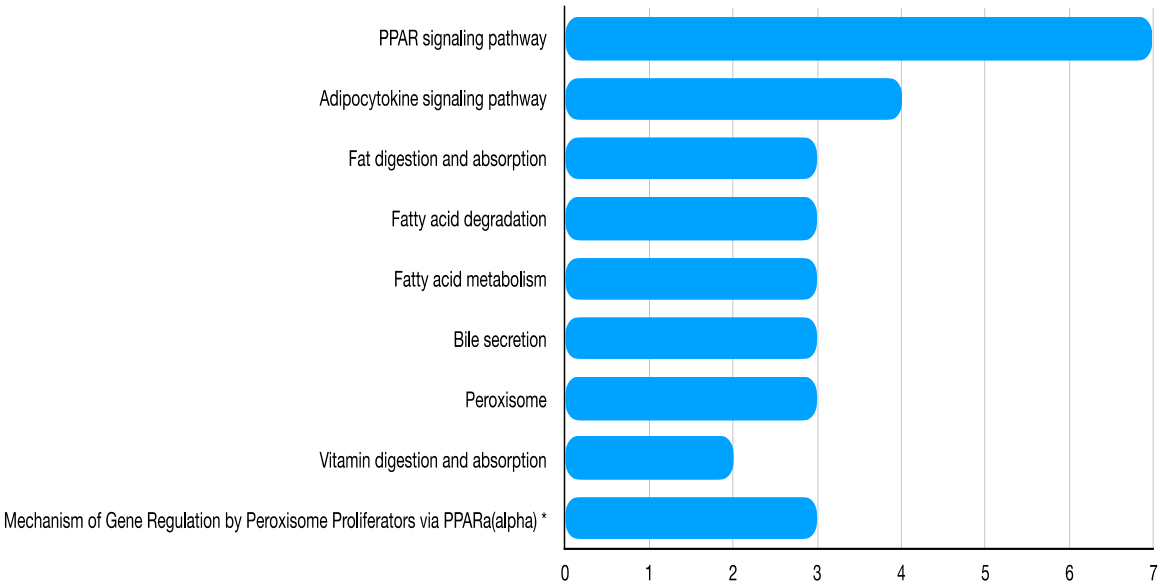

Figure 9A. KEGG and BioCarta (marked with *) pathways for the intestinal cells exposed to flavanols.

