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(-)-Epicatechin exerts positive effects on anxiety in high fat diet-induced obese mice through multi-genomic modifications in the hippocampus

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Abstract

Obesity is associated with increased occurrence of cognitive and mood disorders. While consumption of high-fat diets (HFD) and associated obesity could have a detrimental impact on the brain, dietary bioactives may mitigate these harmful effects. We previously observed that (-)-epicatechin (EC) can mitigate HFD-induced anxiety-associated behaviors in mice. The aim of our study is to investigate the molecular mechanisms of EC actions in the hippocampus which underlies its anti-anxiety effects in HFD-fed mice using a multi-genomic approach. Healthy eight-weeks old male C57BL/6J mice were fed for 24 weeks either: A) a control diet containing 10% total calories from fat; B) a HFD containing 45% total calories from fat; or C) the HFD supplemented with 20 mg EC/kg body weight. Hippocampi were isolated for genomic analysis using Affymetrix arrays, followed by in-depth bioinformatic analyses. Genomic analysis demonstrated that EC induced significant changes in mouse hippocampal global gene expression. We observed changes in the expression of 1001 protein-coding genes, 241 miRNAs, and 167 long non-coding RNAs. Opposite gene expression profiles were observed when the gene expression profile obtained upon EC supplementation was compared to the profile obtained after consumption of the HFD. Functionality analysis revealed that the differentially expressed genes regulate processes involved in neurofunction, inflammation, endothelial function, cell-cell adhesion, and cell signaling. In summary, the capacity of EC to mitigate anxiety-related behaviors in HFD-induced obese mice can be in part explained by its capacity to exert complex genomic modifications in the hippocampus, counteracting changes driven by consumption of the HFD and/or associated obesity.

1. Introduction

More than 2.1 billion adults are estimated to be overweight or obese worldwide, of which 640 million are obese [1]. It is predicted that in the US nearly 1 in 2 adults will be obese and 1 in 4 adults will be severely obese by 2030 [2]. Obesity is a serious public health concern given that it raises risks for several diseases including type 2 diabetes, cardiovascular diseases, nonalcoholic fatty liver disease, and certain types of cancer. Obesity is also a risk factor for the development of metabolic and vascular disorders, which have emerged as risk factors to mood and cognitive disorders [3-5]. HFD and/or associated obesity induce chronic low-grade inflammation and circulating cytokines can cross the blood-brain-barrier (BBB), eliciting neuroinflammation [3, 6]. Systemic inflammation can also disrupt the BBB, leading to infiltration of inflammatory molecules into the central nervous system and subsequently neuroinflammation, which can contribute to alterations in cognition and mood [5, 7].

While HFD and obesity can have detrimental effects on the brain, consumption of select flavonoids have neuroprotective effects [4, 8, 9]. (-)-Epicatechin (EC) is one of the most widely consumed flavan-3-ols, being abundantly found in cocoa, berries, apples, and tea [10]. A considerable body of evidence supports the beneficial effects of dietary EC at the nervous system, which includes its capacity to improve cognition and mood [9, 11-14]. For example, in healthy adults (50-75 years), EC improves hippocampal-dependent learning suggesting that EC consumption may be associated with increased memory function in age-related cognitive decline [15]. In a randomized, double-blind, placebo-controlled, two-by-two factorial trial, that took place from June 2015 through December 2020, aimed to evaluate the effect of cocoa extract supplementation and multivitamins on prevention of cardiovascular disease (CVD) and cancer among 21,442 U.S. adults free of major CVD and recently diagnosed cancer. The participants were randomly assigned to a cocoa extract supplement (500 mg/d flavanols, including 80 mg (–)-

epicatechins) or placebo. This large-scale study showed that cocoa extract supplementation reduced CVD death by 27%, and total number of major cardiovascular events (hearth attacks, strokes, and deaths by 24% [16].

We recently observed that HFD-fed obese mice show significantly increased anxiety-related behaviors which were mitigated by EC consumption (under revision). The capacity of EC to improve anxiety was in part explained by its capacity to modulate brain-derived neurotrophic factor (BDNF)- and glucocorticoids (GC)-regulated signaling and to mitigate HFD-induced dysbiosis. However, a full understanding of EC actions at the hippocampus is missing. Previous evidence showed that EC protects brain vascular endothelial cell integrity and reduces the risk of neurodegenerative conditions by exerting multi-genomic actions, modulating the expression of protein-coding genes as well as non-coding genes, particularly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) [17, 18].

The use of cutting edge untargeted genomic methodologies represents a significant breakthrough in nutrigenomics, as these methods enable detailed insights into the involved molecular mechanisms. Moreover, the implementation of multi-omics approaches allows integration of different levels of regulation of cellular functions and to obtain a comprehensive understanding of the molecular mechanisms of action of polyphenols. miRNAs are small noncoding RNA molecules, in average 22 nucleotides long, that act as post-transcriptional regulators of gene expression, in most cases by degradation or inhibition of translation of their target mRNAs. miRNAs regulate a wide spectrum of biological processes and are therefore involved in the physiopathology of diseases, including age-related and neurodegenerative diseases [19]. LncRNAs are single strand RNAs with over 200 nucleotides in length. Although they do not directly encode proteins, LncRNAs are key regulators of expression of genes by interacting with DNA, RNA and proteins, also by modulating chromatin function, altering the

stability and translation of cytoplasmic mRNAs as well as sequestering miRNAs and subsequently reducing the number of miRNAs available for their target mRNAs [20]. LncRNAs can consequently impact many cellular functions [21]. LncRNAs play an important role in the pathophysiology of diseases, such as cardiovascular [22], cardiometabolic [23], and neurodegenerative diseases [24]. The expressions of lncRNAs can be affected by diet. For example, in mice fed a HFD, changes in the expression of 52 lncRNAs are proposed to play a significant role in diabetes mellitus [25]. Recent studies also showed that polyphenols can affect the expressions of non-coding genes [17, 18, 26]. Taken together, integrated multi-genomic analysis coupled with bioinformatics represent an insightful approach to obtain detailed information about underlying molecular mechanisms of action of dietary components.

In the present work, we investigated the molecular mechanisms underlying the neuroprotective effects of EC against HFD- and obesity-induced anxiety behaviors in mice. For this purpose, we evaluated EC-mediated multi-genomic modifications, including changes in the expression of protein-coding and non-coding genes in the hippocampus. We characterized affected molecular pathways and key regulators to better understand the neuroprotective actions of EC.

2. Material and methods

2.1. Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were administered under the auspices of the Animal Resource Services of the University of California, Davis. Experimental protocols were approved before implementation by the University of California, Davis Animal Use and Care Administrative Advisory Committee. Healthy 8 weeks

old male C57BL/6J mice (20–22 g) were fed for 24 weeks either: a diet containing 10% total calories from fat (C group) (TD.06416, Envigo, Indianapolis, IN), a diet containing 45% total calories from lard fat (HF group) (TD.06415, Envigo, Indianapolis, IN), and the HFD supplemented with 20 mg EC/kg body weight (HFE group). The EC-containing diet was prepared every two weeks to account for changes in body weight and food intake, and to prevent potential EC degradation. All diets were stored at −20 °C until use. Body weight and food intake were measured weekly throughout the study. After 24 weeks on the dietary treatments, and after 4 h fasting, mice were euthanized by cervical dislocation. Blood was collected from the submandibular vein into tubes containing EDTA, and plasma collected after centrifugation at 3,000 x g for 10 min at room temperature. Hippocampi were dissected and flash frozen in liquid nitrogen and then stored at −80 °C for further analysis.

2.2. RNA extraction from the hippocampus

Total RNA was extracted from the right hippocampus from three animals per experimental group (C, HF, HFE) using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), and small residual amounts of DNA were removed using an on-column RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted RNA was assessed by NanoDrop One (Thermo Scientific, Waltham, MA), and integrity by 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quality and integrity assessments showed that A260/A280 ratios were 2 or higher and the RNA integrity number (RIN) was 8 or higher for all samples.

2.3. Microarray hybridization and transcriptome analysis

Clariom D mouse assays (Affymetrix, Santa Clara, CA) were used for transcriptomics analysis. These arrays provide the most intricate transcriptome-wide gene-level expression analysis, including the ability to detect protein coding and non-coding RNAs such as miRNAs, lncRNAs, snoRNAs and others. Total RNA (200 ng per sample) for 9 RNA samples (3 samples per group) was used to prepare cRNA (15 µg) and then single-stranded complementary DNA (sscDNA) (5.5 µg) using GeneChip WT PLUS Reagent Kit (Thermo Scientific, Waltham, MA). Purified sscDNA (5.5 µg) was fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues and labeled by terminal deoxynucleotidyl transferase (TdT) using the DNA Labeling Reagent that is covalently linked to biotin. Fragmented and labeled sscDNA samples were submitted to the UC Davis Comprehensive Cancer Center's Genomics Shared Resource (GSR) core for hybridization, washing, staining, and scanning using the GeneChip Hybridization, Wash, and Stain Kit (Thermo Scientific, Waltham, MA) following the manufacturer's instruction. Hybridization of fragmented and labeled sscDNA samples was done using GeneChip Hybridization oven, and the arrays were washed then stained using GeneChip Fluidics Station. The arrays were scanned using GeneChipTM Scanner 3000 7G (Thermo Fisher Scientific, Santa Clara, CA). Quality control of the microarrays was performed using the Affymetrix Transcriptome Analysis Console (TAC) 4.0.2. (Thermo Fisher Scientific, Santa Clara, CA).

2.4. Bioinformatic analysis

2.4.1. Comparisons of gene expression profiles

The Transcriptome Analysis Console (TAC) software was used to determine differential gene expression of HF vs. C and HFE vs. HF groups using the SST-RMA normalization method and to create volcano plots. All genes from microarray with $p < 0.05$ and ±1.1-fold change was

considered as differentially expressed. Partial Least-Squares Discriminant Analysis (PLS-DA) plot was built using MetaboAnalyst (https://www.metaboanalyst.ca; [27]), and heatmap using Hmisc package in R (https://www.r-project.org/). Gene types of the differentially expressed genes (mRNA, miRNA, and lncRNA) were identified using ShinyGO v0.66 (http://bioinformatics.sdstate.edu/go; [28]). Steps of bioinformatic analyses are presented as a flow chart in **Figure 1**.

2.4.2. Database-predicted miRNA and lncRNA targets

Validated target genes of the identified miRNAs were searched with miRWalk (http://mirwalk.umm.uni-heidelberg.de/; [29]). Network-based visualization of miRNA-gene target enrichment was performed with MIENTURNET (http://userver.bio.uniroma1.it/apps/mienturnet/; [30]). Target genes of the lncRNAs were identified using LncRRIsearch that enables retrieval of lncRNA-gene target interactions (http://rtools.cbrc.jp/LncRRIsearch/; [31]).

2.4.3. Pathway enrichment analysis

The pathways involving differentially expressed genes and targets of significantly regulated miRNAs and lncRNAs from HF vs. C and HFE vs. HF comparisons were obtained using Genetrail2. Over-representation analysis was used to identify statistically significant pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and WikiPathways databases. Additionally, top 30 pathways identified by KEGG database search were added to the list of pathway enrichment analysis. Histograms were generated using Microsoft Excel and Venn diagrams by Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/).

Interaction networks were constructed with Cytoscape software, version 3.7.2. (https://cytoscape.org; [32]).

2.4.4. Transcription factor analysis

Potential transcription factors which activity could be modulated by polyphenols were identified with bioinformatic tool Enrichr (https://amp.pharm.mssm.edu/Enrichr/) [33, 34]. TRRUST [35] and TRANSFAC [36] databases were used to search for the potential transcription factors.

2.4.5. Docking analysis

Potential binding interactions between identified transcription factors, their regulatory cell signaling proteins, and major circulatory EC metabolites were examined by molecular docking using the SwissDock docking analysis tool (http://www.swissdock.ch/docking). Protein 3D structures were obtained from UniProt Data Bank (https://www.uniprot.org) and chemical structures of metabolites from PubChem database (https://pubchem.ncbi.nlm.nih.gov).

2.4.6. Associated and correlated diseases

The association of identified differentially expressed genes with human diseases was analyzed using the Comparative Toxicogenomics Database (https:// ctdbase.org/) [37]. To explore correlations between the identified genes in the study and genes associated with mood disorder, we first searched the GEO (https://www. ncbi.nlm.nih.gov/gds) for suitable dataset of gene expression profiles in human with mood disorder. The available raw datasets were further analyzed with GEO2R. Pearson's correlation coefficients between gene expression profiles in patients with anxiety disorder and genes which expression had been identified as modulated by EC was calculated using R (https://www.r-project.org/).

