Title

Structurally related (−)-epicatechin metabolites and gut microbiota derived metabolites exert genomic modifications via VEGF signaling pathways in brain microvascular endothelial cells under lipotoxic conditions: Integrated multi-omic study

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Integrated multi-omic analyses of the genomic modifications by gut microbiome derived metabolites of epicatechin, 5-(4′-Hydroxyphenyl)-γ-valerolactone, in TNFalpha-stimulated primary human brain microvascular endothelial cells.

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Abstract:	Background : The cerebral blood vessels are lined with endothelial and form the blood-brain barrier which dysfunction constitute key event in physiopathology of most neurodegenerative disorders and is also an early event in the aging brain that can contribute to cognitive impairment. Recent studies have suggested that epicatechin can improve various aspects of cognitive functions, lower the risk for developing Alzheimer's disease, decrease the risk of stroke and improve regional cerebral perfusion. However, cellular and molecular mechanisms of epicatechin on brain vascular endothelial cells is still largely unexplored. The objective of this study was to investigate the biological effects of major gut microbiome derived metabolites of epicatechin, 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide, in TNF-α-stimulated human brain microvascular endothelial cells at physiology-relevant concentrations and time of exposure by evaluating their multi-omic modification, including expression of protein coding genes, microRNA (miRNA), long non-coding RNAs (lncRNAs) and proteins. Results : Using multi-omics analysis, we observed that gut microbiome derived metabolites are biologically active and can simultaneously modulate expression of protein coding and non-coding (miRNAs and lncRNAs) as well as proteins. Integrative bioinformatic analysis of transcriptome, miRNome, lncRNome and proteome revealed complex network on genomics modifications by acting at different levels of regulation. Metabolites affect cellular pathways such as cell-cell adhesion, cytoskeleton organization, focal adhesion and cell signaling pathways, pathways regulating endothelial permeability and interaction with immune cells. Conclusions : This study demonstrates for the first time complex and multimodal mechanisms of action by which gut microbiome derived epicatechin metabolites can preserve brain vascular endothelial cell integrity, presenting mechanisms of action underlying epicatechin neuroprotective properties.
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Integrated multi-omic analyses of the genomic modifications by gut microbiome derived metabolites of epicatechin, 5-(4′-Hydroxyphenyl)-γ-valerolactone, in TNFalpha-stimulated primary human brain microvascular endothelial cells.

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Abstract

Background: The cerebral blood vessels are lined with endothelial and form the blood-brain barrier which dysfunction constitute key event in physiopathology of most neurodegenerative disorders and is also an early event in the aging brain that can contribute to cognitive impairment. Recent studies have suggested that epicatechin can improve various aspects of cognitive functions, lower the risk for developing Alzheimer's disease, decrease the risk of stroke and improve regional cerebral perfusion. However, cellular and molecular mechanisms of epicatechin on brain vascular endothelial cells is still largely unexplored. The objective of this study was to investigate the biological effects of major gut microbiome derived metabolites of epicatechin, 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide, in TNF-α-stimulated human brain microvascular endothelial cells at physiology-relevant concentrations and time of exposure by evaluating their multi-omic modification, including expression of protein coding genes, microRNA (miRNA), long non-coding RNAs (lncRNAs) and proteins.

Results: Using multi-omics analysis, we observed that gut microbiome derived metabolites are biologically active and can simultaneously modulate expression of protein coding and non-coding (miRNAs and lncRNAs) as well as proteins. Integrative bioinformatic analysis of transcriptome, miRNome, lncRNome and proteome revealed complex network on genomics modifications by acting at different levels of regulation. Metabolites affect cellular pathways such as cell-cell adhesion, cytoskeleton organization, focal adhesion and cell signaling pathways, pathways regulating endothelial permeability and interaction with immune cells.

Conclusions: This study demonstrates for the first time complex and multimodal mechanisms of action by which gut microbiome derived epicatechin metabolites can preserve brain vascular endothelial cell integrity, presenting mechanisms of action underlying epicatechin neuroprotective properties.

1. Introduction

Polyphenols are one of the most abundant phytochemicals found in plant foods. They encompass several families of compounds, with most represented in human diet being phenolic acids and flavonoids [1]. Flavanols are a class of flavonoids found in fruits and exist in a monomeric (cathechins and epicatechins), and polymeric (proanthocyanidines) forms. They are commonly found in cocoa, tea and various fruits, such as apple or grape. It has been suggested that they play an important role in the beneficial health effects of fruits, vegetables and derivatives [2].

Numerous studies have shown that epicatechin is highly metabolized following its consumption. Epicatechin is absorbed in the small intestine, rapidly conjugated by phase I and phase II detoxification enzymes and appears in the bloodstream from 1 h to 4 h after ingestion with major metabolites such as E3′G, E3′S, 3′ME5S and 3′ME7S [3]. These metabolites are absent from plasma after 6–8 h, at the point when the ingested epicatechin reaches the gastrointestinal (GI) tract and the colon. There, the microbiota induced opening of the C-ring, resulting in the formation of 5-carbon side chain ring fission metabolites that can be further metabolized by the phase II metabolism by enzymes present in the colon and/or the liver resulting in the sulfated and glucuronidated forms of γ-valerolactones that can be detected in plasma. Two major gut metabolites derived catabolites that were identified in plasma are 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide, at 272 ± 56nM and 125 ± 30nM, respectively [3] and can remain in the circulatory system for over 12 hours. Very few studies suggested that these γ-valerolactones are bioactive, by exerting anti-inflammatory properties or decreasing blood pressure [4] however such studies remain very scarce.

In the context of health, flavonoids are of particular interest because experimental studies and randomized controlled trials (RCT) have shown that the flavanols exert positive effects on different cardiovascular disease risk factors, including blood pressure, vasodilation or vascular stiffness [5,6]. Hypertension and arterial stiffness are also main risk factors for cerebrovascular injury. The role of cerebrovascular dysfunction in cognitive impairment is increasingly recognized [7]. Its dysfunction can lead to accelerated brain atrophy, reduced cognitive ability, an increased risk of stroke and an increased risk of neurodegenerative diseases, such as Alzheimer's disease (AD), and dementia. Aging impairs the increase in cerebral blood flow triggered by neural activation, known as neurovascular coupling, a complex functional impairment of cerebral microvessels and astrocytes, which likely contribute to neurovascular dysfunction and cognitive decline in aging and in age-related neurodegenerative diseases [8].

Several studies suggest that epicatechin can improve various aspects of cognitive function in animals and humans. Flavonoids can preserve cognitive abilities during ageing in rats, lower the risk for developing AD, decrease the risk of stroke in humans, and can exert beneficial effects on cerebral blood flow [9,10]. Protective effects of long-term flavanol consumption on neurocognition and behavior, including age- and disease-related cognitive decline, were shown in animal models of normal aging, dementia, and stroke [11]. A recent systematic review has suggested a positive effect of cocoa polyphenols on memory and executive function [12]. It has also been suggested that consumption of cocoa flavanol may improve regional cerebral perfusion [13] and can also enhance dentate gyrus function and improves cognition in older adults [14].

The cerebral blood vessels are lined with endothelial cells (EC) and sealed by tight junctions. Together with astrocytes, pericytes, microglial cells and the basement membrane, they form the blood-brain barrier (BBB) and represent an interactive cellular complex that regulates the entry of

blood products, pathogens and cells into the brain, which is essential for normal neuronal functioning, thus playing an important role in the protection and homeostasis of the brain. Dysfunction of the BBB also plays a key role in most neurodegenerative disorders as BBB dysfunction results in increased permeability of EC, which results in neuro-inflammation that contributes to the neurodegeneration process [15]. BBB degradation is also an early event in the aging human brain that begins in the hippocampus and can contribute to cognitive impairment [16]. It has therefore been suggested that the degradation of BBB is a sensitive and early measure of cognitive dysfunction in Alzheimer's, Parkinson's and even multiple sclerosis [7,17]. We have previously described that epicatechin metabolites can prevent endothelial dysfunction by reducing interactions between monocytes and TNF-α-stimulated vascular EC [18] and also decreasing endothelial permeability [19]. These observations suggest that the observed cognitive and neurological-protective effects of flavanols may be due to their capacity to protect brain-endothelial integrity and BBB permeability.

The objective of this study was to investigate the biological effects of major gut microbiome derived metabolites of epicatechin, 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide, in TNF-α-stimulated human brain microvascular endothelial cells at physiology-relevant concentrations and time of exposure by evaluating their multi-omic modification, including changes in the expression of protein coding genes, non-coding microRNA (miRNA) and long non-coding RNAs (lncRNAs) genes together with proteomics modifications.

2. Materials and methods

2.1 Compounds

Epicatechin gut microbiota metabolites: 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate (γVL3′G) and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide (γVL3′S) were gifted by Mars, Inc. Chemical structures are presented in the Supplemental Figure 1. Stock solutions of γ-valerolactones were prepared by dissolving them in 50% ethanol at 2mM and stored at -80°C until assayed. For the cell treatments, a mixture of compounds was used in proportion of 0.65/0.35 for γVL3′S and γVL3′G, respectively. Once dissolved in the culture medium, the final concentration for γVL3′S was 650nM and γVL3′G was 350nM, with total final concentration of 1μM.

2.2 Cell culture

Human brain microvascular endothelial cells (HBMEC) were obtained from Angio-Proteomie (Boston, MA, USA). Cells were cultured in the EBM-2 Endothelial Cell Growth Basal Medium supplemented with 2% fetal bovine serum, 0.4 % human fibroblast growth factor, 0.1% human epidermal growth factor, 0.1% insulin-like growth factor, 0.1% vascular endothelial growth factor, 0.1% heparin, 0.1% ascorbic acid, 0.1% gentamicin/amphotericin-B and 0.04% hydrocortisone, all from Lonza (Walkersville, MD, USA). Cell cultures were maintained at 37°C and 5% CO_2. HBMECs were used at the passage 4. The cells, 50000 cells/well, were seeded on 24-well plates (Becton Dickinson, USA) that were coated with collagen (Cell Applications, San Diego, CA, USA). At 80% of confluence, cells were exposed to the mixture of gut metabolites for 20h. Cells treated with medium containing 0.01% ethanol final concentration was used as controls. After the incubation, the medium was discarded, and inflammatory stress was induced by 4h-incubation with 1ng/ml of TNF-α (VALs group) (R&D Systems, MN, USA). Cells treated with medium only

and incubated with TNF-α were used as control (TNF-α group). Negative control cells (control group) were treated with medium and no TNF-α incubation.

2.3 RNA extraction

Total RNA, including short RNAs as miRNAs, were extracted using Monarch® Total RNA Miniprep Kit (New England BioLabs, USA) following manufacture's instruction. Briefly, cells were lysed using a lysis buffer and genomic DNA was removed by centrifugation in gDNA removal columns. RNA was then fixed to the RNA purification column, a step that was followed by successive steps of washing and centrifugations. At the end, total RNA was eluted using nuclease free water. RNA quality and quantity were checked by agarose gel electrophoresis and determination of the absorbance ratio at 260/280 nm using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The total RNA samples were stored at -80°C until used.

2.4 Microarray analysis of mRNA, miRNA, snoRNA and lncRNA expression

For transcriptomics analysis, we used Affymetrix Clariom D array for human, containing over 6 million probes for protein coding genes but also protein non-coding genes such as miRNAs, lncRNAs and small nucleolar RNAs (snoRNAs) (Thermo Fisher Scientific, Santa Clara, CA). RNA (100 ng) was used to prepare cRNA and sscDNA using Thermo Fisher Scientific GeneChip® WT PLUS reagent Kit. SscDNA (5.5 ug) was fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) and labeled by terminal deoxynucleotidyl transferase (TdT) using the DNA Labeling Reagent that is covalently linked to biotin. Fragmented and labelled ssCDNA samples in triplicate were then submitted to the UC Davis Genome Center

shared resource core for hybridization, staining, and scanning using Thermo Fisher Scientific WT array hybridization protocol following the manufacturer's protocol. Hybridization of fragmented and labelled ssCDNA samples was done using GeneChip™Hybridization Oven and samples were then washed and stained using GeneChip™ Fluidics Station. The arrays were scanned using GeneChip™ Scanner 3000 7G (Thermo Fisher Scientific, Santa Clara, CA). Quality control of the microarrays and data analysis were performed using Thermo Fisher Scientific Transcriptome Analysis Console software version 4.0.2. Pair-wise comparisons between biological conditions were applied using specific contrasts. A correction for multiple testing was applied using Benjamini-Hochberg procedure (BH, Benjamini et al. 1995, pubmed ID 24913697) to control the False Discovery Rate (FDR). Probes with FDR-adjusted $P < 0.05$ were considered to be differentially expressed between conditions. All raw and normalized data are available in GEO database under accession series number: GSE156116.

2.5 Proteomics analysis

The global proteomics analysis was performed as previously described [20]. Briefly, to prepare the samples, 4 per group, cells were homogenized in lysis buffer and the protein concentration of the supernatant was measured using Bicinchoninic Acid (BCA) protein assay. One hundred μg of protein sample was denaturized, precipitated and the supernatant was discarded, and pellet was air dried. The proteins were then digested and concentrated and labelled using TMT 10-plex peptide labeling (Thermo Scientific, Canoga Park, CA, US). All TMT labeled samples were combined in equal amounts. LC separation was done on a Dionex Nano Ultimate 3000 (Thermo Scientific) with a Thermo Easy-Spray source. Mass spectra were collected on a Fusion Lumos mass spectrometer (Thermo Fisher Scientific) in a data-dependent MS3 synchronous precursor selection (SPS)

method. MS1 spectra were acquired in the Orbitrap, 120 K resolution, 50 ms max inject time, 5 ×105 max inject time. MS2 spectra were acquired in the linear ion trap with a 0.7 Da isolation window, CID fragmentation energy of 35%, turbo scan speed, 50 ms max inject time, 1 ×104 automatic gain control (AGC) and maximum parallelizable time turned on17. MS2 ions were isolated in the ion trap and fragmented with a HCD energy of 65%. MS3 spectra were acquired in the orbitrap with a resolution of 50K and a scan range of 100–500 Da, 105 ms max inject time and 1 × 105 AGC.

Quantitative data analysis: The 4 samples of cells treated with metabolites and 4 vehicle treated samples were used for isobaric-labeled LC-MS/MS quantitative measurements. These factors include ratio compression and can cause an underestimation of changes in relative abundance of proteins across samples. Raw files were processed with Proteome Discoverer 2.2 (Thermo Scientific) using the default MS3 SPS method with the following modifications for importation into Scaffold Q+. Target Decoy PSM validator was used instead of Percolator and Maximum Delta Cn was set to 1. All MS/MS samples were analyzed using Sequest HT to search all mouse sequences from Uniprot (https://www.uniprot.org/proteomes/UP000000589) and 110 common laboratory contaminants (http://thegpm.org.crap) plus an equal number of reverse decoy sequences assuming the digestion enzyme trypsin. Sequest-HT was searched with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine and TMT10 plex of lysine were specified in Sequest-HT as fixed modifications. Oxidation of methionine and acetyl of the n-terminus were specified in Sequest-HT as variable modifications.

Scaffold Q+ (version Scaffold_4.9.0, Proteome Software Inc., Portland, OR) was used to quantitate Label Based Quantitation (iTRAQ, TMT, SILAC, etc.) peptide and protein identifications. Peptide identifications were accepted with a decoy false FDR cutoff of less than

0.2%. Protein identifications were accepted if they could be established with at least 2 unique identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Normalization was performed iteratively (across samples and spectra) on intensities, as described in Statistical Analysis of Relative Labeled Mass Spectrometry Data from Complex Samples using ANOVA. Medians were used for averaging and spectra data were log-transformed and weighted by an adaptive intensity weighting algorithm.