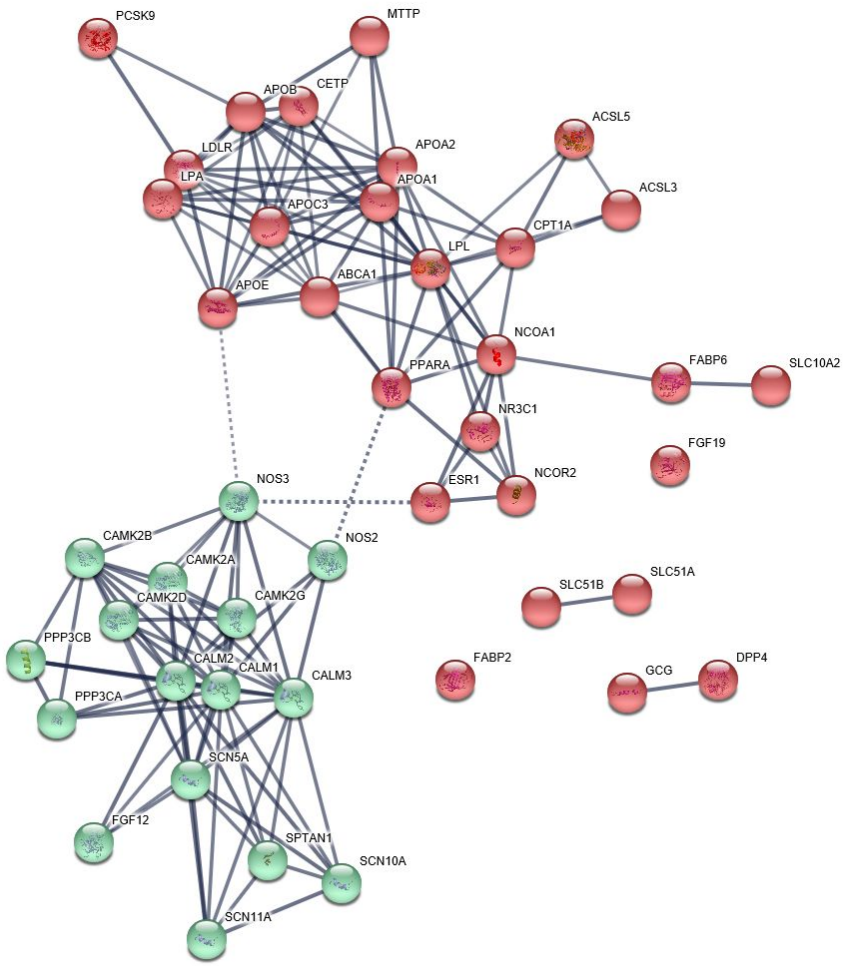

Figure 9B. Protein-protein interactions for the intestinal cells exposed to flavanols. Protein network is organized in two clusters: in red – proteins that are mostly involved in the metabolism of circulating lipoproteins; in green – proteins that are mainly involved in calcium signaling.

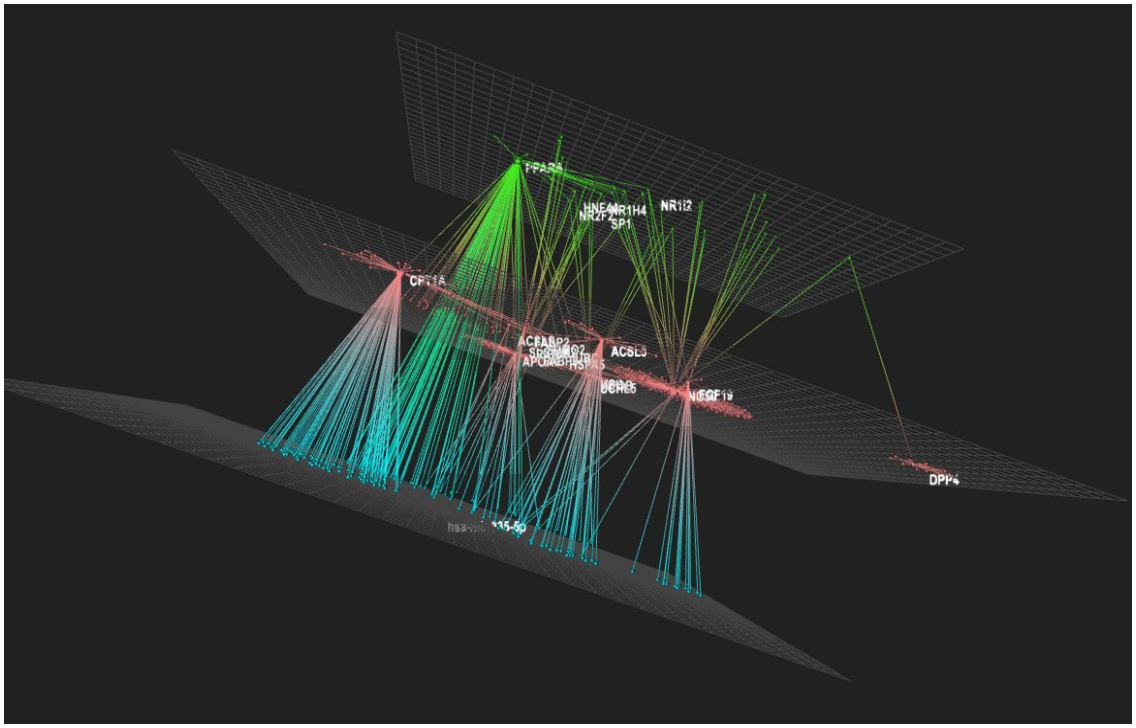

Figure 9C. Regulation of protein-protein interaction network by transcription factors and miRNAs in the intestinal cells exposed to flavanols.