3. Results

3.1. HFD modulates the expression of protein-coding and non-coding genes in the hippocampus.

To assess the molecular mechanisms involved in HFD-induced behavioral changes (under revision), the effects of the HFD on the global expression of genes in the hippocampus was evaluated comparing data from the HF and the C groups. Gene expression analysis showed that the HFD caused significant changes in global hippocampal gene expression. There were 10,778 probes identified as differentially expressed. Volcano plot of the significantly up- or down-regulated genes in the hippocampus of HFD-fed mice showed the upregulation of 3295 probes and downregulation of 7483 probes compared to C mice **(Supplemental Fig. 1A)**. Among them, 6510 genes were identified and classified by ShinyGO v0.66, 47% corresponded to protein-coding genes (3030 mRNAs), 10% to miRNAs (629 miRNAs), and 5% to lncRNAs (353 lncRNAs) **(Supplemental Figure 1B)**.

Gene expression analysis demonstrated that consumption of the HFD induced significant changes in the expression of 3030 hippocampal protein-coding genes. The fold-change values of protein-coding genes varied from -3.26 to 1.97 with an average fold-change of -1.29 for the downregulated genes and 1.24 for the upregulated genes. To understand the biological functions of identified genes, the differentially expressed mRNAs were used to perform enrichment analysis and obtain pathways related to the protein-coding genes. The analysis showed that they are involved in different biological processes such as neurofunction-related pathways, which include pathways involved in Alzheimer's disease, dopaminergic synapse, GABAergic synapse, glutamatergic synapse, and neurodegeneration. Genes identified in these pathways include *Atf4, Calm4, Calm5, Drd1, Gnb2, Gng7, Nos1*, and *Th*. The bioinformatic analysis also revealed that

the differentially expressed protein-coding genes can impact inflammation-related pathways (chemokine signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, NF-κB signaling pathway, TNFα signaling pathway, Toll-like receptor signaling pathway), cell-cell adhesion (adherens junction, focal adhesion, gap junction, PI3K-Akt signaling pathway, tight junction), cell signaling pathways (insulin signaling pathway, MAPK signaling pathway, mTOR signaling pathway, PPAR signaling pathway), metabolic pathways (N-glycan biosynthesis, nuclear receptors involved in lipid metabolism and toxicity, TCA cycle), and other cellular processes (apoptosis, endocytosis, oxidative phosphorylation, regulation of autophagy, ubiquitin mediated proteolysis) **(Supplemental Fig. 1C)**.

The expression of 629 miRNAs was also modulated upon consumption of the HFD. The fold-change values of miRNAs varied from -2.27 to 2.61 with an average fold-change of -1.38 for the downregulated genes and 1.32 for the upregulated genes. The next step of our analysis was to identify target genes of observed differentially expressed miRNAs. MIENTURNET and miRWalk identified 1745 target genes of 68 differentially expressed miRNAs. Networks between differentially expressed miRNAs and their target genes shows a complex interconnectivity **(Supplemental Fig. 1D).** Next, we performed enrichment analysis and obtained pathways in which identified targets of differentially expressed miRNAs are involved in. Pathway enrichment analysis showed that several functional pathways are modulated by the miRNAs target genes, being the most over-represented pathways those involved in axon guidance, neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, cytokine-cytokine receptor interaction, focal adhesion, PI3K-Akt signaling, apelin signaling, foxO signaling, mitogen-activated protein kinases (MAPK) signaling, apoptosis, cellular senescence, endocytosis, and non-odorant GPCRs (**Supplementary Fig. 1E**). Genes identified to

be involved in these pathways include *Akt1, blc2, Ccr9, Cx3cr1, Gabrb2, Gsk-3β, Il1r1, Il18r1, L1cam, Mapk11, Nr3c1, Plcβ1, Sod2*, and *Stat1*.

In addition, consumption of the HFD induced changes in expression of 353 lncRNAs. The fold-change values of lncRNA non-coding genes varied from -1.74 to 1.63 with an average fold-change of -1.26 for the downregulated genes and 1.25 for the upregulated genes. Subsequently, using the differentially expressed lncRNAs identified in the microarray study, we retrieved 2588 target genes of the lncRNAs from LncRRIsearch database. Pathway enrichment analysis of the target genes showed that they can also regulate pathways involved in various biological processes. The most over-represented pathways include neuroactive ligand-receptor interaction, neurodegeneration, cytokine-cytokine receptor interaction, calcium signaling, focal adhesion, PI3K-Akt signaling, regulation of actin cytoskeleton, cAMP signaling, MAPK signaling, endocytosis, and non-odorant GPCRs **(Supplementary Fig. 1F)**. Key genes identified in these pathways include *C5ar1, Calm1, Camk2α, Ccr5, ccr9, Cxcr11, Egfr, Gabra2, Gsk-3β, Igfr1, Mapk10, Mc1r, Nos1*, and *Xcr1*.

There were 43 common genes (**Supplementary table 1**) among the differentially expressed mRNAs, target genes of the differentially expressed miRNAs, and target genes of differentially expressed lncRNAs **(Figure 2A)**. Among the common genes identified are *Stat1, Crp, Tyw3, Gmeb1, Hist4h4, Ceacam20, Ceacam1, Nav2, Ntrk3, Ncan, Ccr9, Neurod2, Cd300a, Zmiz1, Atf7, Rab11b, Sema6a, Wbp1l,* and *Tcf7l2*. Comparison of pathways identified for differentially expressed mRNAs, miRNAs target genes, and lncRNA target genes identified 54 common pathways. These pathways are related to neurofunction (Alzheimer's disease, axon guidance, cholinergic synapse, dopaminergic synapse, glutamatergic synapse, pathways of neurodegeneration), inflammation (chemokine signaling pathway, cytokine-cytokine receptor interaction, Jak-STAT signaling pathway, TNFα signaling pathway, Toll-like signaling

pathway), cell-cell adhesion (adherens junction, focal adhesion, PI3K-Akt signaling pathway, Rap1 signaling pathway, Ras signaling pathway, regulation of actin cytoskeleton), cell signaling (insulin signaling pathway, MAPK signaling pathway, mTOR signaling pathway, Wnt signaling pathway), metabolism (purine metabolism, sphingolipid metabolism), and other cellular processes (apoptosis, endocytosis, non-odorant GPCRs, spliceosome, type II diabetes mellitus) **(Figure 2B).**

3.2. EC modulates the expression of genes in the hippocampus of HFD-fed mice.

A comparison of gene expression profiles in the hippocampus of mice fed the control diet, HFD, and HFD supplemented with 20 mg EC/kg body weight, showed a distinct separation among the three groups, as evaluated by PLS-DA (**Figure 3A**). Gene expression analysis also showed that EC supplementation caused significant changes in hippocampal gene expression in mice fed the HFD (HFE vs. HF groups). There were 5085 probes identified as differentially expressed, and of these, 3927 probes were identified as upregulated and 1158 as downregulated. Among them, 2635 genes were identified and classified by ShinyGO v0.66, 38% corresponded to protein-coding genes (1001 mRNAs), 9% to miRNAs (241 miRNAs), and 6% to lncRNAs (167 lncRNAs) (**Figure 3B**).

3.2.1 EC modulates the expression of protein-coding genes in the hippocampus of HFD-fed mice.

Gene expression analysis showed that EC supplementation caused significant changes in expressions of 1001 hippocampal protein-coding genes. The fold-change values of protein-coding genes varied from -1.57 to 18.27, with 4 above fold change of 2, and with an average fold-change of -1.23 for the downregulated genes and 1.33 for the upregulated genes.

Functionality analysis revealed that the differentially expressed mRNAs mainly regulate processes involved in neurofunction (alcoholism, Alzheimer's disease, amyotrophic lateral sclerosis, neuroactive ligand-receptor interaction, neurodegeneration), inflammation (chemokine signaling pathway, complement and coagulation cascade, cytokine-cytokine receptor interaction, NOD-like receptor signaling pathway), cell-cell adhesion (focal adhesion-PI3K-Akt-mTOR signaling pathway), cell signaling (foxO signaling pathway), and other cellular processes (non-odorant GPCRs, phagosome) (**Figure 4)**. Genes involved in the pathways include *Calm5, Ccl2, Ccr4, Drd1, Gabrr1, Gngt2, Irs1, Kras, Slc2a4, Stat1,* and *Psma2.*

3.2.2 EC modulates the expression of miRNAs in the hippocampus of HFD-fed mice.

Modulation of hippocampal miRNA expression was also identified upon EC supplementation. A total of 241 miRNAs were differentially expressed with a fold-change range of -1.49 to 2.41 and an average fold-change of -1.25 for the downregulated genes and 1.38 for the upregulated genes. MIENTURNET and miRWalk database analysis revealed that among the differentially expressed 241 miRNAs, 30 miRNAs have mRNA targets. The analysis further identified a total of 1177 target genes of differentially expressed 30 miRNAs. Networks between differentially expressed miRNAs and their target genes forms a complex interconnectivity **(Figure 5A).** Pathway enrichment analysis of the identified target genes of differentially expressed miRNAs shows that the target genes are involved in the regulation of various processes including Alzheimer's disease, axon guidance, neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, interleukin-3 signaling, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, calcium signaling, focal adhesion, PI3K-Akt signaling, Rap1 signaling, Ras signaling, foxO signaling, MAPK signaling, Wnt signaling,

cellular senescence, endocytosis, and non-odorant GPCRs (**Figure 5B**). Modulated target genes include *Blc2, Ccr9, Cd4, Cnr1,Cx3cr1, Gabra2, Gsk-3β, Il3, Insr, L1cam, Mapk1, Mapk11, Mylk, Nr3c1, Plcβ1, Rac1, Stat1,* and *Tacr1.*

3.2.3 EC modulates expression of lncRNAs in mouse hippocampus

In addition, EC supplementation of HFD-fed mice caused changes in expression of 167 lncRNAs. The fold-change values of lncRNAs varied from -1.60 to 2.06 with an average fold-change of -1.27 for the downregulated genes and 1.26 for the upregulated genes. LncRRIsearch database analysis retrieved 1481 lncRNA target genes from the identified differentially expressed lncRNAs. Pathway enrichment analysis of the target genes reveals that the most over-represented pathways include neuroactive ligand-receptor interaction, neurodegeneration, chemokine signaling, cytokine-cytokine receptor interaction, calcium signaling, focal adhesion, PI3K-Akt signaling, regulation of actin cytoskeleton, cAMP signaling, MAPK signaling, and non-odorant GPCRs **(Figure 6)**. The target genes identified for these pathways include Atp2b2, Bace1, Cacna1c, *Ccr4, Ccr5,* Creb5, *Cx3cr1, Grin2a Gsk-3β, Lpl, Igfr1, Mapk10, Mylk4, Nos1*, Ryr3, *Tacr1, Taok2, Taok3,* and *Tnr.*

3.2.4 Transcription factors affected by EC and their interactions with EC metabolites

Following identification of differentially expressed protein-coding genes, we used bioinformatic tools to identify potential transcription factors, which could be involved in the regulation of our identified genes and therefore could be affected by EC **(Figure 7A)**. Seven significant transcription factors were identified, NR5A2, RBPJ, GATA4, RARα, FLI1, HNF4α and SREBF1. Our next step was to assess if major circulating EC metabolites could interact with these transcription factors and modify their activities, by performing in-silico 3d docking

analysis between the proteins and the metabolites. Using such approach, we observed that (−)-epicatechin-7-*O*-β-D-glucuronide (E7G) presents a high potential binding capacity to RARα transcription factor (-10.1 kcal/mol) **(Figure 7B)**. We also observed that (−)-epicatechin-3′-sulfate (E3′S) also presents binding capacity to RARα (-8.8 kcal/mol). Moreover, the docking analyses revealed that E3′S and (−)-epicatechin-5-*O*-β-D-glucuronide (E5G) could bind to HNF4α transcription factor, with binding capacity of -7.9 kcal/mol and -9.3 kcal/mol, respectively **(Figure 7B**). Taken together, these bioinformatic analyses allowed identification of potential transcription factors involved in the genomic modifications induced by EC in the hippocampus by interaction with major EC metabolites found in mice.