2.6 Bioinformatic analysis and multi-omics integration

2.6.1. Genes, targets and proteins distribution plots

Data visualization was performed in Manhattan plots to show chromosomal localizations of differentially expressed transcripts and Venn Diagrams to show all possible logical relations between multiple omic sets. Venn diagrams were used to visualize the relationships between differentially expressed (DE) protein-coding genes (mRNAs), miRNA targets, lncRNA targets and DE proteins observed in TNF-α vs control and VALs vs TNF-α group comparisons. Both plots, Manhattan and Venn diagram, were built using R software packages, the Sushi package (Phanstiel, 2019, 24903420) and the VennDiagram package (Chen and Boutros, 2011, 21269502), respectively. Conversely, associations between genes and groups were searched using unsupervised hierarchical clustering of the samples and the differentially expressed probes using the Pearson correlation as distance metric and Ward' s method for agglomeration of clusters. The clustering results were illustrated as a heatmap of expression signals. We used the software Permut Matrix to construct the heatmaps (http://www.atgc-montpellier.fr/permutmatrix/, Caraux and Pinloche, 2005).

2.6.2. Databases-predicted miRNA targets

For each miRNA identified as differentially expressed in TNF-α vs control and VALs vs TNF-α group comparisons, we performed a target analysis. miRNA targets were predicted using three prediction databases miRTarBase [21], mirRDB [22], and TargetScan [23], and only targets identified in at least two of the databases were considered as putative targets for our analysis. For each database we always chose the most stringent option among those proposed by the database.

2.6.3. Databases-predicted lncRNA targets

For each lncRNA identified as differentially expressed in TNF-α vs control and VALs vs TNF-α group comparisons, we also performed a target analysis. lncRNA target transcripts were predicted by combining the results of two prediction databases from Rtools web server: lncRRISearch (http://rtools.cbrc.jp/LncRRIsearch) [24] and RNARNA (http://rtools.cbrc.jp/cgi-bin/RNARNA/index.pl). Targets identified in these two databases were considered as putative targets and used in our analysis. For each database we always chose the most stringent option among those proposed by the database.

2.6.4. Protein-protein interactions

In order to perform Protein-Protein Interaction Networks Functional Enrichment Analysis we used STRING database [25] (https://string-db.org/).

2.6.5. Transcription factors analysis

Transcription factors (TFs) potentially involved in the regulation of the expression of identified mRNAs and which activity can be affected by epicatechin metabolites were searched using enrichR online tool (https://amp.pharm.mssm.edu/Enrichr/) [26]. The TFs were also searched within TRRUST (transcriptional regulatory relationships unravelled by sentence-based text-mining) database, a manually curated database of human and mouse transcriptional regulatory networks that contains 8,444 and 6,552 TF-target regulatory relationships of 800 human TFs and 828 mouse TFs, respectively that have been derived from 11,237 pubmed articles.

2.6.6. Docking analysis

Molecular docking analysis was employed to explore the potential interaction/binding between identified transcription factors and cell signaling proteins regulating the activity of the transcription factors identified and the 2 metabolites, 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate (γVL3′G) and 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide (γVL3′S). The 3D structures of metabolites were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov) and the three-dimensional structure of the proteins was obtained from the Protein Data Bank (PDB) database. Docking calculations were carried out using Blind Docking server (https://bio-hpc.ucam.edu/achilles) [27].

2.6.7. Pathway analysis

*Pathway enrichment analysis***.** Cellular pathways from TNF-α vs control and VALs vs TNF-α group comparisons were explored using GeneTrail2 (https://genetrail2.bioinf.uni-sb.de/) [28]. The lists of significantly regulated mRNAs, miRNA targets, lncRNA targets and significantly regulated proteins, separately or together, were used to identify enrichment of biological categories using an

over-representation analysis or gene set enrichment analysis (GSEA) with threshold fixed at $P < 0.05$ using the Benjamini-Yekuteli correction (as recommended by GeneTrail2). KEGG, Biocarta and Wiki pathways were evaluated in the analysis.

Pathways network. The enriched pathways obtained in our previous step were used to build a network of pathways; two pathways were considered interconnected if at least one of the genes or hits involved in them were common to both. Networks were constructed and visualized using Cytoscape software (version 3.7.1; http://www.cytoscape.org/) [29]. Data preparation was performed with the use of several R packages included splitstackshape (https://github.com/mrdwab/splitstackshape), data.table (https://github.com/Rdatatable /data.table), dplyr (http://dplyr.tidyverse.org,https://github.com/tidyverse/dplyr) and string (http://stringr.tidyverse.org, https://github.com/tidyverse/stringr). Pathway networks were built separately for pathways enriched in each omic layer and pathways obtained from a global pathway enrichment analysis, considering all omic layers components together. To obtain the 6 pathways with the highest degree (number of connections of one node to other nodes), the Cytoscape Network Analyzer application was used (http://apps.cytoscape.org/apps/networkanalyzer).

Networks of pathways related with endothelial function. Endothelial function pathways-related were selected to construct a network. Endothelial function pathway-specific network was performed in Cytoscape software. As a first step we obtained a list of genes involved in selected pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG) database [30]. Subsequently, through a series of intersections and non-intersections functions in R, we obtained the mRNAs and proteins involved in the endothelial function pathways, which were also identified to be differentially expressed in our study. Finally, we identified endothelial function pathway components that were targets of miRNAs or lncRNAs.

2.6.8. Multilayer integration and representation

mRNA, miRNA, lncRNA, proteins and targets interaction networks. Visualization of the interactions between mRNA-TFs, miRNAs-targets, lncRNA-targets and protein-protein were performed in Cytoscape software. Separate networks for each omic layer and a global network with all integrated interactions were made. Smaller networks were constructed to represent the miRNA targets, lncRNA targets and DE proteins intersected with mRNAs DE in our study.

mRNA, miRNA, lncRNA, proteins and targets interaction networks of focal adhesion pathway. We identified quantitatively changed mRNAs, miRNAs and their targets, lncRNAs and their targets, together with proteins DE in our study and mapped them to the global map provided in KEGG pathway database, to include a comprehensive pathway topology.

3. Results

3.1 TNF-α modulates expression of protein coding and non-coding genes and proteins in HBMEC cells

Firstly, we aimed to assess the effect of TNF-α, in comparison to control group without TNF-α, on the expression of genes, both protein-coding and non-coding transcripts, using transcriptomics microarray, as well as the effect on protein expression using shotgun proteomics. Regarding the effect on gene expression, from 135,751 total probes, 751 were identified as differentially expressed, at least 0.55%, since some probes were discarded during data cleaning treatment. Manhattan plot showed a uniform distribution of the transcripts on the chromosomes (Figure 1A). We found that of the total differentially expressed transcripts, 85.5% correspond to protein-coding genes (642 mRNAs), 1.89% to miRNAs (23 miRNAs) and 7.07% to lncRNAs (86 lncRNAs) (Figure 1B). Regarding proteomic data, we observed an 8.3% differentiation rate, that is, 464 significantly differentially expressed proteins out of a total of 5565 (Figure 1B).

Subsequently, miRNAs and lncRNAs target gene predictions from the database analysis identified 4125 miRNA targets and 239 lncRNA targets. Thirty-one of these targets were shared between both omic layers (miRNA and lncRNA targets), of which 3 corresponded to mRNA and 1 to protein category. The number of component intersections between all omic layers is shown in Figure 1C. For instance, 175 miRNAs targets belong to mRNAs category, 3 of them are shared by lncRNA targets and 6 by proteins, 6 total lncRNA targets belong to mRNA category, and 24 total proteins belong to the category of mRNAs.

We extrapolated the nutrigenomic modification data to build a network of interactions, that is considering DE mRNAs, DE miRNA and their targets, DE lncRNA and their targets, and DE proteins. The global network with all the interactions is shown in Figure 1D. The simplified version

of global network is shown in Figure 1E, miRNA targets, lncRNA targets and proteins and that belong to DE mRNAs omic layer were considered (at least 196 components as shown in Figure 1C).The components of each category with the highest degree of connections are: (i) miRNAs: hsa-miR-146-3p, hsa-miR-214-3p, hsa-miR-7844-5p, hsa-miR-6732-3p and hsa-miR-155-5p, (ii) lncRNAs: RP11-274H2.3, RP11-258C19.7, AC098617.1, RP11-373D23.3, RP11-661013.1, (iii) mRNAs: ATP6V1C1, QKI, ANTXR2, TRAF3, TNPO1.

For functionality analysis, each omic layer's components were used to perform an enrichment analysis and obtain pathways related to mRNAs, miRNA targets, lncRNA targets and proteins differentially expressed in our study, as presented in the multicolored histogram in Figure 2A. This analysis shows that differentially expressed genes and proteins can impact the cellular functions regulating cell signaling (Toll like receptor signaling pathways, Ras signaling pathway, NF-kappaB (NF-κB) signaling pathway or PI3K-Akt signaling pathway), cell-cell adhesions (cell adhesion molecules pathway or adherent junctions), chemotaxis (chemokine signaling pathway, cytokine-cytokine receptor interaction) or cellular metabolism (citrate cycle, amino acid metabolism or sucrose metabolism). Certain pathways were enriched with miRNA targets such as Notch signaling pathways, RNA transport, spliceosome; or from lncRNA targets such as purine metabolism and tight junction. Some pathways such as TNF-α and NF-κB signaling were enriched by components of mRNAs and proteins categories; 2-Oxocarboxylic acid metabolism by miRNA and lncRNA targets; and regulation of autophagy by mRNA and miRNA components.

Thenceforward, we constructed a network of interactions between mRNAs, miRNA-targets, lncRNA-targets, and proteins mapped to genes of pathways of endothelial related functions, such as focal adhesion, tight/adherent junctions, or actin cytoskeleton organization (Figure 2B). From 1,392 genes totally involved in endothelial functional pathways, 113 mRNAs and 24 proteins were

differentially expressed in our study. Next, endothelial-related genes were used to search for their regulation by miRNAs or lncRNAs, and 402 miRNA regulations and 15 lncRNA regulations were found. Some of these interactions that may be related to endothelial functions were hsa-miR-7844-5p-TRAF3, REL-VCAM1, VCAM1-ICAM1, for instance. These data suggest that TNF-α can affect EC functions, such as cell adhesion, junctions or cell signaling, through multi-level mode of genomic regulations, simultaneously affecting mRNA, miRNA, lncRNAs and proteins.

3.2 γ-valerolactones can modulate expression of genes and proteins in HBMEC

In order to evaluate the effect of γ-valerolactones (VALs) on the transcriptomic and proteomic expression in brain microvascular endothelial cells, a microarray and shotgun analysis were also performed. We compared the global gene expression profiles of the 3 study groups (Figure 3A) using PLSDA analysis and observed that the VALs groups had different expression profiles from the TNF-α and control groups. This observation suggests changes in expression profile in HBMEC following exposure to the VALs.

Statistical analysis was then performed to identify differentially expressed transcripts. The percentage of transcripts DE in cells treated with TNF-α + VALs (VALs group) vs TNF-α alone (TNF-α group) was 0.15%, that is 211 transcripts. Integrally, 19.67% of these modifications correspond to protein-coding genes (61 mRNAs), 1.93% to miRNAs (6 miRNAs), 25.48% to lncRNAs (79 lncRNAs) (Figure 3B). On the other hand, proteomics analysis indicates 2.94% of differentiated proteins, corresponding to 52.90% (164 proteins) of total transcripts and proteins DE (Figure 3B). A Manhattan plot of transcripts shows that differentially expressed genes are localized throughout the genomes (Figure 3C). The expression profile of genes identified in VALs group was compared with expression profile of genes identified as differentially expressed by

TNF-α group and presented by a heat map, Figure 3D. This analysis revealed that over half of VALs genes present opposite expression profile when compared to TNF-α group, that is genes identified as up-regulated by exposure of cells to TNF-α were identified as down-regulated by VALs and inversely, genes identified as down-regulated by TNF-α were identified as up-regulated by VALs. This observation suggests that exposure of HBMEC to VALs can, at least partially, counteract the inflammatory stress induced by TNF-α.

Furthermore, these data suggest for the first time that epicatechin gut microbiota metabolites have capacity to exert complex genomic modification. These effects however seem to be of lesser impact on genomic and proteomics modifications than of TNF-α effect.

3.2.1. γ-valerolactones modulate expression of protein-coding genes in HBMEC

In order to seek the functional pathways that could be modulated by DE protein-coding genes (Supplemental Table 1) in our VALs study group vs TNF-α group, we performed an enrichment analysis in GeneTrial. This analysis showed that mRNAs are involved in different processes regulating cell adhesion and permeability (Gap junction, Tight junction, Focal adhesion, Adherens junction, Leukocyte transendothelial migration pathways), cell signaling (Rap1 signaling, Thyroid hormone signaling, Wnt signaling, PI3K-Akt signaling), cell metabolism (Glycosphingolipid biosynthesis, TCA cycle, Amino sugar and nucleotide sugar) and other pathways, as shown in Figure 4A. Subsequently, we constructed a network in order to integrate and show connections between the pathways resulting from our previous analysis (Figure 4B). Three clusters were observed, the largest including Rap1, Wnt, and PI3K-Akt signaling pathways strongly connected with leukocyte transendothelial migration, adherent junction, tight junction and gap junction. The two smallest consist of a cluster that involves glycosphingolipid biosynthesis, amino sugar and

nucleotide sugar metabolism and glycosaminoglycan degradation. We can clearly observe a separation of the processes related to cellular metabolism and those of cell-cell contact regulation. The pathways with the highest degree of interaction with other pathways are Wnt signaling, Rap1 signaling, tight junction and hippo signaling pathway. Consequently, this observation suggests that γ-valerolactones exposure could modulate endothelial cells function, that is adhesion with immune cells but also endothelial cell permeability, by regulating of mRNAs, miRNAs, lncRNAs and proteins involved in these processes.

3.2.2. γ-valerolactones modulate the expression of miRNA in HBMEC

MiRNA expression was also identified as modulated following exposure of HBMEC to γ-valerolactones, suggesting the capacity of these metabolites at physiologically-relevant concentrations to modulate the expression of small non-coding RNAs (Supplemental Table 2). Search for their putative targets from the databases for 6 differentially expressed miRNAs, hsa-miR-6730-3p, hsa-miR-6730-5p, hsa-miR-3661-3p, hsa-miR-3661-5p, hsa-miR-6746-3p, hsa -miR-6746-5p, allowed us to identify 1360 target genes, including ARSB, CXADR, FREM1 or HAP1. Among them, 5 targets are in common with differential expression mRNA (Supplemental Figure 3A). Figure 5A shows network topology of miRNA-target interactions which revealed at least four miRNA hubs for the hsa-miR-6730-5p, hsa-miR-6730-3p, hsa-miR-6746-5p and has-miR-6746-3p.

Likewise, miRNA targets functionality analysis was performed by placing these genes into cellular pathways. MiRNAs targets have been found to play a role in pathways such as those regulating EC functions (adherent junction, gap junction, focal adhesion), cell signaling (PI3K-Akt, Wnt, Foxo, MAPK, PPAR signaling) or cell metabolism (pyrimidine, purine and protein digestion

metabolism) (Figure 5B). Thirty-six resulting pathways were found in turn in mRNAs enrichment analysis (Supplemental Figure 3B), this suggests that although only few targets are shared in both omic layers (mRNA and miRNA targets), their functionality is similar. In Supplemental Figure 2, the connections between miRNA targets-related pathways are shown, where centrality is one of its characteristics. We observed that the pathways with the highest degree of interaction with others are pathways in Ras signaling pathway, PI3K-Akt signaling, FOXO signaling, viral carcinogenesis and MAPK signaling pathway.