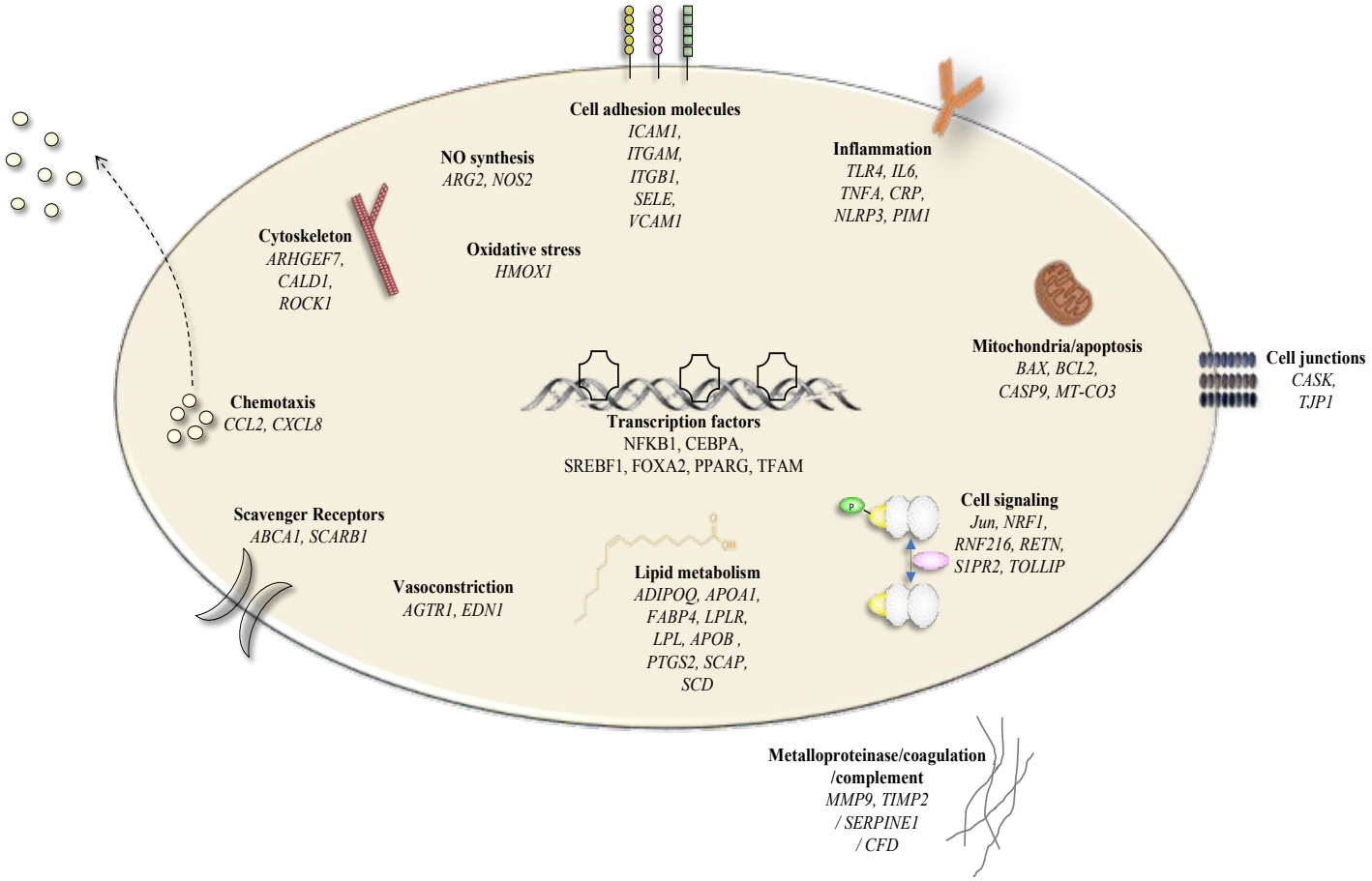

Figure 10. Summary of identified differentially expressed genes modulated by flavanol and related to cardiometabolic health.

Table S2: Proteins with the highest number of interactions within the network (≥ 7), for the intestinal cells.

Gene symbol	Name	Number of interactions
LPL	Lipoprotein lipase	16
APOA1	Apolipoprotein A-I	13
APOA2	Apolipoprotein A-II	12
APOB	Apolipoprotein B-100	11
APOE	Apolipoprotein E	10
APOC3	Apolipoprotein C-III	10
NCOA1	Nuclear receptor coactivator 1	10
ABCA1	ATP-binding cassette sub-family A member 1	9
CETP	Cholesteryl ester transfer protein	9
LDLR	Low-density lipoprotein receptor	9
PPARA	Peroxisome proliferator-activated receptor alpha	9
LPA	Apolipoprotein(a)	8
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform	7
CALM3	Calmodulin-3	15
CALM1	Calmodulin-1	15
CALM2	Calmodulin-2	15
SCN5A	Sodium channel protein type 5 subunit alpha	11
CAMK2B	Calcium/calmodulin-dependent protein kinase type II subunit beta	10
NOS3	Nitric oxide synthase, endothelial	10
CAMK2G	Calcium/calmodulin-dependent protein kinase type II subunit gamma	8

CAMK2A	Calcium/calmodulin-dependent protein kinase type II subunit alpha	8
CAMK2D	Calcium/calmodulin-dependent protein kinase type II subunit delta	8