3.2.5 Integration of multi-genomic modifications by EC in the hippocampus

The next step of our analysis was to integrate data obtained from different genomic analysis. Comparison between differentially expressed protein-coding genes, targets of differentially expressed miRNAs and of differentially expressed lncRNAs identified 5 genes (Zbtb34, Ncan, Ado, Asb13, Eif2ak2) in common **(Figure 8A)**. Also, 28 of differentially expressed protein-coding genes were in common with targets of differentially expressed miRNAs and 26 were identified in common with targets of differentially expressed lncRNAs. These observations suggest that expression levels of the mRNAs of these genes can be affected by the capacity of EC metabolites to regulate the expression of protein non-coding genes. Because EC could affect the activities of transcription factors which will result in changes in expression of genes and non-coding RNAs that can interact with mRNAs, we aimed to build a network of interactions between identified differentially expressed genes, gene targets, and potential transcription factors **(Figure 8B)**. This analysis allowed us to observe global

interactivities between the studied types of RNAs and potential transcription factors, showing complex, multi-omic mode of action of EC in mice hippocampus *in vivo*.

Together with the comparison of identified differentially expressed genes and target genes of non-coding RNAs, we also compared the pathways identified as enriched in the 3 omics analyses. We observed that 18 pathways were identified to be regulated by all three types of RNAs which included pathways related to neurodegenerative diseases, like Alzheimer's disease, pathways regulating endothelial cell functions like focal adhesion, or pathways related to inflammation and cell signaling transduction **(Figure 8C)**. Moreover, 3 pathways were in common between protein-coding genes and targets of lncRNAs and 40 pathways in common between targets of miRNAs and of lncRNAs. The identified pathways were involved in cellular functions including cell signaling pathways, neuronal cell function, and cellular metabolism. This suggests that these common pathways can be affected simultaneously by mRNAs and targets of miRNAs and lncRNAs. An example presented is a pathway related to neurodegeneration where we identified 15 differentially expressed genes, 24 targets of miRNAs, and 17 as targets of lncRNAs **(Figure 8D)**.

To further investigate the observed interactions between different omic effects, we also combined pathways identified for each omic analysis and grouped them into functional groups **(Figure 8E)**. We observed that these pathways are involved in the regulation of neurological functions and diseases, inflammation, cell-cell adhesion, cell signaling, and metabolic pathways. This suggests that, by regulating the expression of different types of RNAs, EC can affect pathways in the hippocampus regulating these cellular functions. For each functional pathway group, we identified differentially expressed genes and target genes involved in each pathway with the aim to construct a network. Example networks of neurofunction-related and cell-cell adhesion functional groups show that pathways and involved genes within each functional group

are interconnected **(Figure 8E)**. This demonstrates that regulation of different types of RNAs can exert multitudinous effects on cellular functions.

3.3 Comparison of the effect of HFD (HF vs. C) and EC (HFE vs. HF)

The expression profile of genes identified in the EC supplemented group was compared to the expression profile identified as differentially expressed by the HFD group. The heatmap analysis showed that the expressions of genes obtained following the EC supplementation have the opposite expression profile when compared to the gene expression profile obtained after consumption of the HFD **(Figure 9A)**. This implies that most of the genes identified as upregulated by the consumption of the HFD were identified as downregulated by the EC consumption and vice versa. This finding suggests that EC consumption can reverse HFD-induced changes of protein-coding and non-coding gene expression profiles in the hippocampus. There was a total of 270 protein-coding genes in common that are differentially expressed by consumption of the HFD and EC. Expression profiles of the common protein-coding genes also demonstrate that EC consumption can reverse the effect of the HFD-induced changes in the protein-coding gene expressions in the hippocampus (**Figure 9B**). In agreement with the finding, the fold changes of the differentially expressed genes induced by the EC consumption show strong inverse correlation with the ones differentially expressed by consumption of the HFD (**Figure 9C).**

The pathway enrichment analysis indicated that 36 pathways were identified to be commonly modulated by both EC and HFD consumption (**Figure 10A** and **B)**. Consistent with the findings observed in the heatmap **(Figure 9A and B)**, expressions of select genes obtained upon EC supplementation show the opposite expression profile when compared to the ones obtained following consumption of the HFD. For example, *Drd1* and *Gngt2* genes implicated in

the pathway related to neuronal consequences of alcoholism were downregulated in the hippocampus of mice fed the HFD while their levels were restored by EC supplementation (**Figure 6D**). Similarly, a couple of genes implicated in the Alzheimer's disease pathway showed opposite expression profiles. *Psme2b* (26S proteasome), *Irs1,* and *Tubb4b* were upregulated and *Calm5* downregulated in the HFD group while they all show opposite expression profiles upon EC supplementation (**Figure 6E**). In the insulin signaling pathway, the HFD downregulated the expression of *Slc2a4* and upregulated the expression of *Irs1* in the hippocampus while EC reversed these effects (**Figure 6F**).

3.4. Association of the genomic data with human diseases

Next, we aimed to identify associations of the highlighted genes in the present study with known human diseases. The Comparative Toxicogenomics Database, a database that interrelates differentially expressed genes with diseases, was used to understand the potential roles of the identified genes in prevention or development of human diseases. Using this approach, we found that genes identified as differentially expressed by the HFD, compared to control diet, are shown to be involved in neurological diseases, such as nervous system malformations, neurodegenerative diseases, and peripheral nervous system diseases. Genes that were identified as modulated by EC on the HFD were also associated with diseases related to neurodegeneration, such as brain injuries, cerebrovascular disorders, intracranial arterial diseases, movement disorders or Parkinson's disease. Comparison of the diseases identified in the 2 groups revealed 4 neurological conditions in common which include nervous system diseases, brain diseases, central nervous system diseases, and neurologic manifestations **(Figure 11A)**.

As cognitive analyses performed on these mice identified anxiolytic effect of EC (under revision), we next aimed to assess how the global gene expression profile in the hippocampus

induced by EC is correlated with anxiety in humans. Correlation analysis of gene expression changes obtained in the hippocampus following EC supplementation and genomic signatures identified in patients with anxiety disorder indicated a significant inverse relationship ($r = -0.15$; $p < 0.05$) **(Figure 11B)**. This suggests that EC-induced changes in hippocampal gene expressions may explain its neuroprotective actions against anxiety-related behavior, an observation that is in accordance with our previous findings for the same animal set (under revision).

4. Discussion

HFD and associated obesity can contribute to cognitive and mood dysfunction [38]. We previously reported that chronic consumption of a HFD induced anxiety-related behavior in mice which was mitigated by EC supplementation [39]. To better understand the mechanisms underlying the anxiolytic effects of EC, the present study conducted multi-genomic analysis that included analyses of mRNA, miRNA, and lncRNA in the hippocampus of HFD-fed obese mice. Both HFD consumption and EC supplementation induced significant changes in the expression of protein-coding and non-coding genes. For the first time, the capacity of EC to regulate the expression of hippocampal protein-coding and non-coding transcripts in a preclinical model of obesity was investigated. We observed a significant inverse relationship between hippocampal gene expression profiles of HFD-fed and EC-supplemented mice. This suggests that EC can counteract changes driven by consumption of the HFD and/or associated obesity in the hippocampus.

Several studies reported that EC can exert beneficial effects by triggering complex multi-omic modifications. Consumption of a cocoa flavanols drink (containing 64 mg EC) for one month improved vascular function in healthy middle-aged volunteers compared to control subjects [40]. This improvement was accompanied by significant changes in whole blood cell

expression profiles of genes involved in the regulation of inflammation, cell adhesion, and chemotaxis of immune cells [41]. Similarly, in a 4-week randomized, double-blind, placebo-controlled crossover trial, EC supplementation (100 mg/day) downregulated groups of genes involved in inflammation, PPAR signaling, and adipogenesis in PBMCs from prehypertensive subjects aged between 30 and 80 [42]. This change in gene expression profiles was accompanied by EC-mediated decrease in plasma insulin and improvement in insulin resistance. Therefore, our study is in line with the capacity of epicatechin to modulate the expression of genes and by counteracting the global gene expression profile induced by HFD, is suggestive of preventing dysfunctions and diseases development.

Our pathway enrichment analysis revealed that differentially expressed mRNAs and targets of differently expressed miRNAs and lncRNAs upon EC supplementation are involved in different biological processes regulating neurofunction, inflammation, and cell-cell adhesion, metabolism, and cell signaling pathways. Interestingly, these transcripts were particularly enriched in the pathways regulating BBB permeability, neurofunction, inflammation, and neurodegeneration in the hippocampus. Recently, it has been shown that a western-type diet given to mice induces not only increased BBB permeability but also decreased cognitive function [43], phenotypic changes found to be associated with significant changes in the expression of protein-coding as well as non-coding RNAs in hippocampal microvasculature of male [44] and female mice [45]. On the other hand, studies have suggested the capacity of EC to mitigate BBB dysfunction and protect from neurodegeneration via multi-omic regulation. For instance, gut microbiome-derived EC metabolites, 5-(4’-hydroxyphenyl)-γ-valerolactone-3’-sulfate and 5-(4’-hydroxyphenyl)-γ-valerolactone-3’-O-glucuronide, exerted neuroprotective action in TNF-α-stimulated human brain microvascular endothelial cell by modulating the expressions of mRNAs, miRNAs, lncRNAs, and proteins involved in the regulation of cell adhesion,

cytoskeleton organization, focal adhesion, and interaction with immune cells [18]. Similarly, in brain microvascular endothelial cells stimulated by lipid stress, a model of BBB dysfunction, structurally related EC metabolites (SREM) and gut microbiome-derived EC metabolites simultaneously modulated the expression of mRNAs, miRNAs, and lncRNAs involved in VEGF signaling, cell adhesion, and permeability [17]. An inverse correlation was identified between gene expression profiles of the lipid-stressed cells and cells exposed to EC metabolites mixtures, suggesting the capacity of EC to counteract the adverse effects of lipotoxic stress on gene expressions [17].

We also identified 36 common enriched pathways comparing HF vs. C and HFE vs. HF, and several genes involved in pathways modulated by HFD consumption that were inversely modulated by EC supplementation. This supports the capacity of EC supplementation to reverse the effect of HFD on gene expressions. In the pathway related to neuronal consequences of alcoholism, *Drd1* and *Gngt2* are downregulated following the consumption of the HFD while both were upregulated by EC supplementation. *Drd1* encodes the D1 subtype of the dopamine receptor. Interestingly, studies report robust antidepressant-like effects upon D1R activation [46]. For instance, levo-stepholidine (l-SPD), an antipsychotic medication, has antidepressant effects on rats with depressive- and anxiety-like behaviors. The antidepressant effect was attributed to activation of D1R that leads to activation of downstream PKA/mTOR signaling pathway, which in turn increases synaptic proteins and enhances synaptic plasticity. Similarly, EC-induced restoration of *Drd1* may in part explain the observed anxiolytic effects of EC in HFD-fed mice. Although a direct relevance of *Gngt2*, a member of Gi/o family, in anxiety has not been established, it has been reported that Gi/o levels decrease with aging, particularly in age-vulnerable brain regions like the hippocampus [47]. Similarly, several genes implicated in the Alzheimer's disease pathway showed opposite expression profiles. *Psme2b* (26S proteasome),

Irs1, and *Tubb4b* were upregulated and *Calm5* downregulated in the hippocampus of HFD group while they all showed opposite expression profiles upon EC supplementation (**Figure 6E**). It has been shown that the proteosome [48] and IRS1 [49] play important role in the development of Alzheimer's disease. In the insulin signaling pathway, consumption of the HFD downregulated hippocampal gene expression of *Slc2a4,* which encodes GLUT4, an insulin-regulated glucose transporter that plays an important role in glucose uptake and utilization in the brain [50]. Reduced hippocampal *Slc2a4* expression has been proposed to be responsible for inducing anxiety-like behavior in diabetic rats [51]. The capacity of EC to reverse HFD-induced downregulation of *Slc2a4* also may in part explain its capacity to protect HFD-fed mice from anxiety. Interestingly, the HFD upregulated the expression of *Irs1* in the hippocampus while EC downregulated its expression. It is possible that the observed downregulation is due to the mitigation of HFD-induced increase in plasma insulin levels upon EC supplementation observed in this set of animals (under revision). Consistent with our result, EC-mediated improvements of plasma insulin were observed in HFD-fed mice [52, 53]. Similarly, downregulation of select genes involved in the insulin signaling cascade was found in immune cells of prehypertensive adults upon EC supplementation [42].