3.2.3. lncRNA expression modulation by γ-valerolactones

Microarray analysis have also shown for the first time that exposure of HBMEC to γ-valerolactones can also modulate expression of another group of protein non-coding RNA, which are long-non-coding RNAs. We observed change in expression of 79 lncRNAs (Supplemental Table 3). Search of databases for their targets allowed us to identify 364 lncRNA-targets. Among these 364 targets, 4 (UBC, C11orf95, PLCB1, BHLHE40) are in common with protein coding genes and 14 (IL6ST, ABHD12, HIPK2, PLEKHG2, PSD4, ZNF37A, AMOT, POU4F1, UBC, CELF3, AGO1, EFNA3, BCL7A, PRDM2) with miRNAs targets (Supplemental Figure 3A). In Figure 6A lncRNA-targets interactions topology is shown which identified several clusters of genes, such as for lncRNA RP11-386G11.10, RP11-192H23.7, AC012668.2, FTX, AC005562.1 or lncRNAs hubs.

In lncRNAs targets functionality analysis, we observed an enrichment of pathways involved in the regulation of EC (gap junction, focal adhesion, ECM-receptor interaction, regulation of actin cytoskeleton, Rap1 signaling pathways), cellular metabolism (PI3K-AKT, calcium, PPAR, NF-κB, JAK-STAT, mTOR signaling pathways) or cell signaling (carbon, purine, pentose phosphate,

glycolysis, gluconeogenesis metabolism) (Figure 6B). We can corroborate that most of the pathways described are themselves the result of mRNA and miRNA targets pathway analysis Sixteen pathways were shared with protein-coding genes enrichment analysis, while 37 are shared with miRNAs pathways analysis (Supplementary Figure 3B).

In order to analyze the connections between these pathways, we built a network of interactions presented in Figure 6C. We observed a strong relationship between intracellular signaling pathways, PPAR, NF-κB, PI3K-Akt, Rap1 signaling, with focal adhesion and gap junction. These pathways were grouped in a large cluster, connected with a smaller cluster in which inflammatory and cytokine pathways were involved. Metabolic processes were grouped in a separate cluster. The pathways with the highest degree of connections with other pathways are Rap1 signaling, Focal adhesion, long term potentiation, microRNAs in cancer, phagosome and gap junction. Taken together, this study shows for the first time the capacity of γ-valerolactones to modulate the expression of miRNAs and lncRNAs, particularly those involved in the regulation of endothelial cell function and permeability.

3.2.4. Proteomics modulation by γ-valerolactones

Use of proteomic untargeted shotgun approach allowed us to demonstrate that exposure of HBMEC to **γ**-valerolactones can also affect the expression of proteins (Supplemetal Table 4). We observed that **γ**-valerolactone metabolites modulated the expression of 164 different proteins. Functional analyses revealed that these proteins play a role in pathways related to cell metabolic pathways, cell signaling pathways and others, as shown in Figure 7A. In metabolic processes we observed that valine, leucine and isoleucine byosynthesis, 2-oxocarboxylic acid, byosynthesis of unsaturated fatty acids, glycosaminglycan degradation were part of this category. The interactions

between pathways is shown in Figure 7B. The pathways with the highest degree of connections with other pathways are renal cell carcinoma, prostate cancer, or natural killer cell mediated cytotoxicity.

3.2.5. Transcription factor and interactome analysis using 3D in-silico modeling for γ-valerolactones

Using gene expression analysis data, Ttrust database was searched using EnrichR tool to identify potential TFs whose activity could be affected by γ-valerolactones and involved in the observed changes in the expression of genes. Among the most significant TFs identified were NF-κB1, cJUN, STAT2, IRF1, or FOXO4 (Figure 8A). Following this analysis, we aimed to identify if γ-valerolactones could have binding affinity with these TFs, binding that may affect their activity and consequently result in changes in expression of genes as we observed. Using this approach, we observed that γ-valerolactones have potential to bind to transcription factor, interaction that could affect their activity and induce changes in expression of genes, as observed using our microarray analysis. The analysis suggests that glucuronidated form of γ-valerolactone has slightly higher potential to bind to protein tested than sulfated metabolite. The highest binding was observed between γVL3′G and RelA protein, with binding free energy identified being -7kcal/mol (Figure 8B), followed by its binding to NF-κB (-6.9kcal/mol). γVL3′S showed highest binding capacity with RelA (binding energy of -6.6kcal/mol) (Figure 8C).

3.3. Integration of multi-omics data of γ-valerolactones treatment in HBMEC cells

As a first step towards data integration, we grouped mRNA, miRNA targets, lncRNA targets, and protein interactions from their individual analyses into a global network of VALs vs TNF-α

comparison. The network presented in Figure 9A shows at least three main clusters, dominated by hsa-miR-6730-3p, hsa-miR-6730-5p, hsa-miR-6746-3p, hsa-miR-6746-5p. Likewise, lncRNA smaller clusters are observed, formed by FTX, RP11-386G11.10, AC012668.2, AC005562.1, CTD-2031P19.5 as central hubs. Moreover, the global network of enriched pathway interactions of our omic layers (mRNAs, miRNAs, lncRNAs and proteins) was grouped and shown in Figure 9B-C, where individual clusters were not differentiated, but rather a centralized network. The pathways with the highest degree of connections with other pathways into the network are pathways in cancer, focal adhesion, thyroid hormone signaling pathway, RAS signaling, PI3K-Akt signaling and Rap1 signaling.

Our next step was to identify common enriched pathways to mRNAs, miRNAs, lncRNAs and proteins. As shown in the heatmap in Figure 10A, pathways specific to one, 2, or 3 omic analyses (mRNA, miRNA, lncRNA) were identified but also a group of pathways, 30, have been identified as common for all the 4 omics analyses (mRNA, miRNA, lncRNA and proteins). Among these pathways are gap junction, regulation of actin cytoskeleton chemokine signaling pathway, PPAR signaling, PI3K signaling, Ras signaling or focal adhesion (Figure 10A, side box). Figure 10B provides an example of integration for the focal adhesion pathway showing the integrated analysis of the four regulatory layers, that is mRNA, miRNA, lncRNA and protein regulation, with their interactions, revealing how this pathway can be modulated by γ-valerolactones. Some genes involved in focal adhesion are regulated by miRNAs and lncRNAs, such as Integrin beta (ITGB), found in protein, mRNA and miRNA map and regulated by hsa-miR-6746-3p. Integrin-linked protein kinase (ILK) found in the protein and lncRNA map and being regulated by RP11-732A19.2. Phosphatase and tensin homolog (PTEN) is found in the miRNA map and regulated by miR-6730-5p and miR-6730-3p. Protein kinase (PAK) protein is regulated by miR-6730-5p.

Taken together, this integrated multi-omic analysis suggests that genomic modifications induced by γ-valerolactones highly impact pathways regulating endothelial cell function and permeability.

4. Discussion

The BBB plays an important role in brain health and is often compromised in disease. The BBB is composed of BMECs and other cells such as astrocytesand matrix molecules that help regulate the flow of immune cells and molecules into and out of the brain. Complex intercellular tight junctions limit the passive diffusion of molecules into the brain [31]. Neuroinflammation and mitochondrial dysfunction are common features of chronic neurodegenerative diseases of the central nervous system. Both conditions can lead to increased oxidative stress by excessive release of harmful reactive oxygen and nitrogen species, which further promote neuronal damage and subsequent inflammation resulting in a feed-forward loop of neurodegeneration. The cytokine TNF-α, a master regulator of the immune system, plays an important role in the propagation of inflammation due to the activation and recruitment of immune cells via its receptor TNF receptor 1 (TNFR1). TNF-α may play a dual role in neurodegenerative disease, since stimulation via its second receptor, TNFR2, is neuroprotective and promotes tissue regeneration [32]. However, it has been reported that the levels of both TNFR1 and TNFR2 were significantly correlated with amyloid-β oligomers (AβO) levels, considered as the most proximal neurotoxic species, in amnestic mild cognitive impairment and mild AD patients [33]. TNF-α mediates double-stranded RNA-dependent protein kinase (PKR)-dependent memory impairment and brain insulin receptor substrate 1 inhibition induced by Alzheimer's AβOs in mice and monkeys [34]. Natural compounds, especially polyphenols, have proven capable of modifying different neuropathological features, such as in AD. Special attention, in this context, has been given to

flavan-3-ols, polycyclic flavonoids that are particularly abundant in cocoa, tea, berries, red wine and other plant-derived foods and beverages. Phenyl-valerolactones (PVL) are the major group of circulating flavan-3-ol metabolites in humans [3]. AβO inhibition may indicate a mechanism by which **γ**-valerolactones could inhibit inflammatory processes in neurodegenerative diseases and subsequently protect endothelial integrity, however, the direct effect of **γ**-valerolactones on HBMEC has not been elucidated.

In this study we assessed the effect of **γ**-valerolactones treatment on HBMEC cells on the inhibition of TNF-α -generated stress *in vitro*, through the multi-omic data analysis that included mRNAs, miRNAs, lncRNAs and proteins layers integration (**Figure 11**). We observed that TNF-α alone exerted greater genomic modifications on transcript and protein modulation than treatment with TNF-α + **γ**-valerolactones (VALs), which could indicate that the pro inflammatory effect of TNF-α could be corrected by these metabolites. We performed an analysis of differential expression and pathways associated for each omic data category individual and globally. After the treatment with VALs, we assessed that miRNA targets-mapped pathways are more associated with cell signaling and those protein-mapped pathways are more related with metabolism. However, when we performed the omic integration analysis, we observed a strong association between cell adhesion and permeability pathways, such as, focal adhesion, tight junction, gap junction, adherent junctions with cell signaling pathways, such as, Rap1, PI3K-Akt, Wnt, and thyroid hormone signaling. Signaling pathways such as Rap1, Wnt and PI3K-Akt, has been found to play a crucial role in the maintenance of cell-cell regulation [35,36].

Our analysis allowed us to extract some key elements and their targets interactions in the modulation of EC functions pathways. Here, we identified mRNAs, miRNAs, lncRNAs and proteins involved in cell adhesion and permeability functions. miRNAs are short ncRNAs with a

length of 19–23 nucleotides. MiRNAs have two main functions: post-transcriptional gene regulation and RNA silencing. Consequently, the mRNAs are regulated by one or more mechanisms that include the inhibition of mRNA translation to proteins by ribosomes and by mRNA strand cleavage into two fragments and poly(A) tail shortening that results in mRNA disruption [37]. Clues about brain endothelial function modulated by miRNAs such as hsa-miR-146a-3p, hsa-miR-214-3p in the TNF-α group and hsa-miR-6746 and miR-6730-5p in the VALs group were obtained in our analysis. Deng S et al. (2017), showed that miR-146a was upregulated in lineage negative bone marrow cells in aged mice, which were enriched in endothelial progenitor cells (EPCs) [38]. Overexpression of miR-146a enhanced senescence and augmented apoptosis, suggesting that miR-146a inhibition improves the capacity of vascular repair in EPCs [38]. On the other hand, stimulation of primary Human Umbilical Vein Endothelial Cells (HUVECs) with lipopolysaccharide (LPS) promoted the production of miR-146a in replicative senescent HUVECs [39]. Since pro-inflammatory conditions, such as chronic heart failure, accelerated senescence of ECs, the increase of miR-146a in ECs might represent senescence-associated pro-inflammatory conditions in the vasculature. *In vitro*, TNF-α and IFN*γ* treatment of human cerebral microvascular endothelial cells (hCMEC/D3) led to upregulation of miR-146a, what agrees with our data [40]. On the other hand, miR-214, other miRNA found in TNF-α group is suggested as a biomarker to detect early stages of Parkinson's disease [41].

Hsa-miR-6746 is a miRNA found upregulated in VALs group, this miRNA has been studied in different contexts, but scarce information exists about its relationship with EC function. It would be interesting to extrapolate the epigenetic mechanisms attributed to this miRNA to study the function of brain ECs. In this regard, it has been reported that the splicing activator protein SRSF2 and the splicing inhibitor protein HNRNPD may be implicated in EC senescence. EC senescence

has also been associated with vascular dysfunction and increased vascular risk. Our observations demonstrated that SRSF2 can be targeted by miR-6730-5p and miR-6746-5p, two miRNAs differentially expressed in VALs group, which could indicate a VALs prevent EC dysfunction mechanism.

LncRNAs are all ncRNAs larger than 200 nucleotides and are classified according to their proximity to protein-coding genes as intergenic, intronic, bidirectional, sense, and antisense lncRNAs. The main function of a signal lncRNA is to serve as a molecular signal to regulate transcription in response to various stimuli [42]. LncRNAs show tissue-specific expression patterns and are predominantly located in the nucleus rather than the cytoplasm. In fact, there are several lines of evidence that suggest that lncRNAs are significantly more enriched in chromatin than miRNAs [37]. In our analysis, we identified lncRNAs differentially expressed, such as FTX, RP11-386G11.10, RP11-192H23.7, AC012668.2, AC005562.1 found in VALs group, and searched their connections in brain EC function. For instance, five primes to Xist (FTX) is downregulated in VALs group, and it was shown that overexpression of FTX inhibited apoptosis in H2O2 treated cardiomyocyte and ischemia- reperfusion (I/R) injury mice model by negatively regulating miR-29b-1-5p [43]. miR-29 is required for normal endothelial function in humans and animal models and has therapeutic potential for cardiometabolic [44]. Therefore, downregulation of FTX could contribute to upregulation of miR-29 and subsequently to an improvement in EC function.

Mechanistically, peroxisome proliferator-activated receptor γ (PPARγ) expression in human hepatocellular carcinoma tissues and cell lines was positively correlated with lncRNA FTX [45]. PPARγ overexpression in brain EC results in selective abrogation of inflammation-induced ICAM-1 and VCAM-1 upregulation and subsequent adhesion and transmigration of T cells. Therefore, it

has been proposed that PPARγ in brain EC may be exploited to target detrimental EC-T cell interactions under inflammatory conditions [46]. Moreover, HIV-1 neuropathogenesis, enhanced adhesion and migration of HIV-1 infected monocytes across the BBB were significantly reduced when bovine brain microvascular endothelial cells (BMVEC) were treated with PPARγ agonist. These findings indicate that PPARγ agonists could be a new approach for treatment of neuroinflammation by preventing monocyte migration across the BBB [47]. FTX appears to be related to PPARγ, although FTX was down-regulated in our study, PPARγ signaling was one of the enriched pathways in our global enrichment analysis. Therefore, diverse mechanisms must converge for PPARγ to be activated in the VALs group.

Likewise, we identified TFs regulating our mRNA omic layer. NF-κB, it was one of the transcriptional factors found in our analysis, regulating some of the differentially expressed transcripts. NFκB activation initiates the canonical and non-conical pathways that promote activation of TFs leading to inflammation, such as leukocyte adhesion molecules, cytokines, and chemokines. However, flavonoids may modulate the expression of pro-inflammatory genes leading to the attenuation of the inflammatory responses underlying various pathologies [48]. It has been corroborated that small molecules, derived from dietary (poly)phenols may cross the BBB, reach brain cells, modulate microglia-mediated inflammation and exert neuroprotective effects, with potential for alleviation of neurodegenerative diseases. The (poly)phenol metabolites attenuated neuro-inflammatory processes via regulation of nuclear factor (NF)-κB translocation into the nucleus and modulation of IκBα (inhibitor of NF-κB) levels [49]. Our in-silico docking analysis also suggests that valerolactones metabolites present capacity to bind to this transcription factor, binding that could affect its activity and consequently expression of related genes. This analysis also suggested that the studied valerolactone metabolites present potential binding

capacity to different transcription factors. Earlier studies have suggested that epicatechin secondary metabolites can bind to these cell signaling proteins [19] but it is first to suggest such interaction for gut metabolites.

By omic layers integration, our analysis allowed us to find relationships between HBMEC and focal adhesion pathways. We were able to identify components mapped to the focal adhesion network, which were differentially regulated in our study (Figure 10). For instance, alpha integrin (ITGA) and ITGB and their relationship with Platelet endothelial cell adhesion molecule (PECAM1-1). PECAM-1 is critically involved in regulating BBB permeability and although not required for T-cell diapedesis itself, its presence or absence influences the cellular route of T-cell diapedesis across the BBB [50]. In ECs, this molecule controls junctional and adhesive properties. It is reported that although in physiological conditions, PECAM-1 supports the endothelial barrier function, in inflammation that is observed in vessels affected by atherosclerosis, the function of PECAM-1 is impaired, an event that leads to increased adhesion of neutrophils and other leukocytes to ECs, decreased vascular integrity, and higher leukocyte transmigration to the intima media. PECAM-1 contains six extracellular Ig-like domains that mediate the attraction and adhesion of leukocytes to EC, for example by enhancing eosinophil adhesion to IL-4-stimulated HUVECs in an α4β1 integrin-dependent manner [51]. Therefore, searching for mechanisms that mediate PECAM-1 interactions with beta or alpha integrins is of importance. Hsa-miR-6746-3p and hsa-miR-6730-5p were two differentially regulated miRNAs in the VALs group and whose targets include ITGA and ITGB. In conditions of stress, such as TGF-β stimulus, the inhibition of these two integrins, could prevent leukocyte transendothelial migration (TEM) under inflammatory conditions.

Another possible mechanism of VALs function against TNF-α inflammation could be mediated by Integrin-linked kinase (ILK). An ILK knockdown significantly decreased β1-integrin expression by 24 h after transfection of confluent primary cerebral microvessel endothelial cells that was accompanied by a significant decrease in claudin-5 expression, and a small significant change in F-actin. ILK is essential for EC survival, vascular development, and cell integrin–matrix interactions in mice. Yet, there is little information about the role(s) of ILK in tight junction protein expression or permeability in the CNS in inflammatory processes [52]. In a study aimed to explore the mechanism of the function of TGF-β signaling in dermal lymphatic endothelial cells (LECs) epithelial–mesenchymal transition (EMT) it was found that TGF-β treatment increased the expression level of ILK Human lens epithelial cells (HLEC-h3), promoted migration of HLEC-h3 cells, increased the expression level of E-cadherin protein, and decreased the expression level of α-SMA protein, playing an important role in fibrogenesis. However, treatment with ILK siRNA, ILK inhibitor, and NF-κB inhibitor reversed the effects of TGF-β on HLEC-h3 cells [53]. In our study, ILK was a target of the lncRNA RP11-732A19.2, differentially expressed in our VALs group which could indicate a possible mechanism, by which γ-valerolactones inhibit the inflammatory process of TNF-α. Preventing neuronal damage and neuronal death have a huge clinical benefit. Flavonoids are key compounds for the development of a new generation of therapeutic agents that could be clinically effective in treating neurodegenerative diseases. Regular consumption of flavonoids has been associated with a reduced risk of neurodegenerative diseases (Solanki et al, 2015). The molecular targets identified in our study will supply the basis for the development of future therapeutic targets.

Figure legends

Figure 1. TNF-α modulates the expression of mRNAs, miRNAs, lncRNAs and proteins in human brain microvascular endothelial cells. HBMEC cells induced under stress by 4h-incubation with TNF-α were used in microarray analysis and proteomic analysis to determine the differential expression of transcripts and proteins between groups. A) Manhattan plot of total transcripts, 983 transcripts positioned above the line were considered differentially expressed. B) Histogram of the number of differentially expressed mRNAs, miRNAs, lncRNAs and proteins. C) Venn diagram to indicate the relations between the number of mRNAs, miRNAs targets, lncRNA targets and proteins differentially expressed. D) Global network of interactions modulated by TNF-α treatment in HBMEC cells. The total interactions between mRNAs, miRNAs and targets, lncRNAs and targets and protein-protein interactions were used to build a network with Cytoscape software. Nodes colored labels. Red-lncRNAs, blue marine-miRNAs, green-miRNAs targets and mRNAs DE, pink-lncRNA targets and mRNA DE, yellow-lncRNA and miRNA targets and mRNA no DE, purple-mRNAs DE, melon-proteins and mRNA DE, clear purple -proteins and mRNAs DE and regulated by miRNAs or lncRNAs, blue clair-proteins and mRNAs no DE. DE = differentially expressed. E) Zoom network of interactions between the components of each omic layer and also corresponding to differentially expressed mRNAs. Nodes colored labels. Red-lncRNAs, blue marine-miRNAs, green-miRNA targets, pink-lncRNA targets, yellow-lncRNA and miRNA targets, melon-proteins, clear purple-proteins regulated by miRNAs and lncRNAs.

Figure 2. Enriched pathways of differentially expressed transcripts and proteins of human brain microvascular endothelial cells treated with TNF-α vs control. Differentially expressed transcripts and proteins between study groups were used in a GeneTrial analysis to obtain the list

of enriched pathways. Subsequently, a set of genes involved in KEGG endothelial pathway functions was used to map against our differentially expressed transcripts and proteins. A) Histogram that shows the number of transcripts and proteins mapped to the enriched pathways. The blue bars represent the enriched pathways of mRNAs, the green bars of miRNAs, the yellow bars of lncRNAs and the red bars of proteins. B) Network of interactions between mRNAs, miRNAs and targets, lncRNAs and targets, protein-protein interactions related to a set of genes involved in endothelial cell functions. Node colored labels. Red-lncRNAs, blue marine-miRNAs, green-miRNAs targets and mRNAs DE, yellow-lncRNA and miRNA targets and mRNA no DE, melon-proteins and mRNA DE, clear purple-proteins and mRNAs DE and regulated by miRNAs or lncRNAs, blue clair-proteins and mRNAs no DE, black-miRNA and lncRNA target and mRNA DE, white-miRNA target or lncRNA target and mRNA no DE. DE= differentially expressed.

Figure 3. Genomic modifications induced by γ-valerolactones treatment in human brain microvascular endothelial cells. HBMEC cells exposed to the mixture of gut metabolites (γ-valerolactones) for 20h and induced under stress by 4h-incubation with TNF-α were used in microarray analysis and proteomic analysis to determine the differential expression of transcripts and proteins between groups, respectively. A) A 3D Principal component analysis (PCA) plot of the data that characterizes the trends exhibited by the expression profiles of HBMEC cell no treated with TNF-α (control, blue), cells treated with TNF-α (TNF-α, red) and cells treated with VALs + TNF-α (VALs, purple). Each dot represents a sample and each color represents the type of the sample. B) Histogram of the number of differentially expressed mRNAs, miRNAs, lncRNAs and proteins. C) Manhattan plot of total transcripts, 211 transcripts positioned above the line were considered differentially expressed. D) Heatmap shows transcript abundance (expression level) in

each group. In red are upregulated classes of genes (>2-fold standard deviation); in green are downregulated classes of genes (<2-fold standard deviation). Classes were grouped by a pattern of transcription, genes that are upregulated in both tissues; genes that are differentially expressed, genes that are downregulated in both tissues.

Figure 4. Enriched pathways of protein-coding genes differentially expressed in human brain microvascular endothelial cells treated with γ-valerolactones. Differentially expressed protein-coding genes between VALs and TNF-α groups were used in a GeneTrial analysis to obtain the list of enriched pathways. A) Histogram that shows the number of genes mapped to the enriched pathways classified in cell adhesion and permeability, cell signaling, cell metabolism and other pathways. B) Pathway interactions network built in Cytoscape software to show the connections (edges) between enriched pathways (nodes) by differentially expressed protein-coding genes.

Figure 5. MiRNAs expression mediated by γ-valerolactones treatment in human brain microvascular endothelial cells and their relation with endothelial cell functions. HBMEC cells exposed to the mixture of gut metabolites (γ-valerolactones) for 20h and induced under stress by 4h-incubation with TNF-α were used in microarray analysis to determine the differential expression of miRNAs between groups. Subsequently, the miRNAs targets were obtained from an analysis of databases (miRTarbase, TargetScan and miRDb). An enrichment pathway analysis of miRNAs targets in GeneTrial was also performed. A) Network of miRNAs differentially expressed and their targets. Network built in Cytoscape software. Hubs network components are highlighted by red squares. B) Histogram that shows the number of genes mapped to the enriched pathways

classified as endothelial cells related (blue), cell signaling (green), cell metabolism (yellow) and other pathways (red).

Figure 6. LncRNAs expression mediated by γ-valerolactones treatment in human brain microvascular endothelial cells and their relation with endothelial cell functions. HBMEC cells exposed to the mixture of gut metabolites (γ-valerolactones) for 20h and cells induced under stress by 4h-incubation with TNF-α were used in microarray analysis to determine the differential expression of lncRNAs between groups. Subsequently, the lncRNAs targets were obtained from an analysis of databases (RNARNA, LncRRIsearch). An enrichment pathway analysis of miRNAs targets in GeneTrial was also performed. A) Network of lncRNAs differentially expressed and their targets. Network built in Cytoscape software. Hubs network components are highlighted by red squares. B) lncRNA targets were overrepresented by genes coding for KEGG, Biocarta and Wiki modules associated with endothelial cell related, cellular metabolism, cell signaling and other pathways. C) lncRNA-enriched pathway interactions network. Pathway interactions network built in Cytoscape software to show the connections (edges) between enriched pathways (nodes) by lncRNAs differentially expressed. Hubs network components are highlighted by yellow circles.

Figure 7. Protein expression mediated by γ-valerolactones treatment in human brain microvascular endothelial cells and their relation with endothelial cell functions. HBMEC cells exposed to the mixture of gut metabolites (γ-valerolactones) for 20h and cells induced under stress by 4h-incubation with TNF-α were used in shotgun proteomic analysis to determine the differential expression of proteins between groups. An enrichment pathway analysis of miRNAs targets in GeneTrial was also performed. A) Proteins differentially expressed mapped onto KEGG,

Biocarta and Wikipathways modules associated with endothelial cell function, cell metabolism, cell signaling pathways and others. B) Proteins-enriched pathway interactions network, pathway interactions network built in Cytoscape software to show the connections (edges) between enriched pathways (nodes) by proteins differentially expressed. Hubs network components are highlighted by yellow circles.

Figure 8. Transcription factors potentially involved in the nutrigenomic modifications induces by γ-valerolactones and their *in-silico* interaction with RelA subunit of NF-κB. A) List of potential transcription factors identified using bioinformatic analysis. Binding mode obtained by computational docking for: (B) 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate and (C) 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide.

Figure 9. Global network of interactions modulated by γ-valerolactones treatment in human brain microvascular endothelial cells and their relation with endothelial function pathways. A) The total interactions between mRNAs, miRNAs and targets, lncRNAs and targets and protein-protein interactions were used to build a network with Cytoscape software. Nodes colored labels. Red-lncRNAs, blue marine-miRNAs, green-miRNAs targets and mRNAs DE, pink-lncRNA targets and mRNA DE, yellow-lncRNA and miRNA targets and mRNA no DE, purple-mRNAs DE, blue clair-proteins and mRNAs no DE, black-miRNA and lncRNA target and mRNA DE, clair green-proteins and mRNA no DE and regulated by miRNA or lncRNA, olive-TFs, white-components not belonging to any of the previous color categories. DE = differentially expressed. Hubs network components are highlited by bigger squares. B) mRNA, miRNA targets, lncRNA targets and proteins were used to perform a global GeneTrial enrichment analysis and obtained the

pathways related. A pathway-connections network was built in Cytoscape. Node colored labels represent the omic layers (mRNA, miRNA targets, mRNA targets, proteins or combinations) from which the pathways were enriched. Yellow-miRNA, green-proteins, pink-mRNA+lncRNA+miRNA, blue marine-miRNA+proteins, clair blue-mRNA+miRNAs, clair purple-lncRNA+miRNA, red-mRNA+miRNA+lncRNA+proteins, melon-lncRNA+miRNA+proteins, clair green-mRNA+miRNA+proteins. C) Small network of focal adhesion components and regulated by miRNAs and lncRNAS differentially expressed in our VALs vs TNF-α groups comparison.

Figure 10. Integration of differentially expressed mRNAs, microRNA target gene, lncRNA target gene and protein modulating focal adhesion function in human brain microvascular endothelial cells treated with γ-valerolactones and stressed with TNF-α. A) Heatmap of differentially expressed gene pathways, miRNA target gene pathways, lncRNA gene pathways and protein pathways. Comparisons of pathways between biological categories identified a group of pathways in common including chemokine signaling pathway, cytokine cytokine receptor, focal adhesion, gap junction, FOXO signaling, fatty acid metabolism and pathways that regulate endothelial cell interaction and permeability. B) A representative integrated analysis of differentially expressed genes and proteins, and target genes of differentially expressed miRNAs, lncRNA is shown for Focal adhesion. Yellow= differentially expressed genes; Red= target genes of differentially expressed miRNAs, Green=target genes of differentially expressed lncRNAs, Blue= differentially expressed proteins. Color gradations= genes identified in both omic maps.

Supplemental figure legends

Supplemental Figure 1. Chemical structures of microbiota epicatechin metabolites.

Supplemental Figure 2. miRNA-enriched pathway interactions network. Pathway interactions network built in Cytoscape software to show the connections (edges) between enriched pathways (nodes) by miRNAs differentially expressed.

Supplemental Figure 3. Venn diagrams of mRNAs, miRNAs targets, lncRNAs targets, proteins and pathways related in human brain microvascular endothelial cells- γ-valerolactone treatment cell. A) Venn diagram indicates the relationships between the number of mRNAs, miRNAs targets, lncRNA targets and proteins differentially expressed. B) Venn diagram indicates the relationships between the number of enriched pathways-mRNAs, miRNAs targets, lncRNA targets and proteins differentially expressed.

Supplemental table legends

Supplemental Table 1. Effect of the γ-Valerolactones treatment on the expression of protein coding genes in human brain microvascular endothelial cells.

Supplemental Table 2. Effect of the γ-Valerolactones treatment on the expression of miRNAs in human brain microvascular endothelial cells.

Supplemental Table 3. Effect of the γ-Valerolactones treatment on the expression of lncRNAs in human brain microvascular endothelial cells.

Supplemental Table 4. Effect of the γ-Valerolactones treatment on the expression of proteins in HBMEC cells.

Competing Interests

Dragan Milenkovic initiated the study that was partially funded through an unrestricted research grant that he received from Mars Inc. Mars, Inc. also provided the epicatechin metabolites used in this study. H.S. is employed by Mars Inc., a company engaged in flavanol research and flavanol-related commercial activities.

The authors' roles

KF, SN and DM performed bioinformatic analyses and wrote the manuscript; SN and DM performed microarray analysis; DM designed the research and had primary responsibility for final content; All authors participated in the interpretation of the data and also read and approved the final manuscript.