In addition to the modulation of protein-coding genes, we observed that EC modulated the expression of non-coding RNAs, including miRNAs and lncRNAs. Studies have shown that several miRNAs are modulated in patients with neurodegenerative diseases suggesting that non-coding RNAs also play a role in the pathogenesis of neurodegeneration [19]. Indeed, miRNAs such as *miR-15b, miR-127-3p, miR-let-7f-5p, miR-124, miR-219, miR-342-3p,* and *miR-455-3p* are involved in the development of Alzheimer's diseases [19]. Similarly, several miRNAs, including *miR-34a, miR-16, miR-34c, miR-182, and miR-124*, are associated with the pathogenesis as well as treatment of depression and anxiety [54-58]. For instance, targeted deletion of the *miR-34*-family

resulted in increased resilience to stress-induced anxiety in mice [55]. Similarly, chronic corticosterone administration induced depressive behavior and increased *miR-34a* levels in the hippocampus while *miR-34a* downregulation exerted antidepressant-like effects [56]. Among the miRNAs identified as differentially expressed by EC, *miR-467* has been reported to promote inflammation and insulin resistance in mice [59]. EC supplementation downregulated *miR-467* expression in the hippocampus of HFD-fed mice which may partly explain the beneficial effect of EC. Moreover, *miR-669*, which plays a protective role in ischemic stroke in mice [60], was increased by EC. EC also modulated lncRNA expressions. Although the role of many lncRNAs in regulating biological functions are unknown, studies have suggested that lncRNAs are involved in the pathogenesis of Parkinson's disease [61], Alzheimer's [62], and depression [63]. Interestingly, a study reported that expression of the lncRNA Gm15628, which we identified as modulated by EC, was altered in the brain of mice following a stroke [64]. Taken together, our findings suggest that mRNAs, miRNAs and for the first time lncRNAs, may contribute to the neuroprotective properties of EC in the hippocampus.

In-silico docking analysis suggests that certain EC metabolites could bind to some of the identified transcription factors, potentially affecting their activity and the expressions of related genes. Among the identified transcription factors, RARα demonstrated energetically favorable binding to (-)-epicatechin-7-O-glucuronide (E7G) and (-)-epicatechin-3′-sulfate (E3'S), and HNF4α to E3'S and (-)-epicatechin-5-O-β-D-glucuronide (E5G). RARα modulates several pathways crucial for synaptic plasticity [65], which when disrupted increase susceptibility to depression [66]. It has been proposed that RARα has anti-inflammatory effects and can promote clearance of amyloid beta (Aβ) [67]. HNF4α also plays a critical role in emotional behaviors. A TNFα inhibitor, infliximab, was effective in ameliorating depressive symptoms in patients, especially in those with a high inflammatory condition [68]. Genomic studies proposed that HNF4α

anti-depressive effects are linked to the regulation of gluconeogenesis, lipid homeostasis, and serotonin metabolism [69, 70]. Moreover, it has been shown that binding of drugs to this transcription factors can impact its activity and consequently modulate cell functions [71]. Similarly, several studies also identified several molecules presenting high affinity for HNF4α affecting its activity [72]. Therefore, it could be suggested that epicatechin metabolites by interacting with these proteins will modulate their activities, resulting in changes in the expression of identified genes and exert their health properties.

In conclusion, our multi-genomic data including transcriptomic, microRNomic, and lncRNomic of the hippocampus of EC supplemented HFD-fed mice revealed that EC can exert neuroprotective effects by regulating pathways that modulate BBB permeability, neurofunction, inflammation, and neurodegeneration in the hippocampus. HFD and associated obesity can be detrimental to the brain, eliciting BBB dysfunction and neuroinflammation, and consequently cognitive impairment and mood disorders. Our results indicate that EC can counteract HFD-induced alterations of hippocampal gene expression profiles. Additionally, we observed that gene expression profiles upon EC supplementation are negatively correlated with gene expression profiles associated with symptoms of anxiety disorder in humans. This suggests that EC could elicit neuroprotective action and reduce the risk of HFD-induced or obesity-associated mood disorders. The present study does not establish a direct causal link between the observed changes in gene expression levels of protein-coding and non-coding genes and alteration in cognitions and mood. However, the bioinformatic analyses provide valuable insights and biological networks possibly affected by consumption of EC. Although the causal relationship was not investigated, protein-coding genes, miRNAs, lncRNAs, and transcription factors that have been described in neurofunction were identified. Further studies elucidating the direct role

of the identified transcripts and proteins in mood and cognition upon EC consumption are warranted.

Figure legends.

Figure 1. A flow-chart describing the steps implemented in the genomic and bioinformatic analysis. The hippocampus (n = 3/group) was isolated from control, HFD, and HFE groups for microarray analysis. For the differentially expressed mRNAs, functional pathways analysis was conducted followed by identification of transcription factors and docking analysis. Next, target genes of the differentially expressed miRNAs and lncRNAs were identified. Subsequently, network and functional analysis were performed with the identified targets. Integrating them together, network analysis of mRNAs, miRNAs, lncRNAs, target genes, and transcription factors was conducted. Differentially expressed genes and pathways in the hippocampus of the HFD-fed and EC supplemented animals were also compared. Lastly, the identified genomic profile upon EC supplementation was compared with genomic signatures identified in patients with generalized anxiety disorder.

Figure 2. Functional pathway analysis of differentially expressed genes in the hippocampus of HFD-fed mice compared to controls. A) Venn diagram of the differentially expressed mRNAs and target genes of miRNAs and lncRNAs identified from comparing HFD-fed mice to control diet-fed mice. B) Histogram of over-represented pathways obtained from differentially expressed protein-coding genes, target genes of differentially expressed miRNAs and target genes of differentially expressed lncRNAs in the hippocampus. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

Figure 3. Global genomic modifications induced by EC supplementation in the hippocampus of HFD-fed mice. A) 3D Partial least squares discriminant analysis (PLS-DA) plot of the genomic profiles obtained in samples from 3 experimental groups: control, HFD, and HFD supplemented with 20 mg EC/kg body weight. B) Volcano plot representing the distribution of differentially expressed genes, mapping the up- (red) and down-regulated (green) genes in the hippocampus of EC supplemented and HFD-fed mice when compared to the genes obtained from the hippocampus of HFD-fed mice. C) Pie chart of types of RNAs that are identified as differentially expressed.

Figure 4. Histogram of significantly over-represented pathways identified from differentially expressed protein-coding genes in the hippocampus of HFD-fed mice supplemented with EC. Pathways were identified using differentially expressed genes in the hippocampus from the EC supplemented mice compared to the hippocampus from HFD-fed mice. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

Figure 5. Functional analysis of differentially expressed miRNAs in the hippocampus of HFD-fed mice upon EC supplementation. A) Network presentation of differentially expressed miRNAs (blue circles) and their potential target genes (yellow circles). B) Histogram of subset of significant gene pathways of the identified miRNA target genes. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

Figure 6. Functional analysis of differentially expressed lncRNAs in the hippocampus of HFD-fed mice upon EC supplementation. A) Network presentation of differentially expressed lncRNAs (purple rectangles) and their potential target genes (blue circles). B) Histogram of subset of significant gene pathways of the identified lncRNA target genes. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

Figure 7. Transcription factors involved in the observed genomic modifications and their potential interactions with EC metabolites. A) List of identified potential transcription factors involved in the observed global genomic modifications upon EC supplementation. B) In-silico docking analysis of interactions between EC metabolites and transcription factors. RARα demonstrated energetically favorable binding to (-)-epicatechin-7-O-glucuronide (E7G) and (-)-epicatechin-3′-sulfate (E3'S), and HNF4α to E3'S and (-)-epicatechin-5-O-β -D-glucuronide (E5G).

Figure 8. Integrative analyses of multi-genomic data and networks of genes present in pathways of functional groups. A) Venn diagram of the differentially expressed mRNAs, target genes of miRNAs, and target genes of lncRNAs identified from comparing EC supplemented HFD-fed mice to the HFD-fed mice. B) Integrative network showing interactions of differentially expressed protein-coding genes, transcription factors, miRNAs and lncRNAs. Gray rectangles = lncRNAs; Red rectangles = transcription factors; Green rectangles = miRNAs; White circles = differentially expressed mRNAs, and miRNA and lncRNA targets. C) Venn diagram comparison of common pathways obtained from differentially expressed protein-coding genes, target genes of differentially expressed miRNAs and target genes of differentially

expressed lncRNAs. D) A representative integrated analysis of differentially expressed genes and target genes of differentially expressed miRNAs and lncRNAs indicated in a pathway related to neurodegeneration. Blue = differentially expressed genes; Yellow = target genes of differentially expressed miRNAs, Green = target genes of differentially expressed lncRNAs. E) Histogram of subset of significant gene pathways of differentially expressed protein-coding genes, targets of differentially expressed miRNAs and lncRNAs, with networks of pathways and associated genes related to specific cellular processes. Pathway and gene interaction networks implicated in neurofunction-related and cell-cell adhesion functional group show the interconnections between enriched pathways and differentially expressed protein-coding genes and non-coding target genes. The most enriched top 30 pathways were identified using KEGG (*). Additional KEGG (**), Biocarta (***), and WikiPathway (****) pathways were identified using the Genetrial2 online database.

Figure 9. Comparison of genomic modifications induced by the HFD and by EC supplementation in the hippocampus. A) Heatmap analyses comparing the global gene expression profiles obtained from HF vs. C and HFE vs. HF comparisons. B) Venn diagram comparison of differentially expressed protein-coding genes induced by the HFD when compared to control diet (HF vs. C) and by EC supplementation when compared to non-supplemented HFD (HFE vs. HF). C) Correlation plot of differentially expressed protein-coding genes identified from HF vs. C and HFE vs. HF comparisons showing a significant inverse relationship ($r = -0.89$, $p < 2.2x10^{-16}$).

Figure 10. Comparison of enriched pathways and protein-coding genes modulated by the HFD and by EC supplementation in the hippocampus. A) Venn diagram and lists of the

common enriched pathways identified in HF vs. C and HFE vs. HF comparison. Opposite gene expression profiles indicated in pathways associated with B) alcoholism, C) Alzheimer's diseases, and D) insulin signaling.

Figure 11. Association of identified differentially expressed genes with human neurological disorders. A) Comparison of neurological disorders in humans associated with differentially expressed genes following HFD and following EC diet. B) Correlation analysis between gene expression changes obtained in the hippocampus following EC supplementation and genomic signatures identified in patients with anxiety disorder. The correlation plot indicates a significant inverse relationship ($r = -0.15$; $p < 0.05$).

Figure 12. Summary of functional analysis of multi-genomic data. EC can modulate the expression of protein-coding and non-coding genes that are involved in various processes such as pathways regulating BBB permeability, neuroinflammation, neurodegeneration, and neuronal function which may in part explain the neuroprotective capacity of EC.

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Conflict of Interest

Authors have no conflict of interest.

The authors' roles

J.K. and D.M. performed bioinformatic analyses and wrote the manuscript. J.K., P.O. and D.M. designed the research; had primary responsibility for final content; participated in the interpretation of the data and editing of the manuscript. All authors read and approved the final manuscript.

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Figure 1

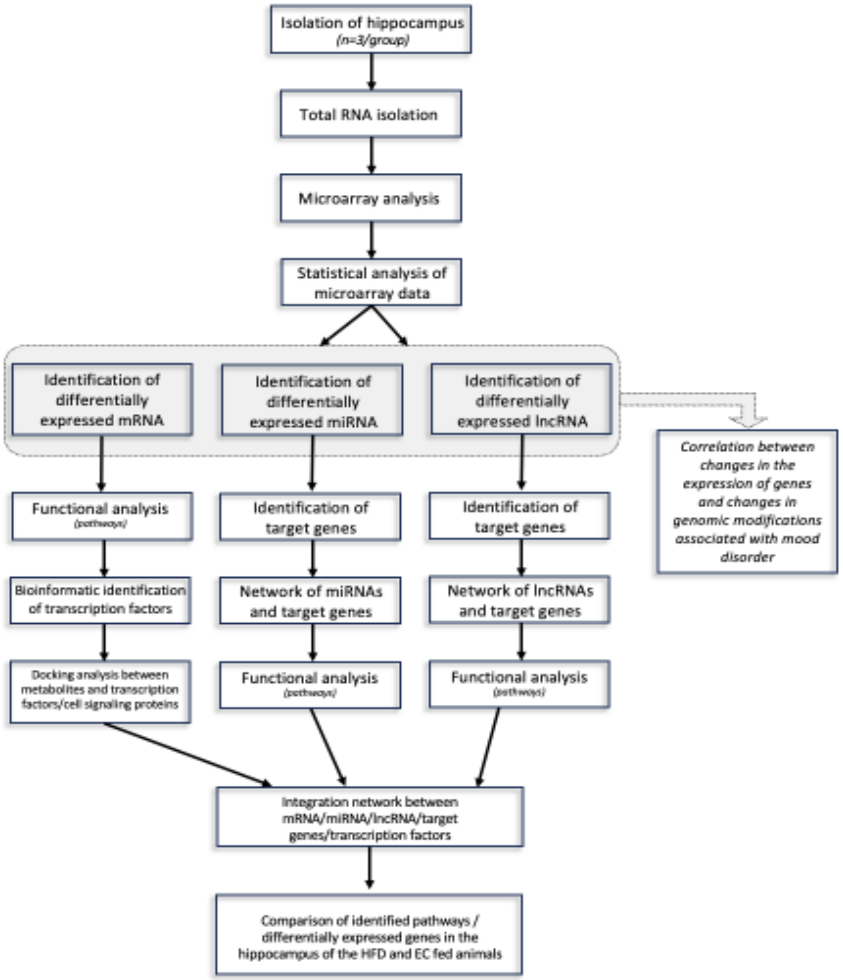
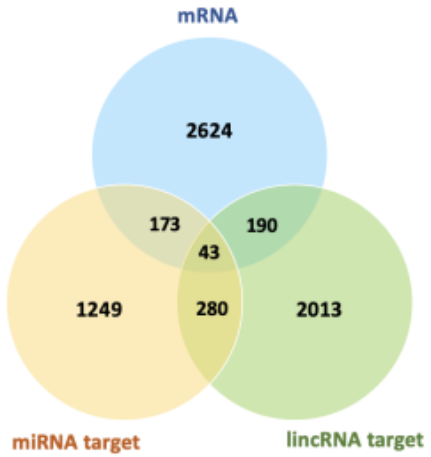