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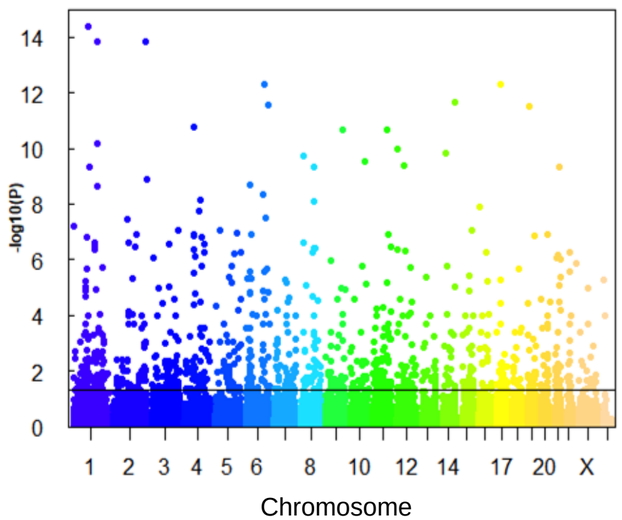
Figure 1. TNFa modulates the expression of mRNAs, miRNAs, lncRNAs and proteins in human brain microvascular endothelial cells.

A)



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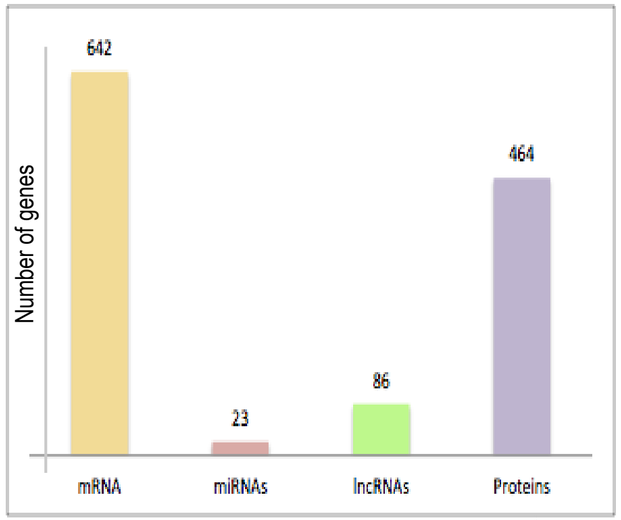

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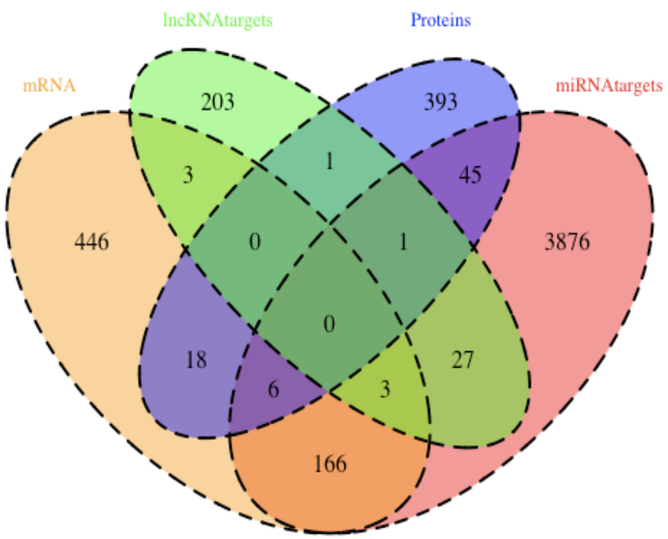

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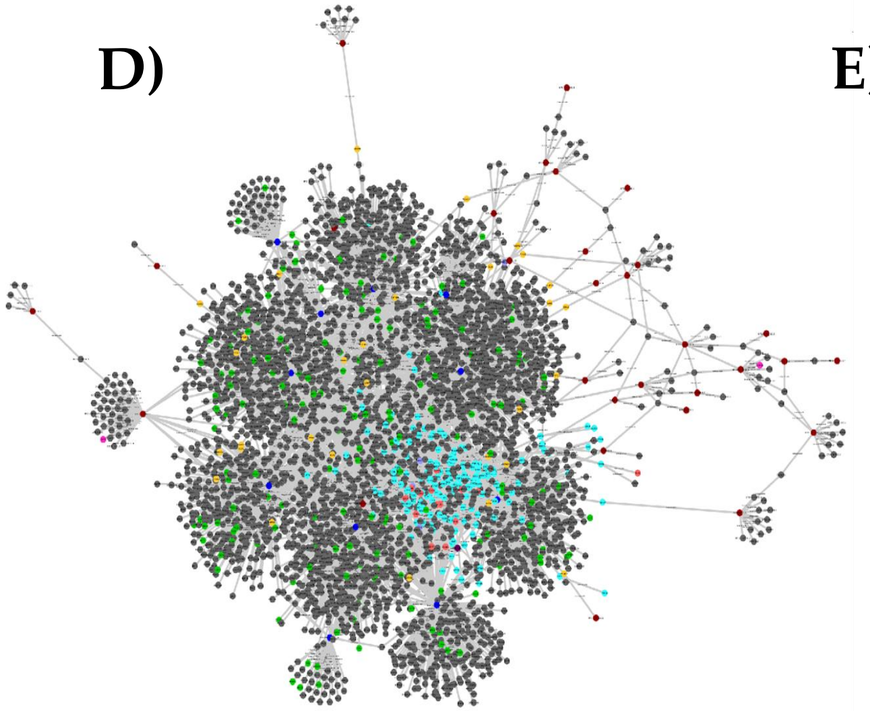

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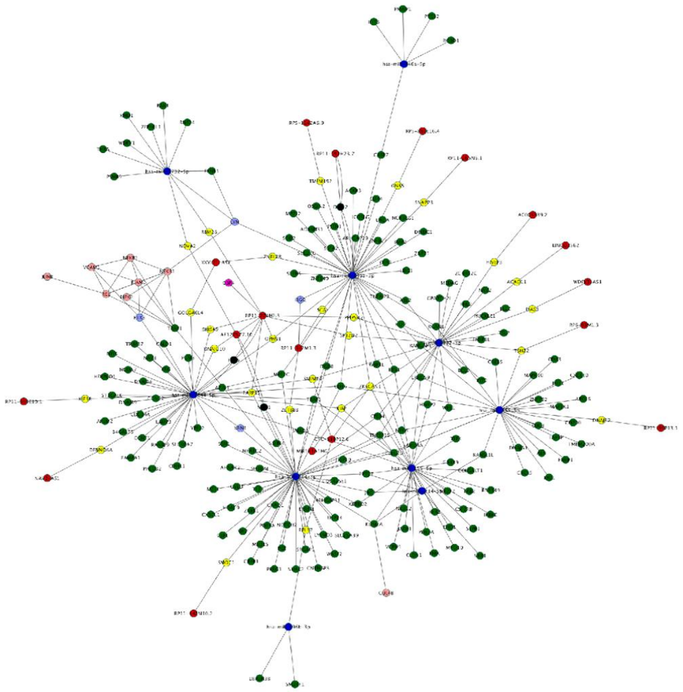
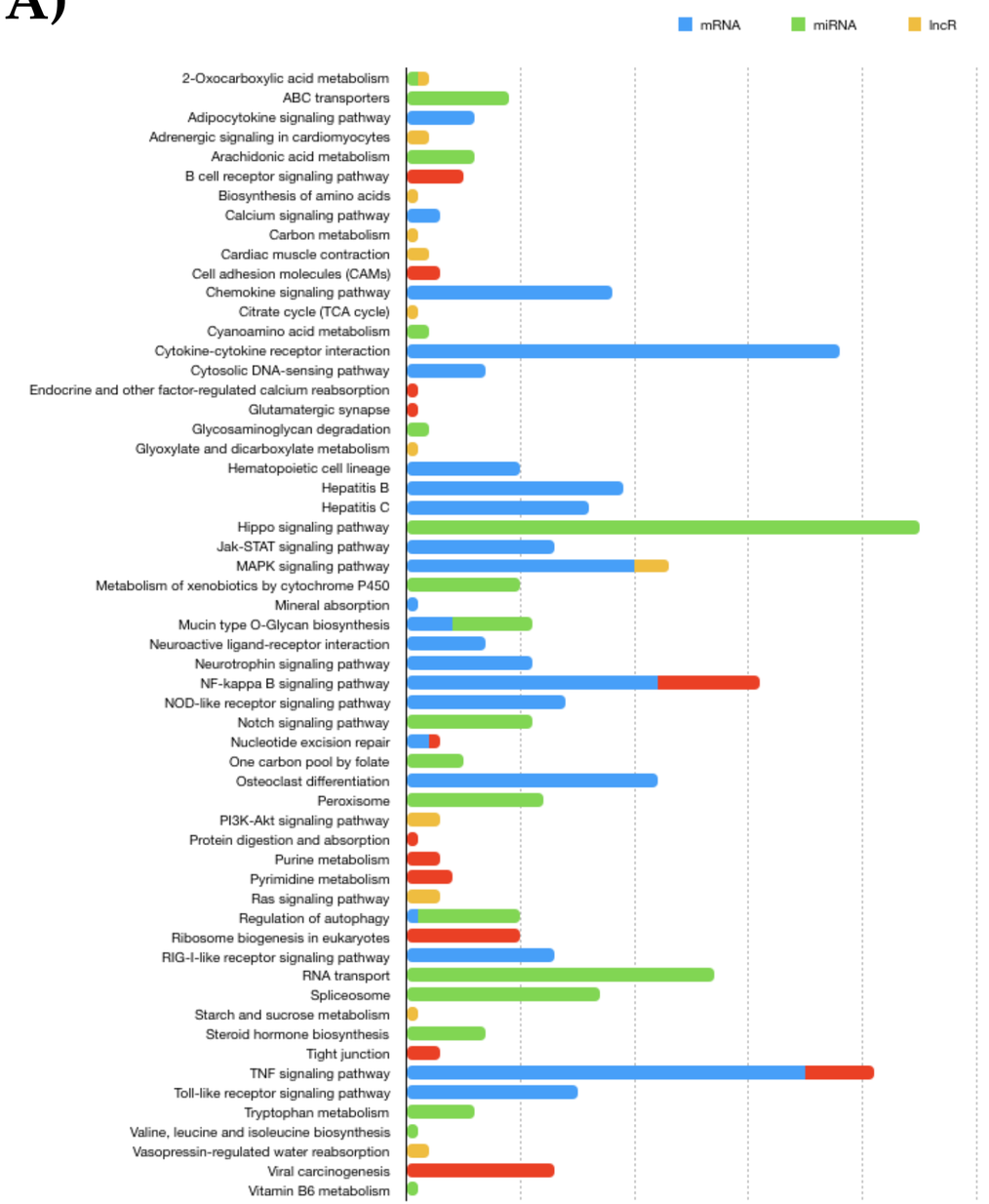


Figure 2. Enriched pathways of differentially expressed transcripts and proteins of HBMEC cells treated with TNFa vs control.

A)



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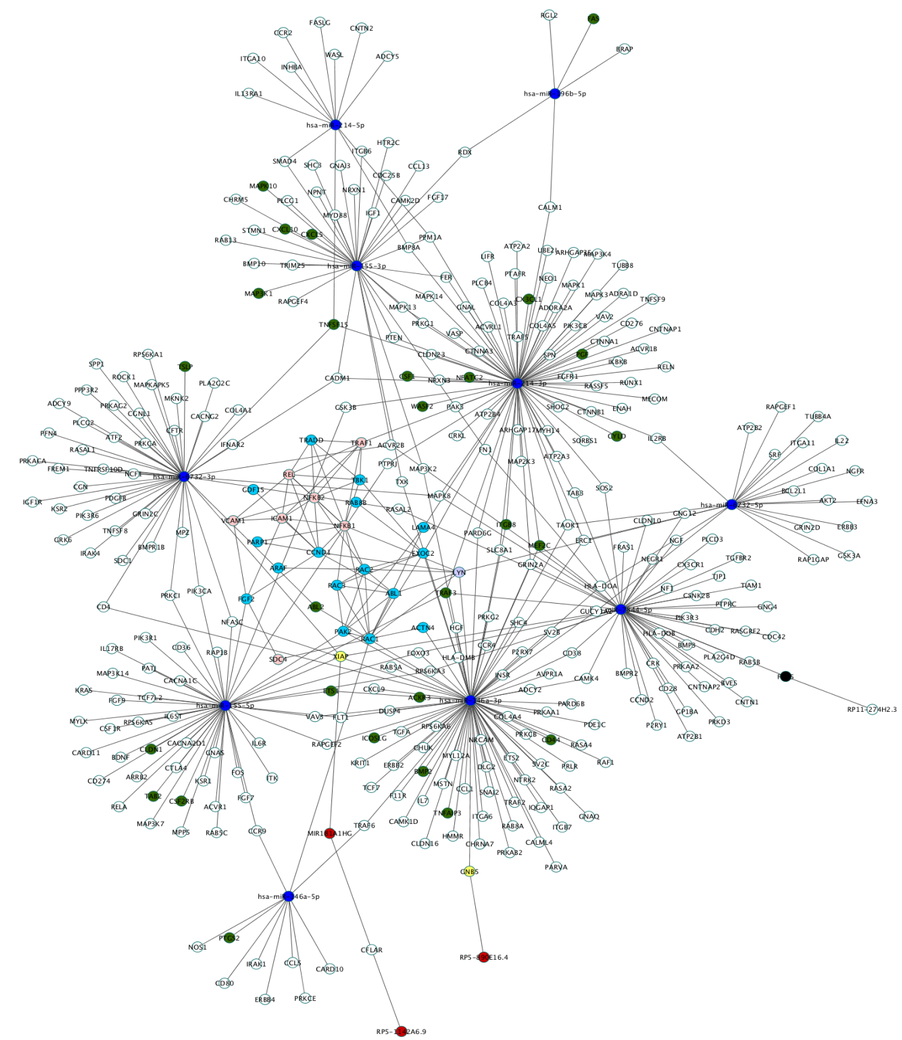
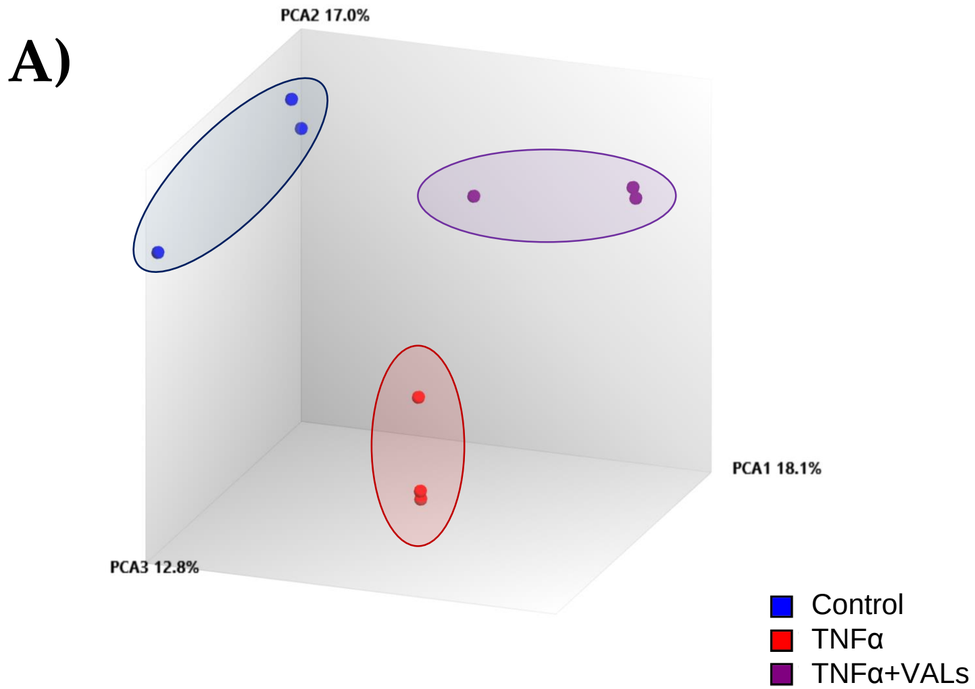
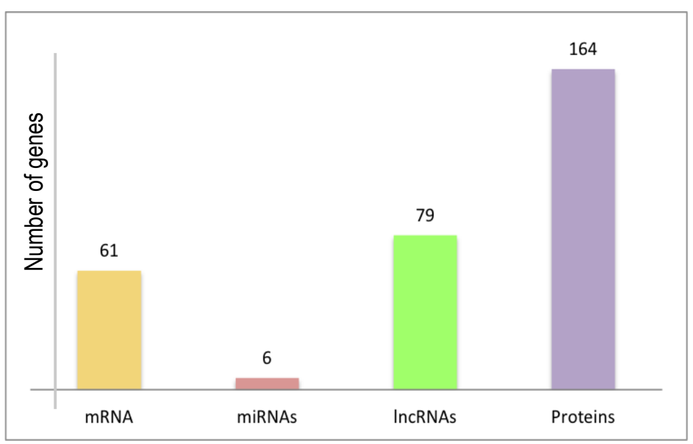
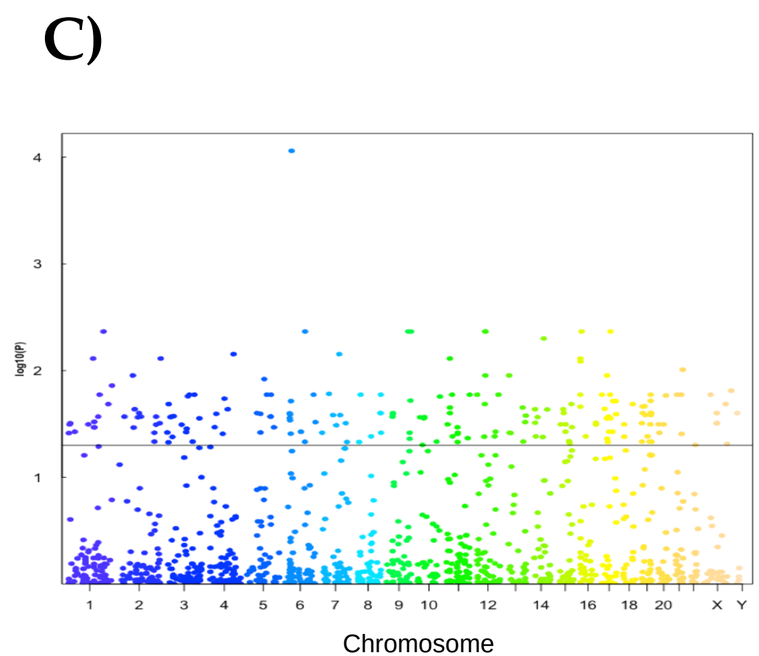