Isolation of hippocampus
(n=3/group)
Total RNA isolation
Microarray analysis
Statistical analysis of microarray data
Identification of differentially expressed mRNA
Identification of differentially expressed miRNA
Identification of differentially expressed lncRNA
Correlation between changes in the expression of genes and changes in genomic modifications associated with mood disorder
Functional analysis
(pathways)
Identification of target genes
Identification of target genes
Bioinformatic identification of transcription factors
Network of miRNAs and target genes
Network of lncRNAs and target genes
Docking analysis between metabolites and transcription factors/cell signaling proteins
Functional analysis
(pathways)
Functional analysis
(pathways)
Integration network between mRNA/miRNA/lncRNA/target genes/transcription factors
Comparison of identified pathways / differentially expressed genes in the hippocampus of the HFD and EC fed animals

Figure 2

A


mRNA
2624
173
190
43
1249
280
2013
miRNA target
lincRNA target

B

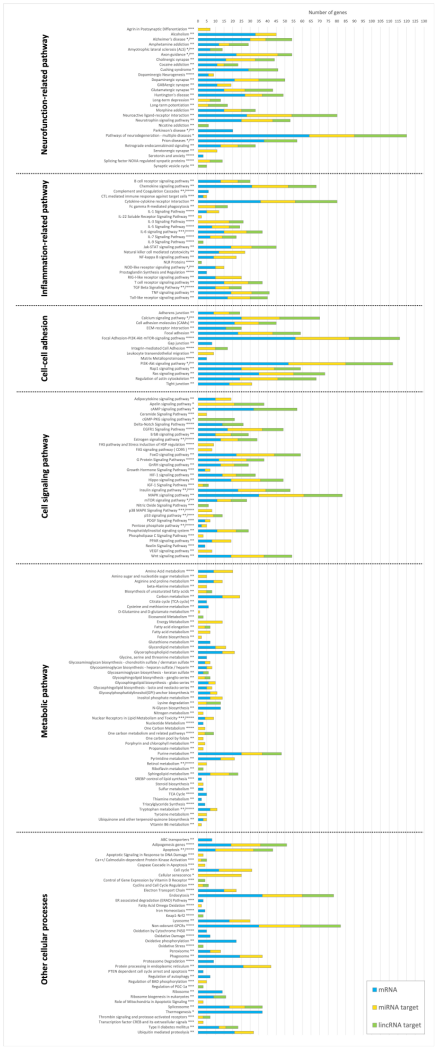
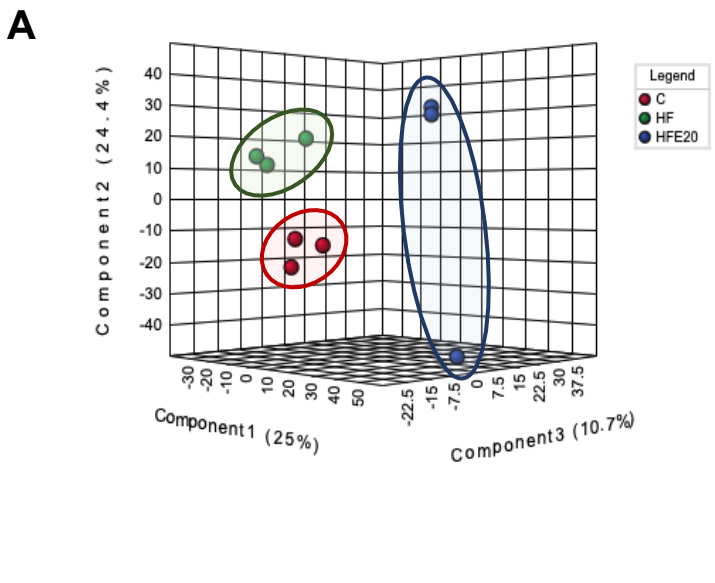

Number of genes
Neurofunction-related pathway
Inflammation-related pathway
Cell-cell adhesion
Cell signaling pathway
Metabolic pathway
Other cellular processes
mRNA
miRNA target
lincRNA target

Figure 3


A
Component2 (24.4%)
40
30
20
10
0
-10
-20
-30
-40
-30 -20 -10 0 10 20 30 40 50
Component1 (25%)
-22.5 -15 -7.5 0 7.5 15 22.5 30 37.5
Component3 (10.7%)
Legend
C
HF
HFE20

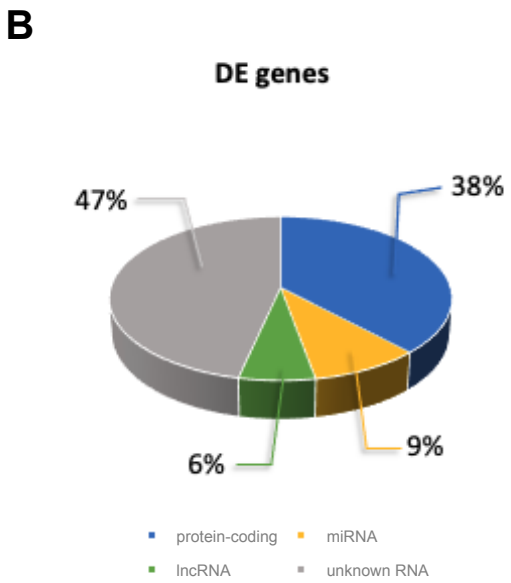

B
DE genes
47%
38%
6%
9%
protein-coding
miRNA
lncRNA
unknown RNA

Figure 4

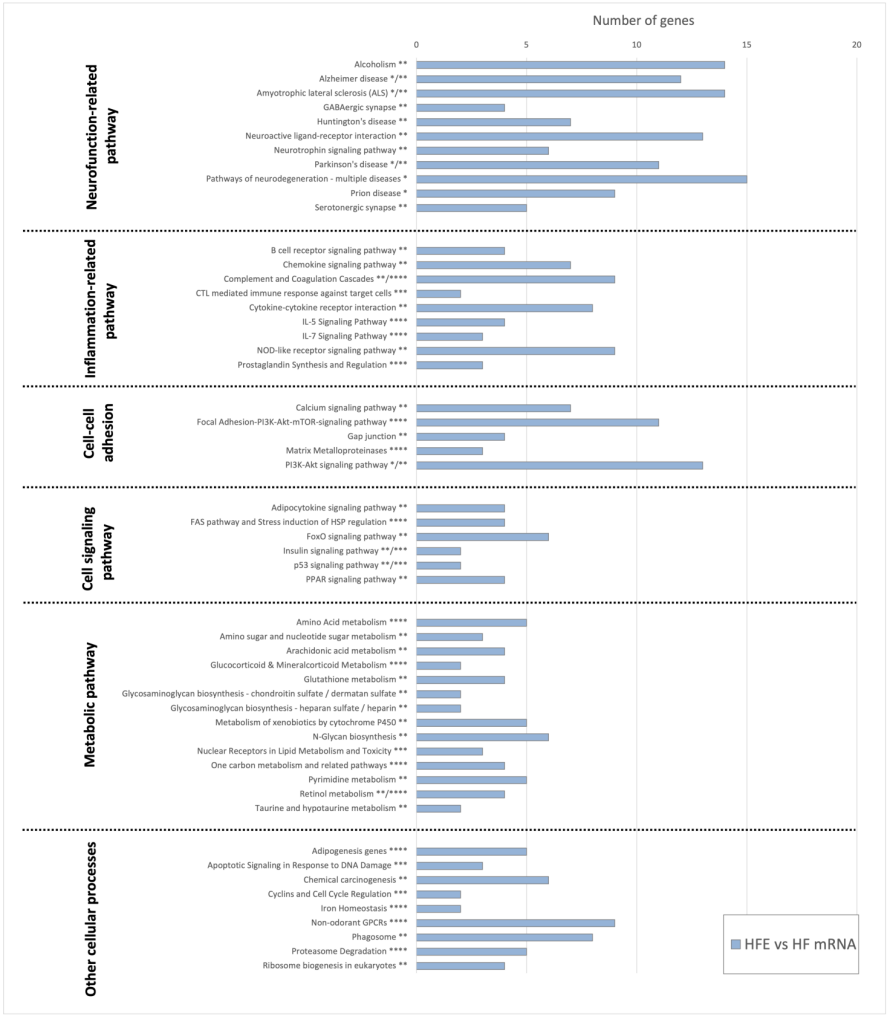
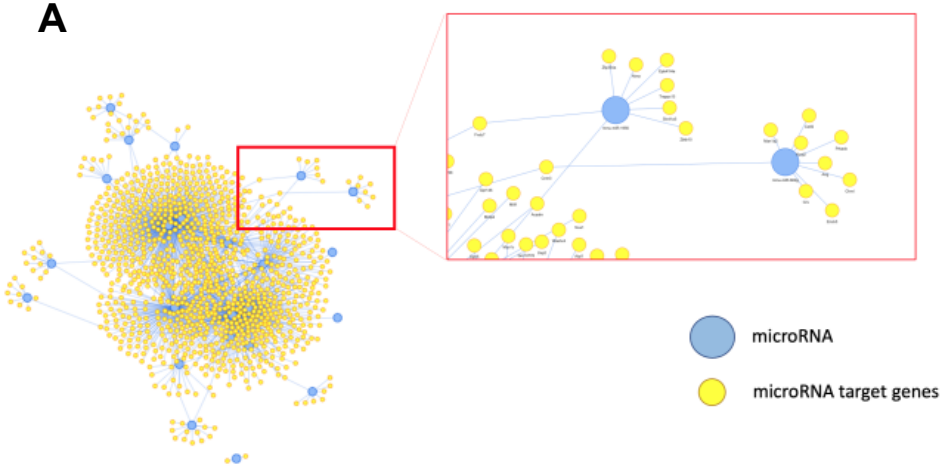

Number of genes
0
5
10
15
20
Neurofunction-related pathway
Alcoholism **
Alzheimer disease */**
Amyotrophic lateral sclerosis (ALS) */**
GABAergic synapse **
Huntington's disease **
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Parkinson's disease */**
Pathways of neurodegeneration - multiple diseases *
Prion disease *
Serotonergic synapse **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Complement and Coagulation Cascades **/****
CTL mediated immune response against target cells ***
Cytokine-cytokine receptor interaction **
IL-5 Signaling Pathway ****
IL-7 Signaling Pathway ****
NOD-like receptor signaling pathway **
Prostaglandin Synthesis and Regulation ****
Cell-cell adhesion
Calcium signaling pathway **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
Gap junction **
Matrix Metalloproteinases ****
PI3K-Akt signaling pathway */**
Cell signaling pathway
Adipocytokine signaling pathway **
FAS pathway and Stress induction of HSP regulation ****
FoxO signaling pathway **
Insulin signaling pathway **/***
p53 signaling pathway **/***
PPAR signaling pathway **
Metabolic pathway
Amino Acid metabolism ****
Amino sugar and nucleotide sugar metabolism **
Arachidonic acid metabolism **
Glucocorticoid & Mineralcorticoid Metabolism ****
Glutathione metabolism **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
Metabolism of xenobiotics by cytochrome P450 **
N-Glycan biosynthesis **
Nuclear Receptors in Lipid Metabolism and Toxicity ***
One carbon metabolism and related pathways ****
Pyrimidine metabolism **
Retinol metabolism **/****
Taurine and hypotaurine metabolism **
Other cellular processes
Adipogenesis genes ****
Apoptotic Signaling in Response to DNA Damage ***
Chemical carcinogenesis **
Cyclins and Cell Cycle Regulation ***
Iron Homeostasis ****
Non-odorant GPCRs ****
Phagosome **
Proteasome Degradation ****
Ribosome biogenesis in eukaryotes **
HFE vs HF mRNA

Figure 5


A
microRNA
microRNA target genes

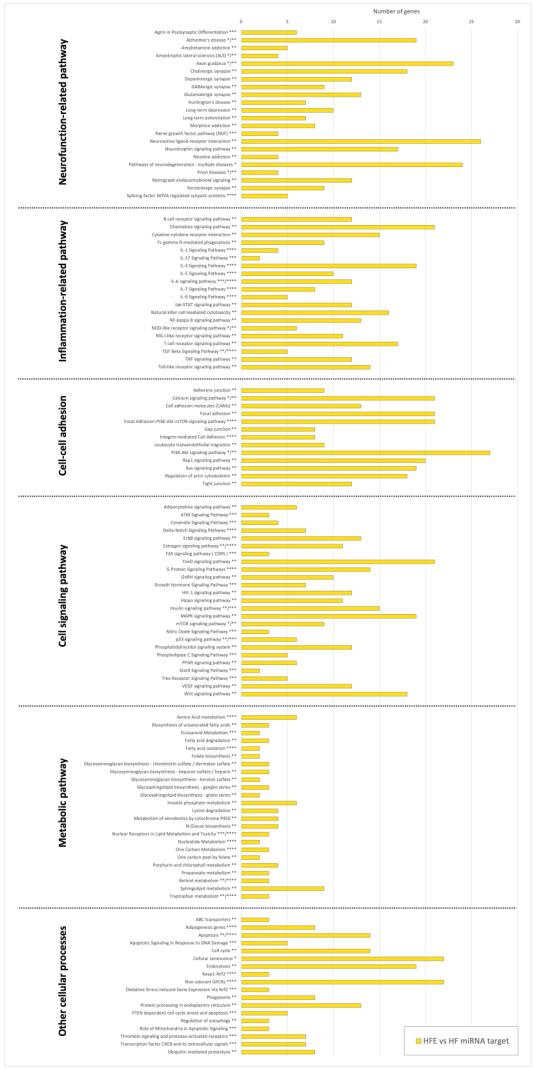

B
Number of genes
Neurofunction-related pathway
Inflammation-related pathway
Cell-cell adhesion
Cell signaling pathway
Metabolic pathway
Other cellular processes
HFE vs HF miRNA target

Figure 6

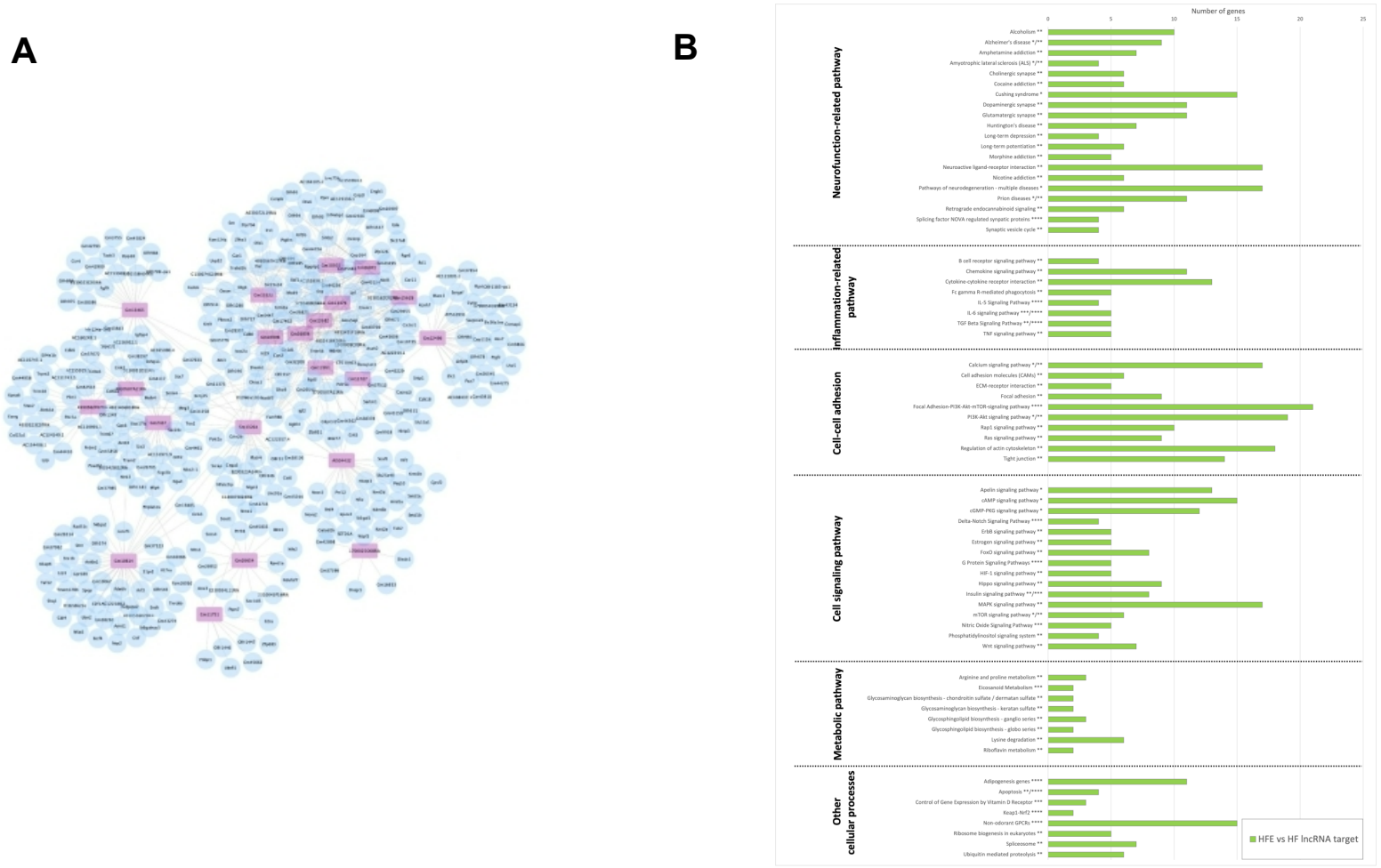

A
B
Number of genes
0
5
10
15
20
25
Neurofunction-related pathway
Alcoholism **
Alzheimer's disease */**
Amphetamine addiction **
Amyotrophic lateral sclerosis (ALS) */**
Cholinergic synapse **
Cocaine addiction **
Cushing syndrome *
Dopaminergic synapse **
Glutamatergic synapse **
Huntington's disease **
Long-term depression **
Long-term potentiation **
Morphine addiction **
Neuroactive ligand-receptor interaction **
Nicotine addiction **
Pathways of neurodegeneration - multiple diseases *
Prion diseases */**
Retrograde endocannabinoid signaling **
Splicing factor NOVA regulated synaptic proteins ****
Synaptic vesicle cycle **
Inflammation-related pathway
B cell receptor signaling pathway **
Chemokine signaling pathway **
Cytokine-cytokine receptor interaction **
Fc gamma R-mediated phagocytosis **
IL-5 Signaling Pathway ***
IL-6 signaling pathway ***/****
TGF Beta Signaling Pathway **/****
TNF signaling pathway **
Cell-cell adhesion
Calcium signaling pathway */**
Cell adhesion molecules (CAMs) **
ECM-receptor interaction **
Focal adhesion **
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
PI3K-Akt signaling pathway */**
Rap1 signaling pathway **
Ras signaling pathway **
Regulation of actin cytoskeleton **
Tight junction **
Cell signaling pathway
Apelin signaling pathway *
cAMP signaling pathway *
cGMP-PKG signaling pathway *
Delta-Notch Signaling Pathway ****
ErbB signaling pathway **
Estrogen signaling pathway **
FoxO signaling pathway **
G Protein Signaling Pathways ***
HIF-1 signaling pathway **
Hippo signaling pathway **
Insulin signaling pathway **/***
MAPK signaling pathway **
mTOR signaling pathway */**
Nitric Oxide Signaling Pathway ***
Phosphatidylinositol signaling system **
Wnt signaling pathway **
Metabolic pathway
Arginine and proline metabolism **
Eicosanoid Metabolism ***
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - keratan sulfate **
Glycosphingolipid biosynthesis - ganglio series **
Glycosphingolipid biosynthesis - globo series **
Lysine degradation **
Riboflavin metabolism **
Other cellular processes
Adipogenesis genes ****
Apoptosis **/****
Control of Gene Expression by Vitamin D Receptor ***
Keap1-Nrf2 ****
Non-odorant GPCRs ****
Ribosome biogenesis in eukaryotes **
Spliceosome **
Ubiquitin mediated proteolysis **
HFE vs HF lncRNA target

Figure 7

A

Transcription factor	Name
NR5A2	Nuclear receptor subfamily 5, group A, member 2
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
GATA4	GATA binding protein 4
RARα	Retinoic acid receptor, alpha
FLI1	Friend leukemia integration 1
HNF4α	Hepatic nuclear factor 4, alpha
SREBF1	Sterol regulatory element binding transcription factor 1
NKX2-5	NK2 Homeobox 5
SRF	Serum Response Factor
CBEPA	CCAAT Enhancer Binding Protein Alpha
TBP	TATA-binding *protein*
NFYB	Nuclear Transcription Factor Y Subunit Beta
CPEB1	Cytoplasmic Polyadenylation Element Binding *Protein* 1
SOX2	SRY-Box Transcription Factor 2
SP1	specificity *protein* 1
NFKB1	Nuclear Factor Kappa B
RELA	Nuclear Factor NF-Kappa-B P65 Subunit
TRP53	Tumor Protein P53