Figure 3. Genomic modifications induced by valerolactones treatment in HBMEC cells.







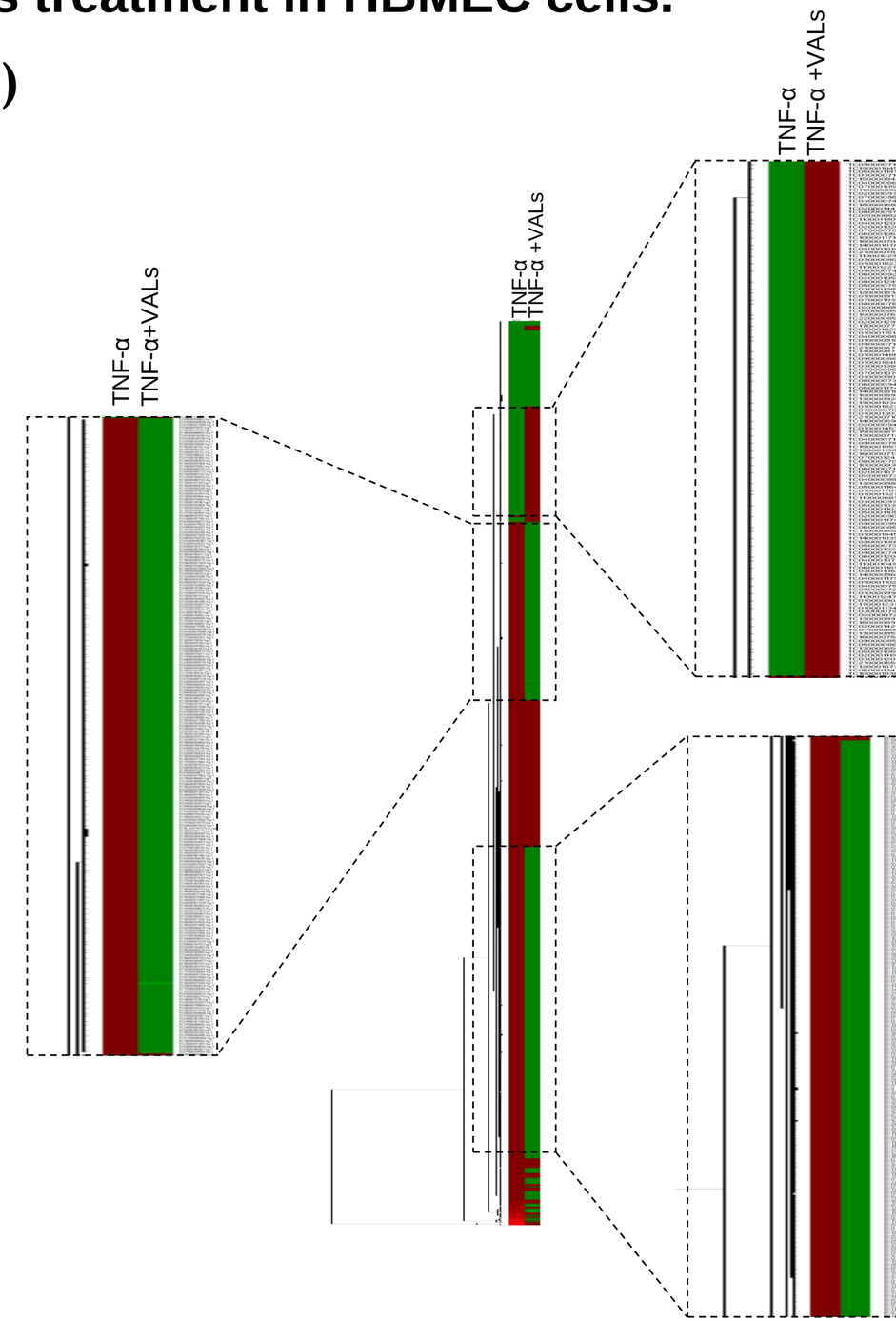


Figure 4. Enriched pathways of protein-coding genes differentially expressed in HBMEC cells treated with valerolactones.

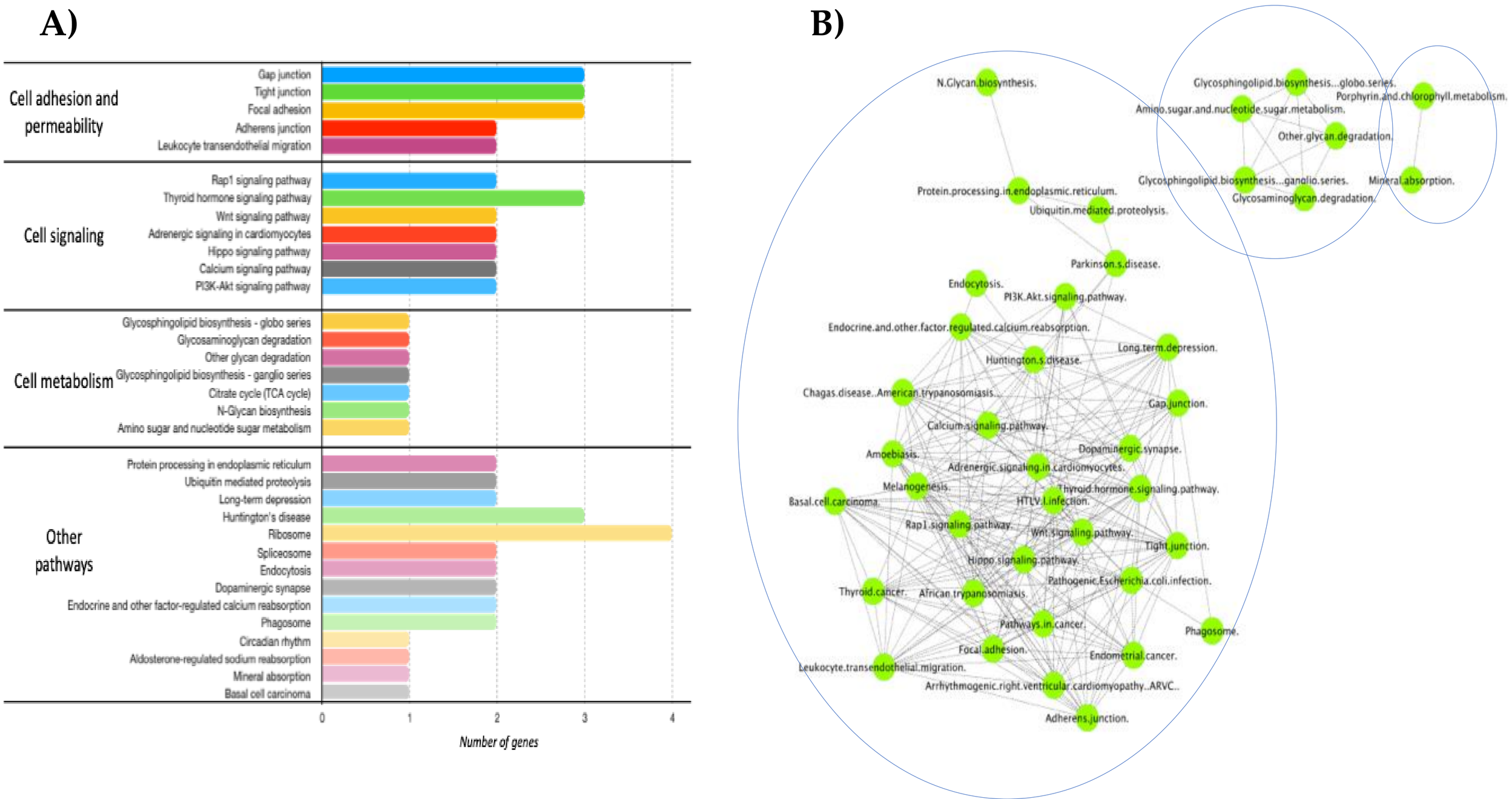
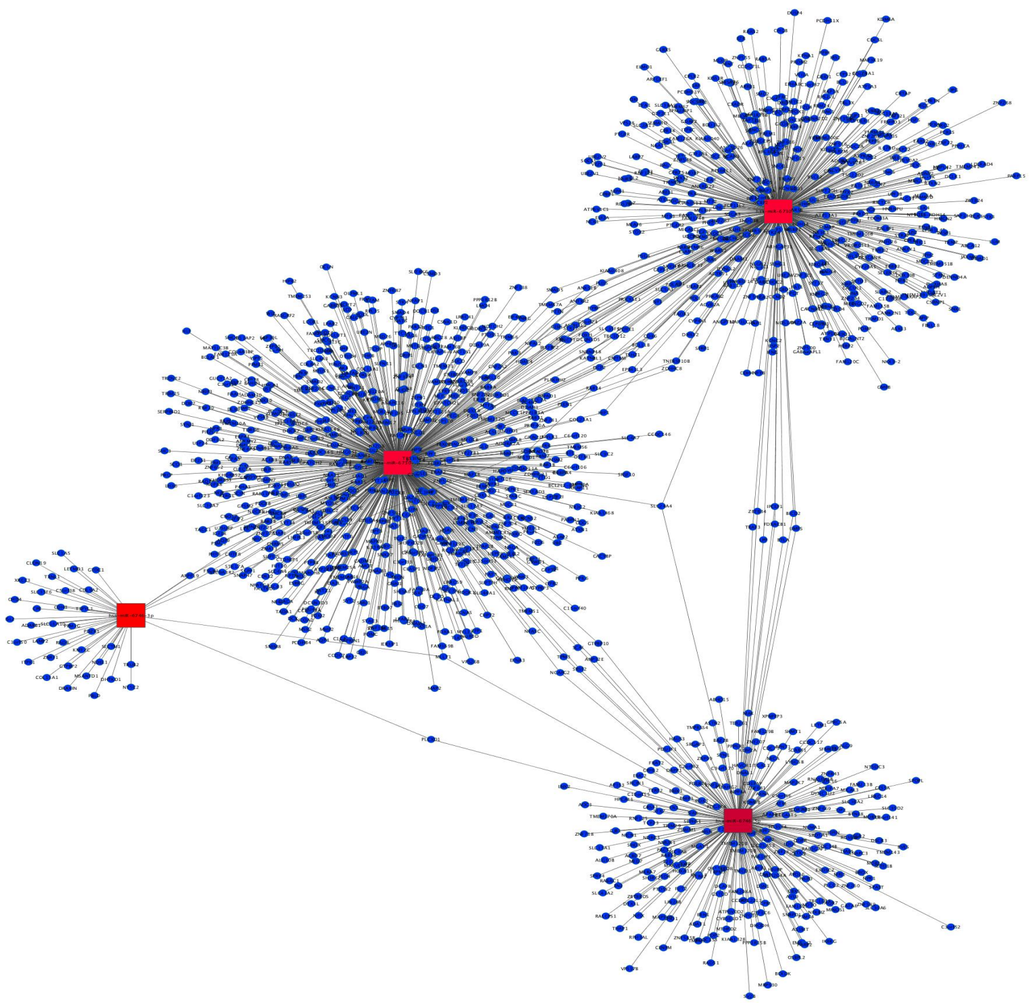


Figure 5. miRNAs expression mediated by valerolactones treatment in HBMEC cell and their relation with endothelial functions.