B

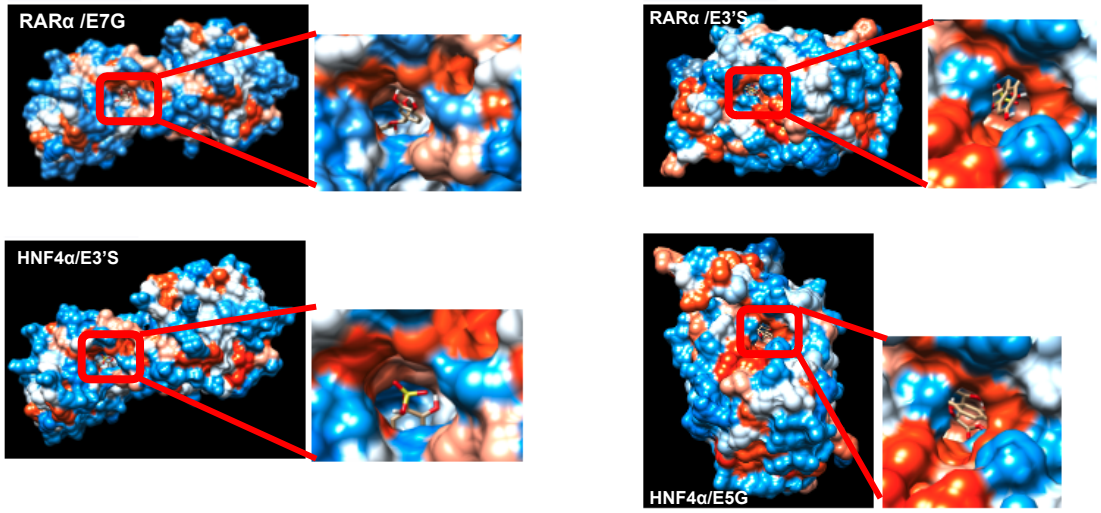


Figure 8

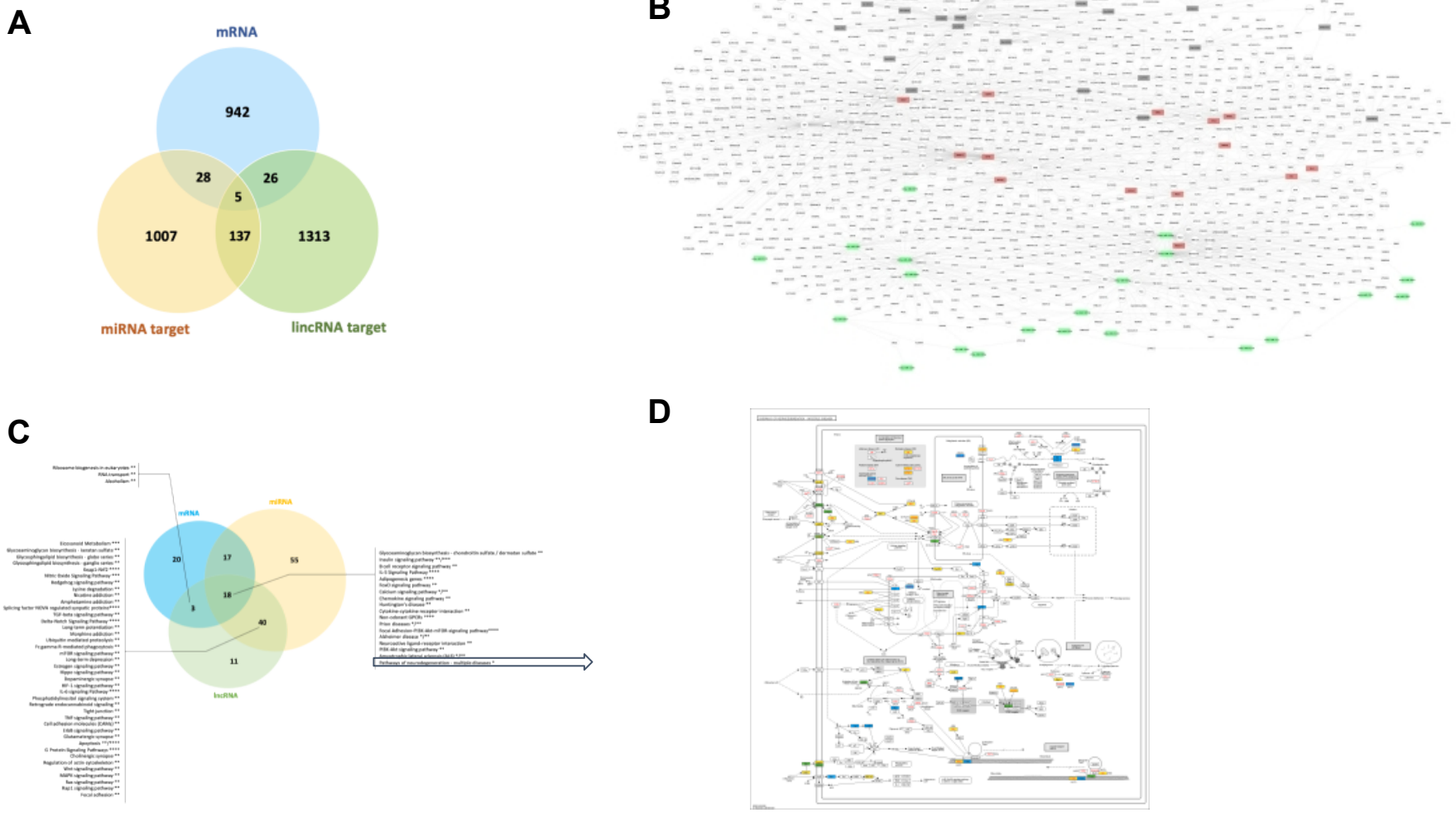

A
mRNA
942
28
26
5
1007
137
1313
miRNA target
lincRNA target
B
C
mRNA
miRNA
lncRNA
20
17
55
18
1
40
11
D

Figure 8

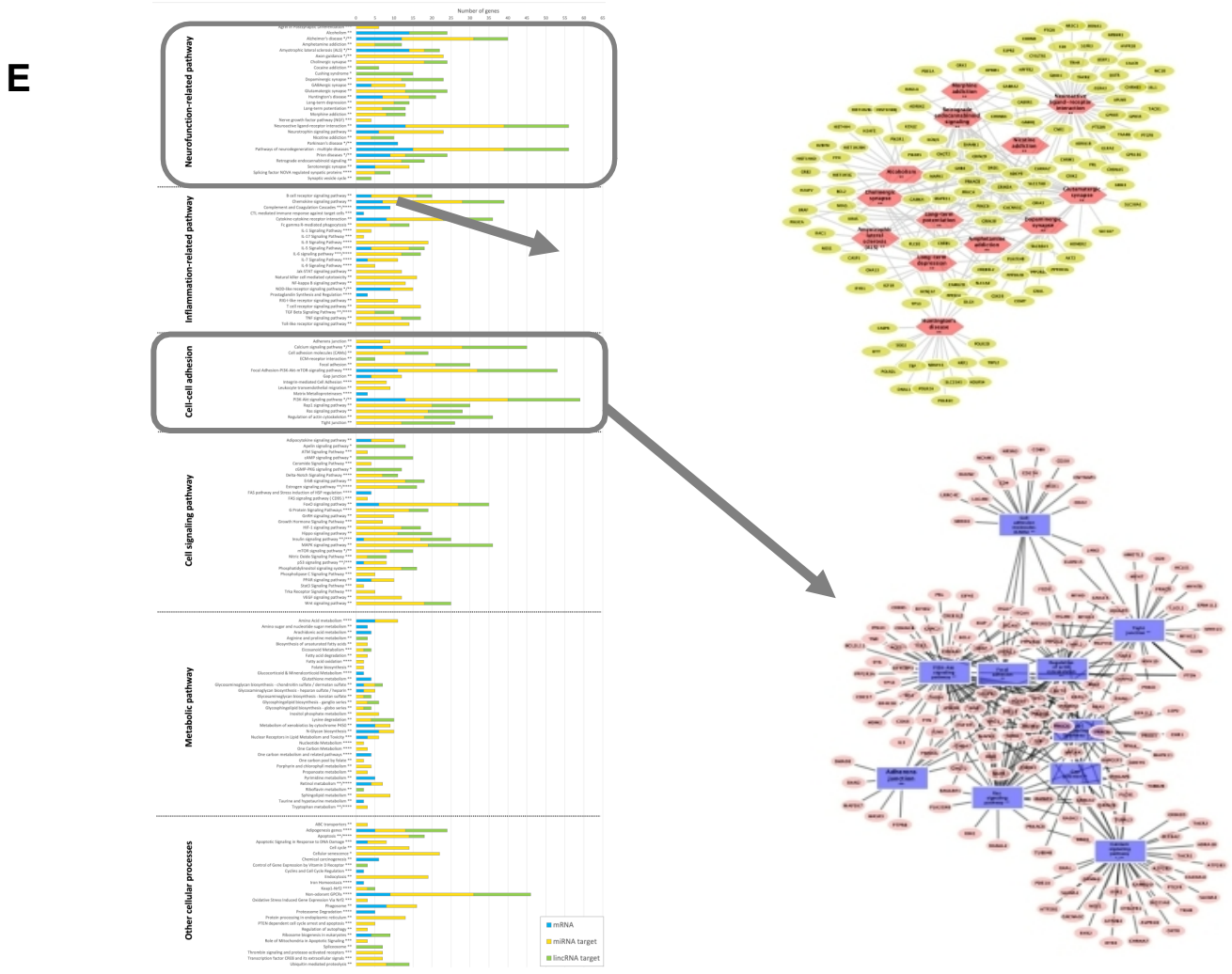

E
Number of genes
Neurofunction-related pathway
Inflammation-related pathway
Cell-cell adhesion
Cell signaling pathway
Metabolic pathway
Other cellular processes
mRNA
miRNA target
lincRNA target

Figure 9

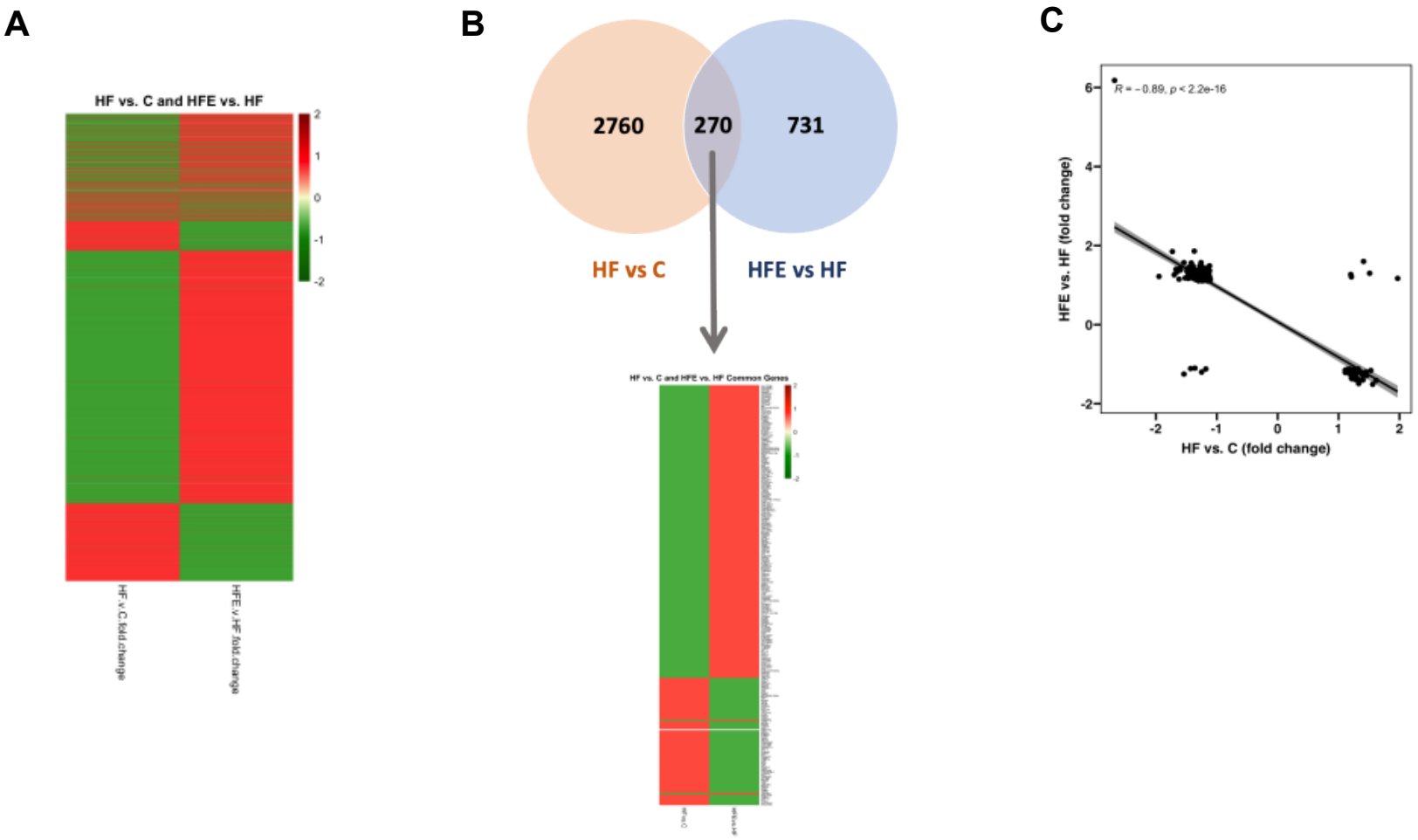
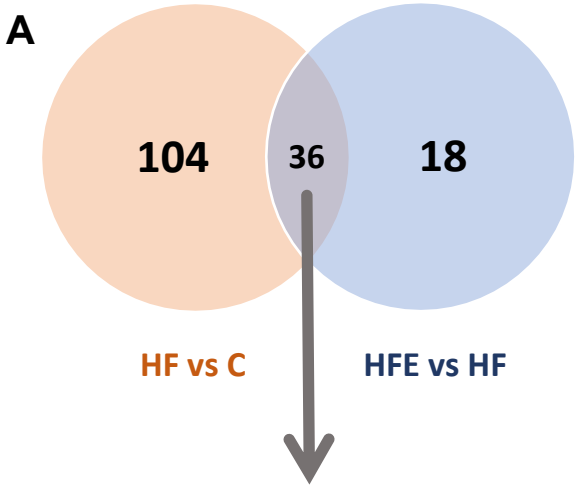
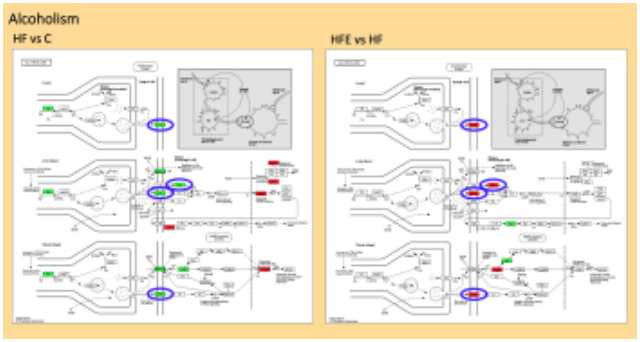
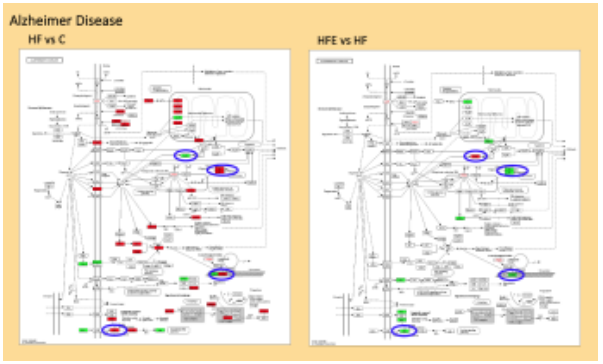
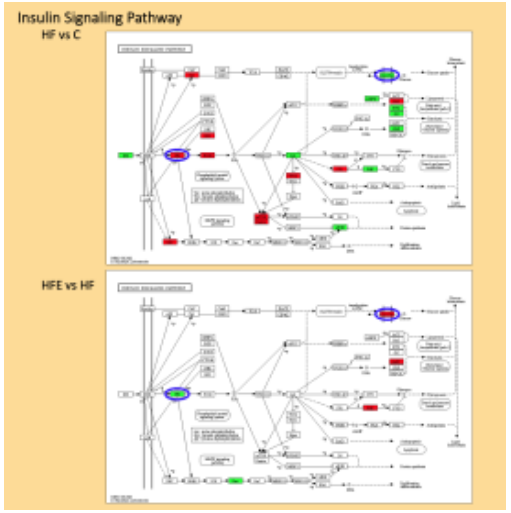