A)



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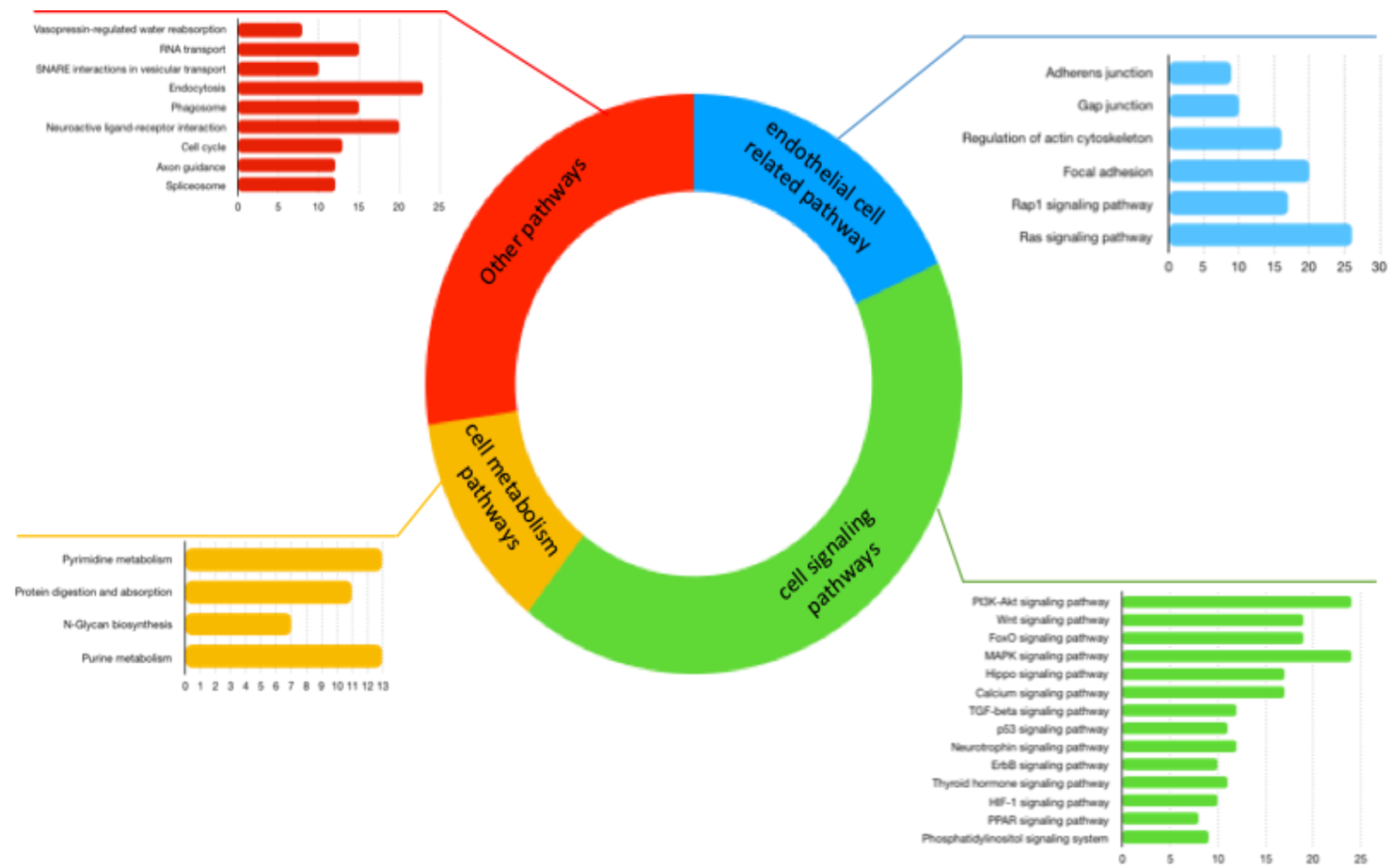
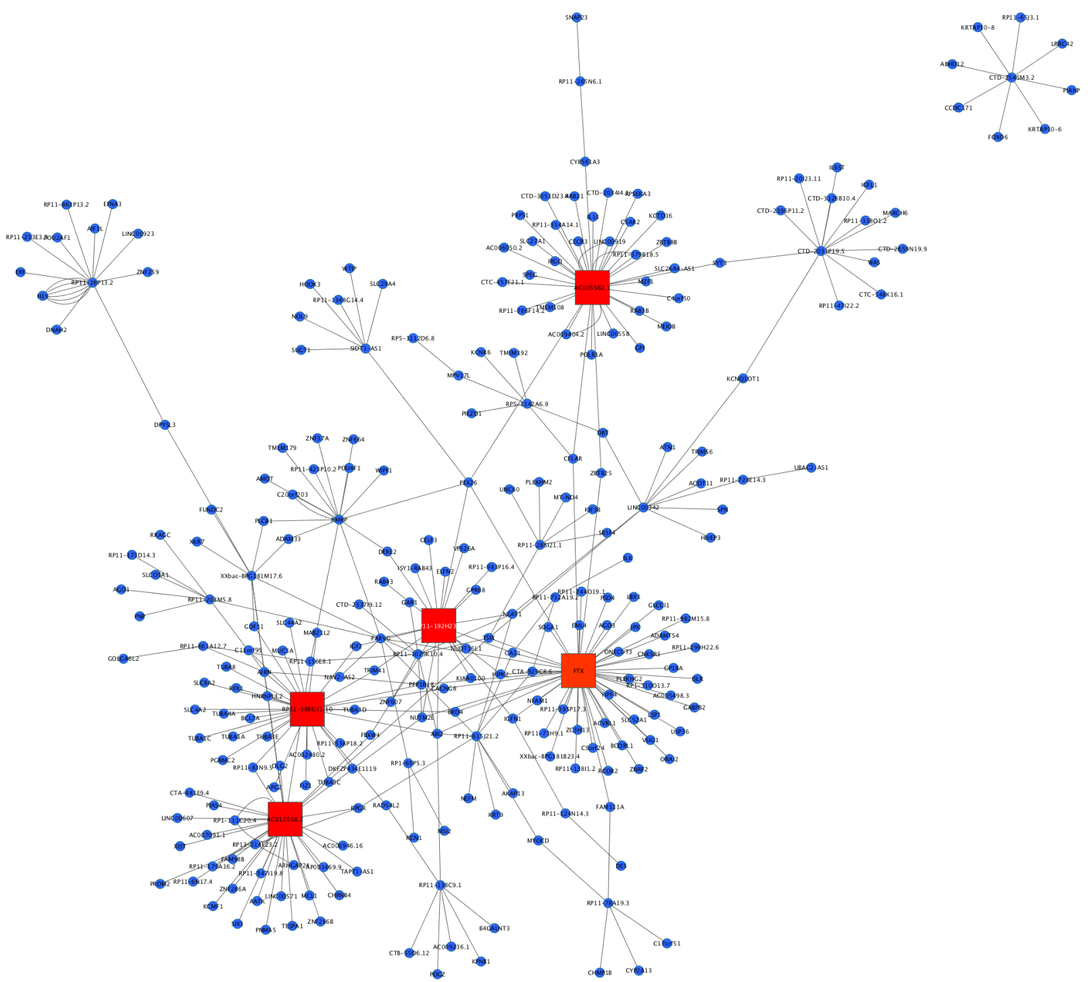


Figure 6. lncRNAs expression mediated by valerolactones treatment in HBMEC cell and their relation with endothelial cell functions.

A)



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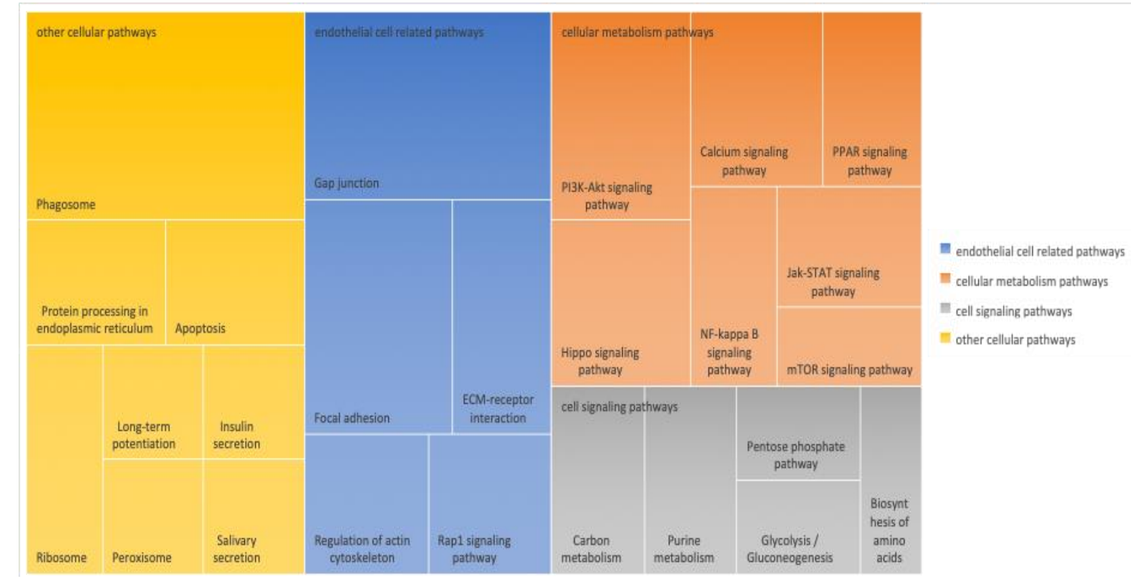

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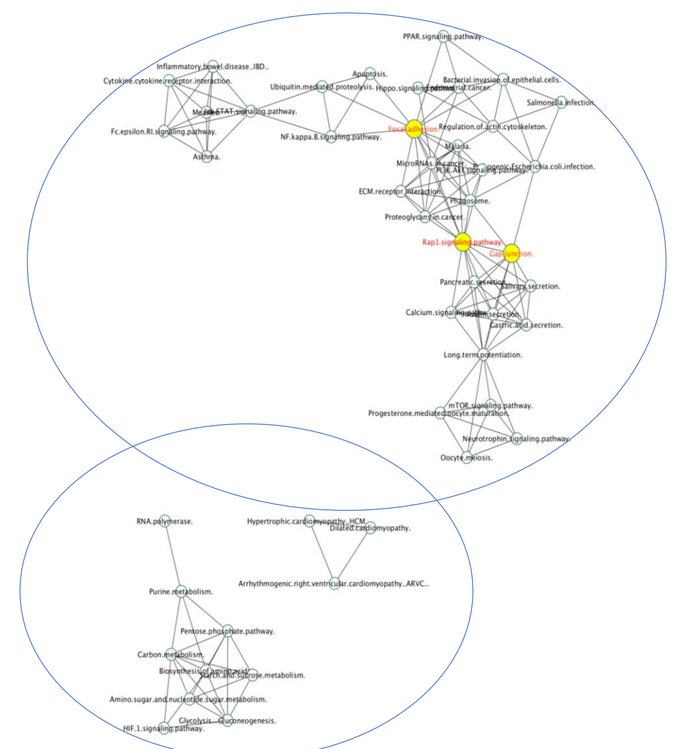
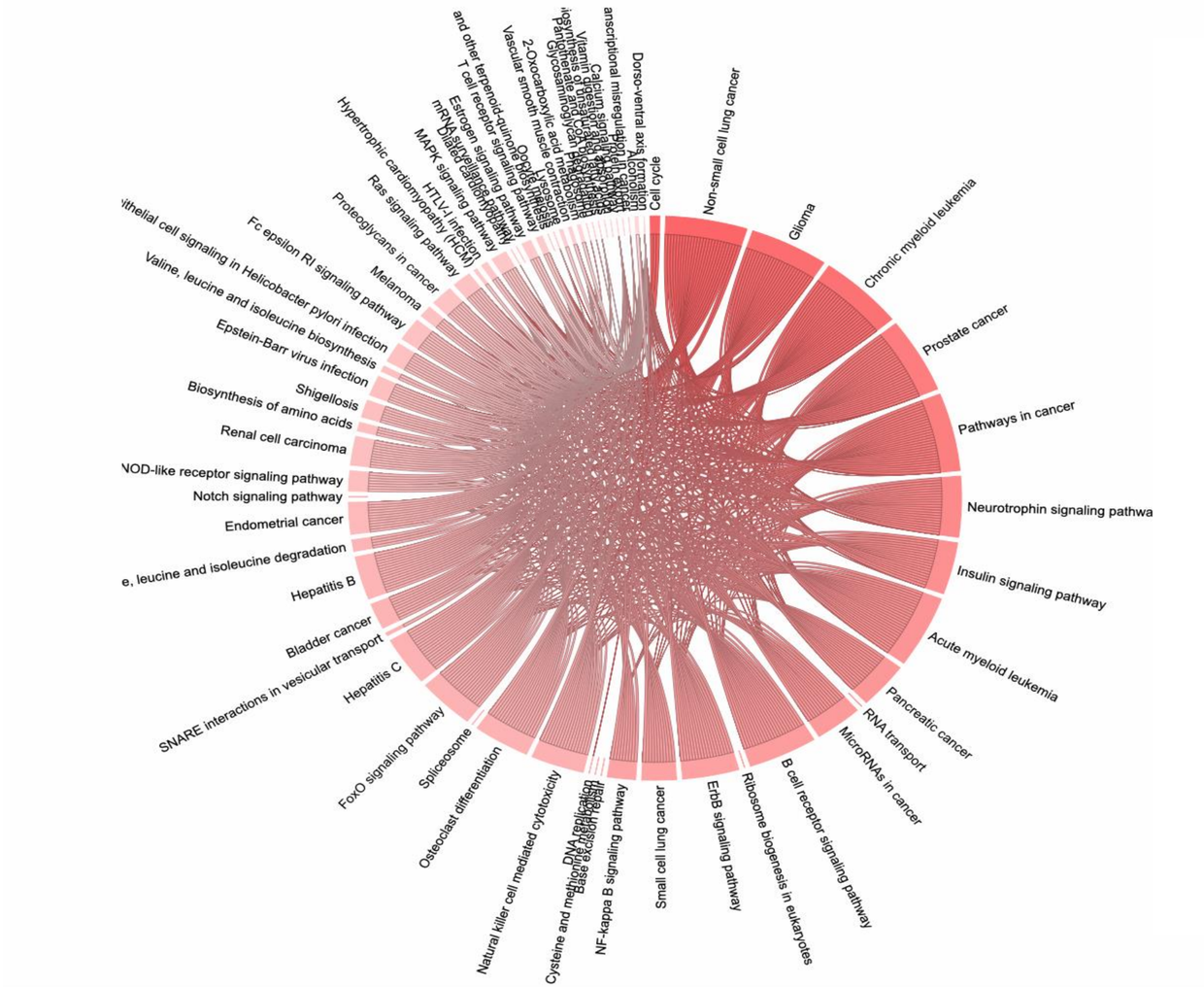


Figure 7. proteins modulation

A)



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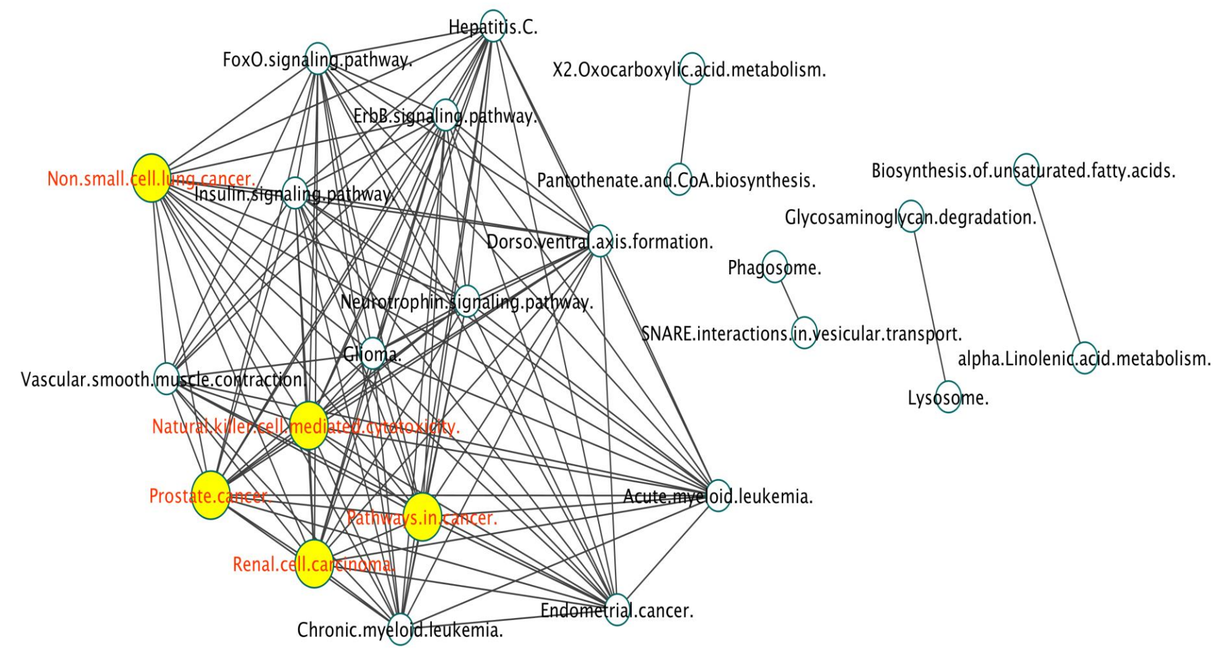
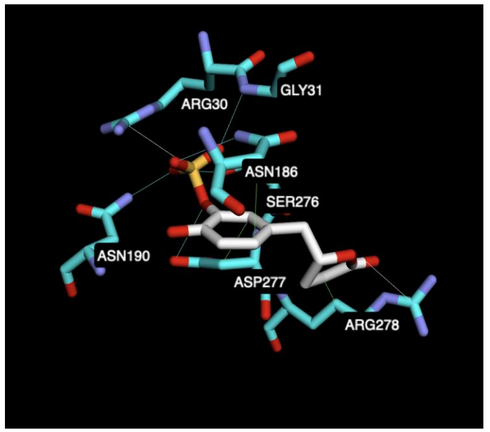