A
HF vs. C and HFE vs. HF
2
1
0
-1
-2
HF v.C fold change
HFE v.HF fold change
B
2760
270
731
HF vs C
HFE vs HF
HF vs. C and HFE vs. HF Common Genes
C
R = −0.89, p < 2.2e-16
HFE vs. HF (fold change)
HF vs. C (fold change)
6
4
2
0
-2
-2
-1
0
1
2

Figure 10









Alcoholism **
Alzheimer's disease */**
GABAergic synapse **
Huntington's disease **
Neuroactive ligand-receptor interaction **
Neurotrophin signaling pathway **
Pathways of neurodegeneration - multiple diseases *
Serotonergic synapse **
B cell receptor signaling pathway **
Chemokine signaling pathway **
CTL mediated immune response against target cells ***
Cytokine-cytokine receptor interaction **
IL-5 Signaling Pathway ****
IL-7 Signaling Pathway ****
NOD-like receptor signaling pathway */**
Calcium signaling pathway */**
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway ****
PI3K-Akt signaling pathway */**

Adipocytokine signaling pathway **
FAS pathway and Stress induction of HSP regulation ****
FoxO signaling pathway **
Insulin signaling pathway **/***
p53 signaling pathway **/***
PPAR signaling pathway **
Amino Acid metabolism ****
Amino sugar and nucleotide sugar metabolism **
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate **
Glycosaminoglycan biosynthesis - heparan sulfate / heparin **
One carbon metabolism and related pathways ****
Pyrimidine metabolism **
Retinol metabolism **/****
Adipogenesis genes ****
Apoptotic Signaling in Response to DNA Damage ***
Cyclins and Cell Cycle Regulation ***
Non-odorant GPCRs ****
Phagosome **

Figure 11

A

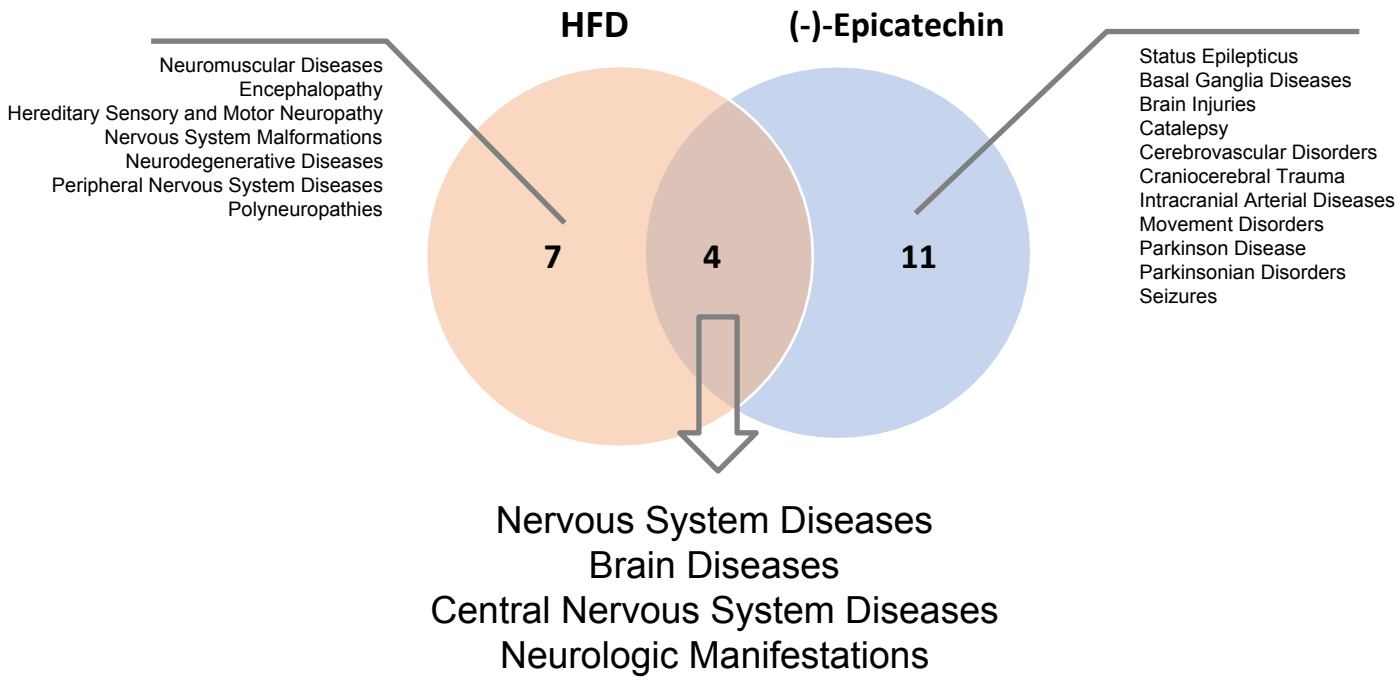

HFD
(-)-Epicatechin
Neuromuscular Diseases
Encephalopathy
Hereditary Sensory and Motor Neuropathy
Nervous System Malformations
Neurodegenerative Diseases
Peripheral Nervous System Diseases
Polyneuropathies
7
4
11
Status Epilepticus
Basal Ganglia Diseases
Brain Injuries
Catalepsy
Cerebrovascular Disorders
Craniocerebral Trauma
Intracranial Arterial Diseases
Movement Disorders
Parkinson Disease
Parkinsonian Disorders
Seizures
Nervous System Diseases
Brain Diseases
Central Nervous System Diseases
Neurologic Manifestations

Figure 11

B

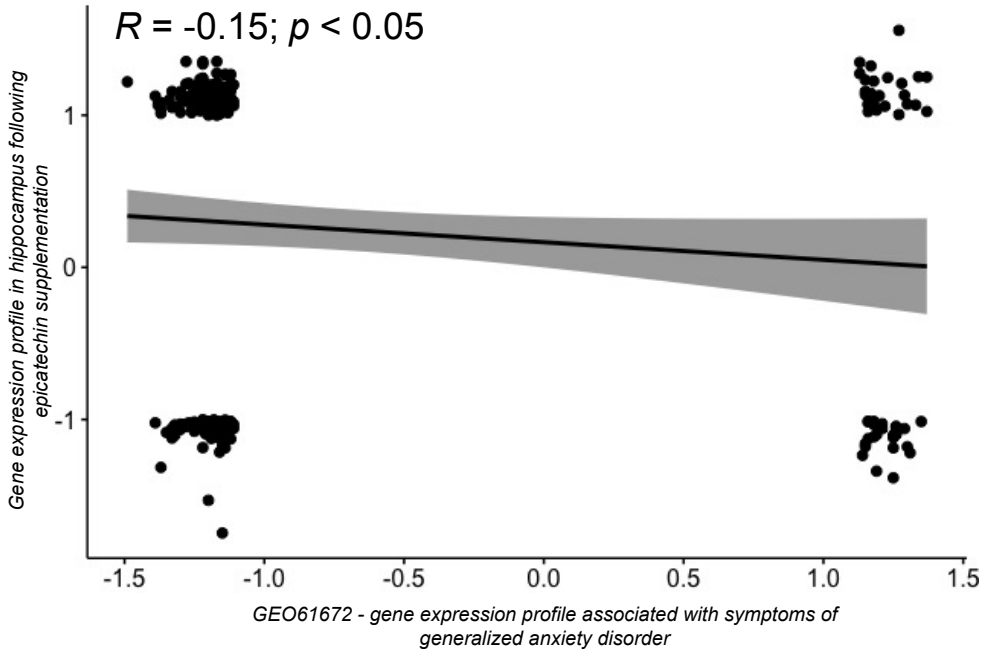
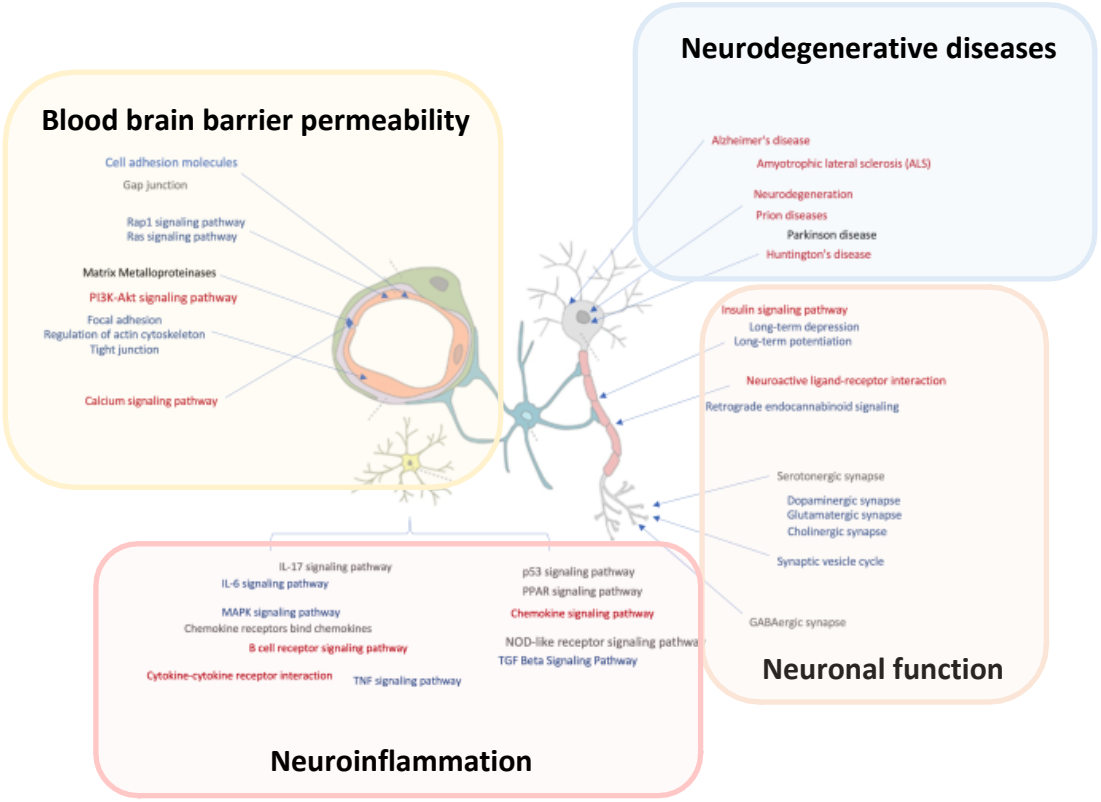

R = -0.15; p < 0.05
Gene expression profile in hippocampus following epicatechin supplementation
1
0
-1
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
GEO61672 - gene expression profile associated with symptoms of generalized anxiety disorder

Figure 12



Black: only RNA
Red: all 3
Blue: miRNA/lncRNA
Green: mRNA/lncRNA
Grey: mRNA/miRNA