Figure 8. Docking

A)

Transcription factor	P-value	Adjusted p-value
NFKB	7.64E-21	2.18E-18
RELA	5.39E-21	3.08E-18
JUN	2.41E-07	0.00001966
STAT2	0.000004974	0.0003156
IRF1	0.000004636	0.0003309
STAT1	0.00001196	0.0006207
FOXO4	0.00007812	0.003717
ATF2	0.0001153	0.005064
SRF	0.0001429	0.00583
STAT3	0.0001939	0.00738
EGR1	0.0003295	0.01176
ZNF382	0.0009342	0.02963
JUND	0.00109	0.03276
ATF4	0.001306	0.03551
HIVEP2	0.002073	0.04933

B)



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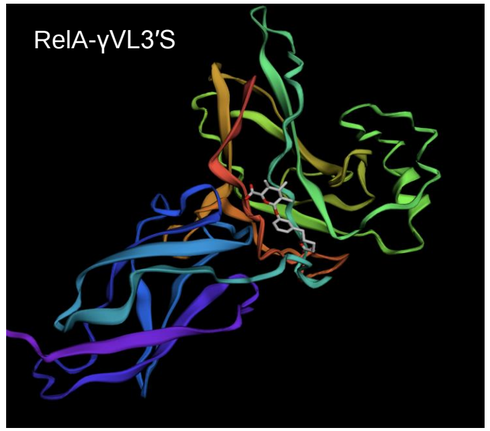


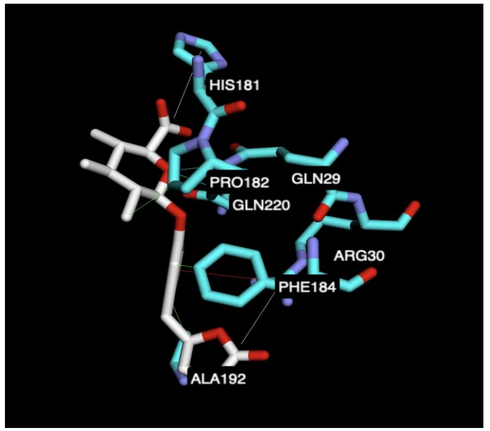


Figure 9. Global network of interactions modulated by valerolactones treatment in HBMEC cells and their relation with endothelial function.

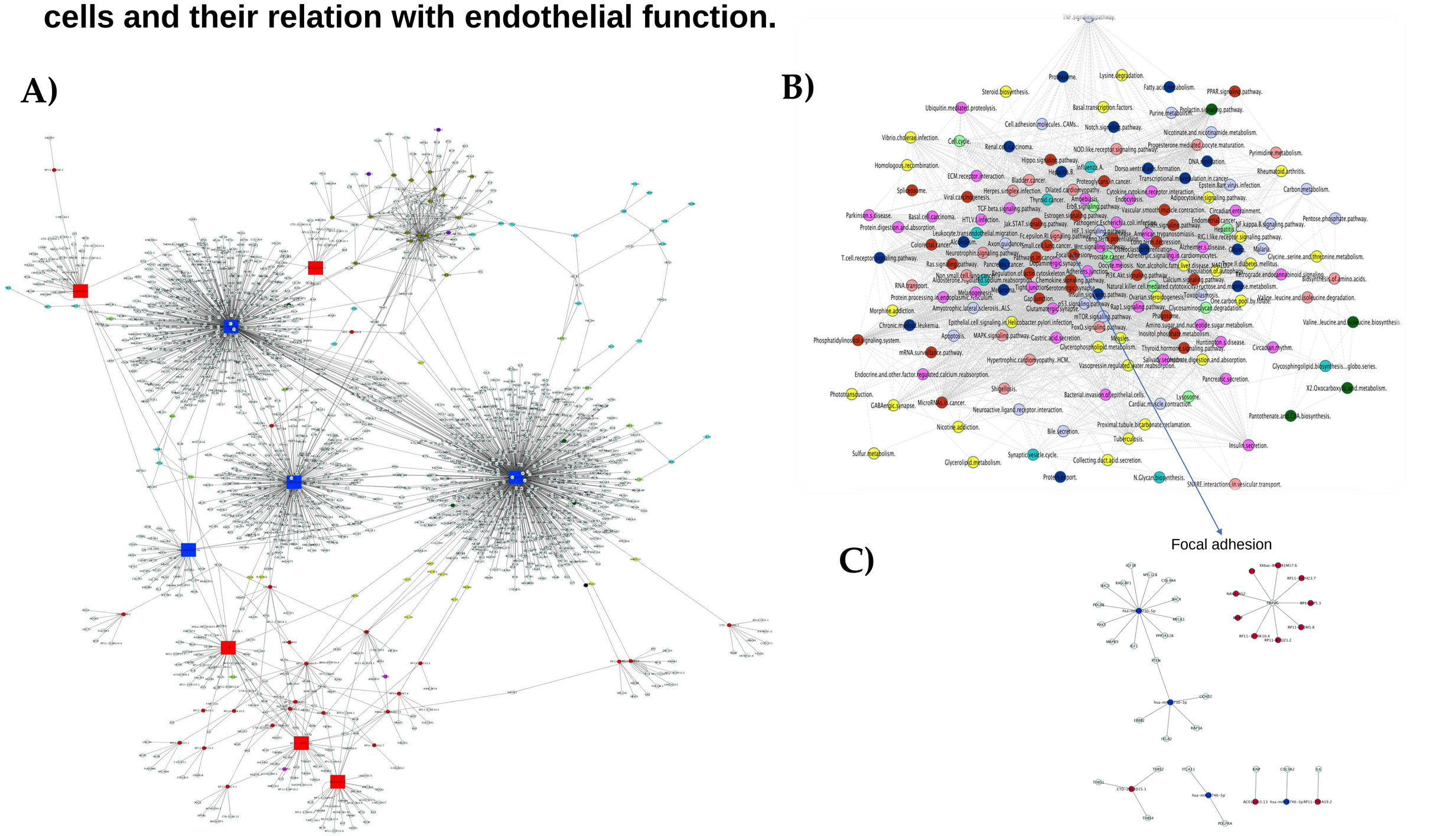


Figure 10. Integration of differentially expressed mRNAs, microRNA target gene, lncRNA target gene and protein modulating focal adhesion function in HBMEC cells treated with valerolactones.

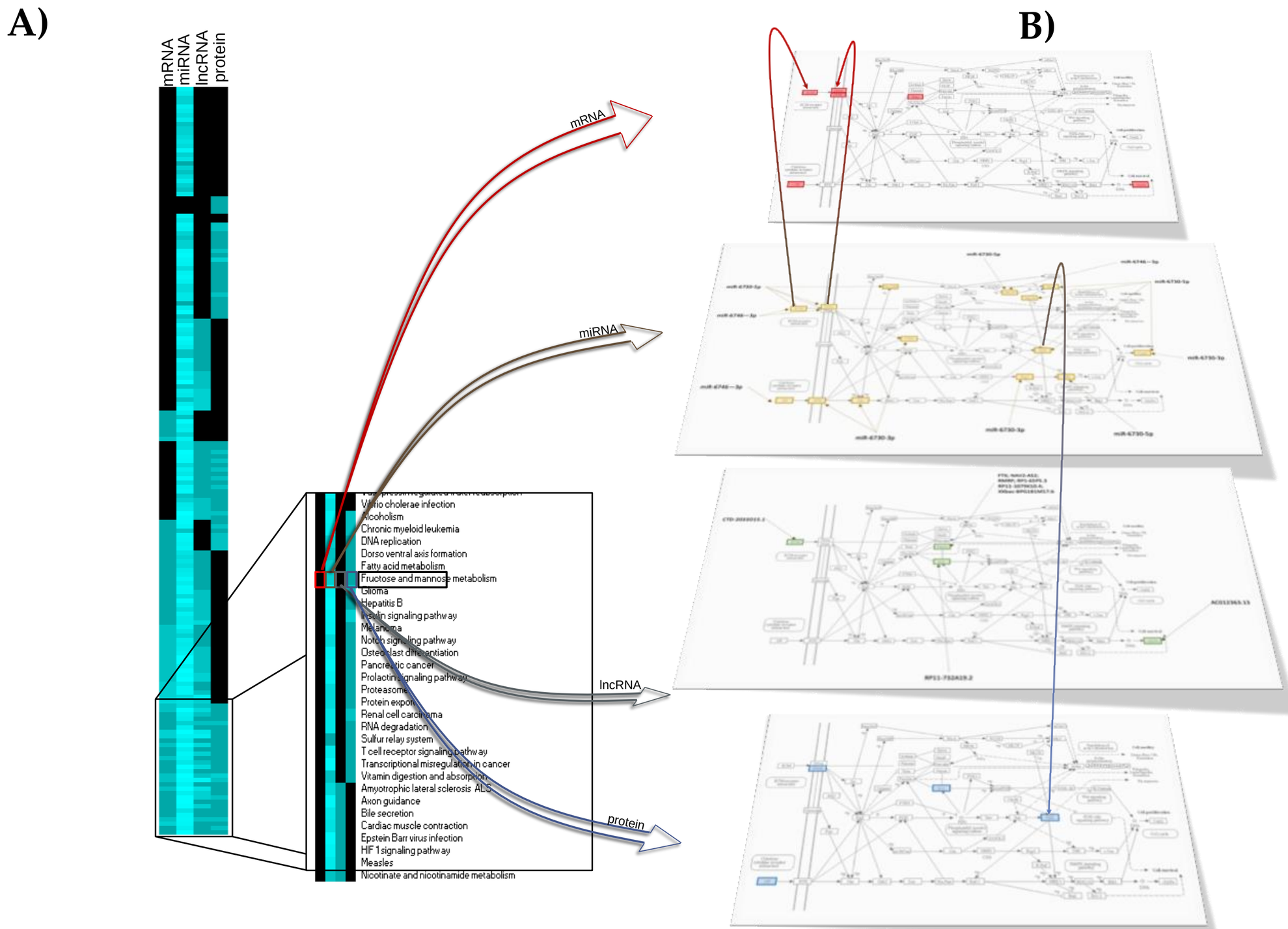
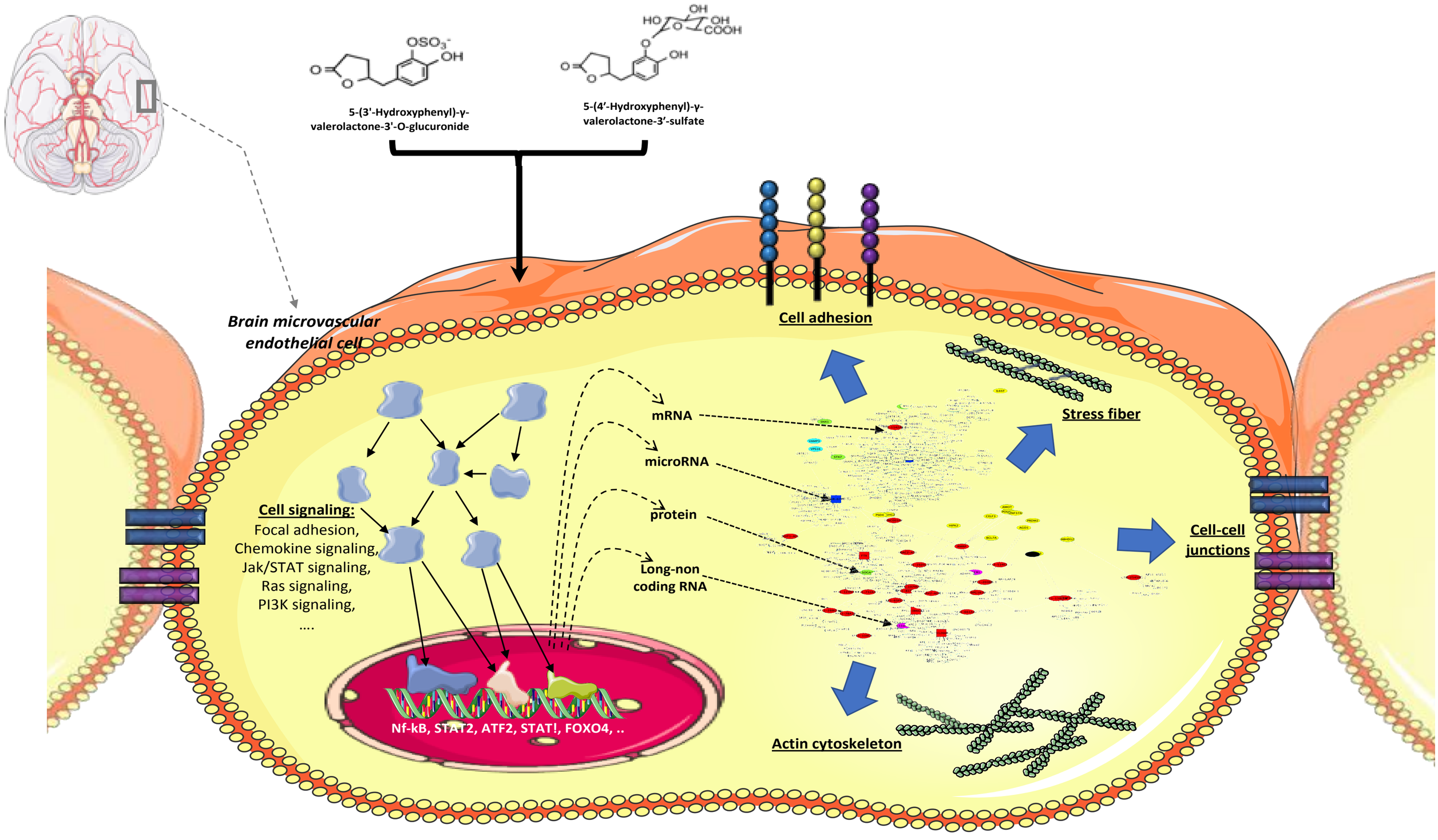


Figure 11


5-(3'-Hydroxyphenyl)-γ-valerolactone-3'-O-glucuronide
5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate
Brain microvascular endothelial cell
Cell adhesion
Stress fiber
Cell-cell junctions
Actin cytoskeleton
mRNA
microRNA
protein
Long-non coding RNA
Cell signaling:
Focal adhesion,
Chemokine signaling,
Jak/STAT signaling,
Ras signaling,
PI3K signaling,
....
Nf-kB, STAT2, ATF2, STAT!, FOXO4, ..