Comprehensive Study of Advanced Literature

Course Code: BMB 519

Dept. of Biochemistry and Molecular Biology
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MS Level-1, Semester-1 Final Exam.
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Novel Genes In Brain Tissues Of EAE-induced Normal And Obese Mice: Upregulation Of Metal Ion-binding Protein Genes In Obese-EAE Mice

Neuroscience 2016 Dec 10; 343:322-336

Contents

1. Introduction

- Multiple Sclerosis (MS), autoimmune inflammatory disorder, result of demyelination of neurons of CNS.
- Transfer of immune cells into the CNS through the BBB.
- The inflammatory cells interact with astrocytes and microglia and induce demyelination.

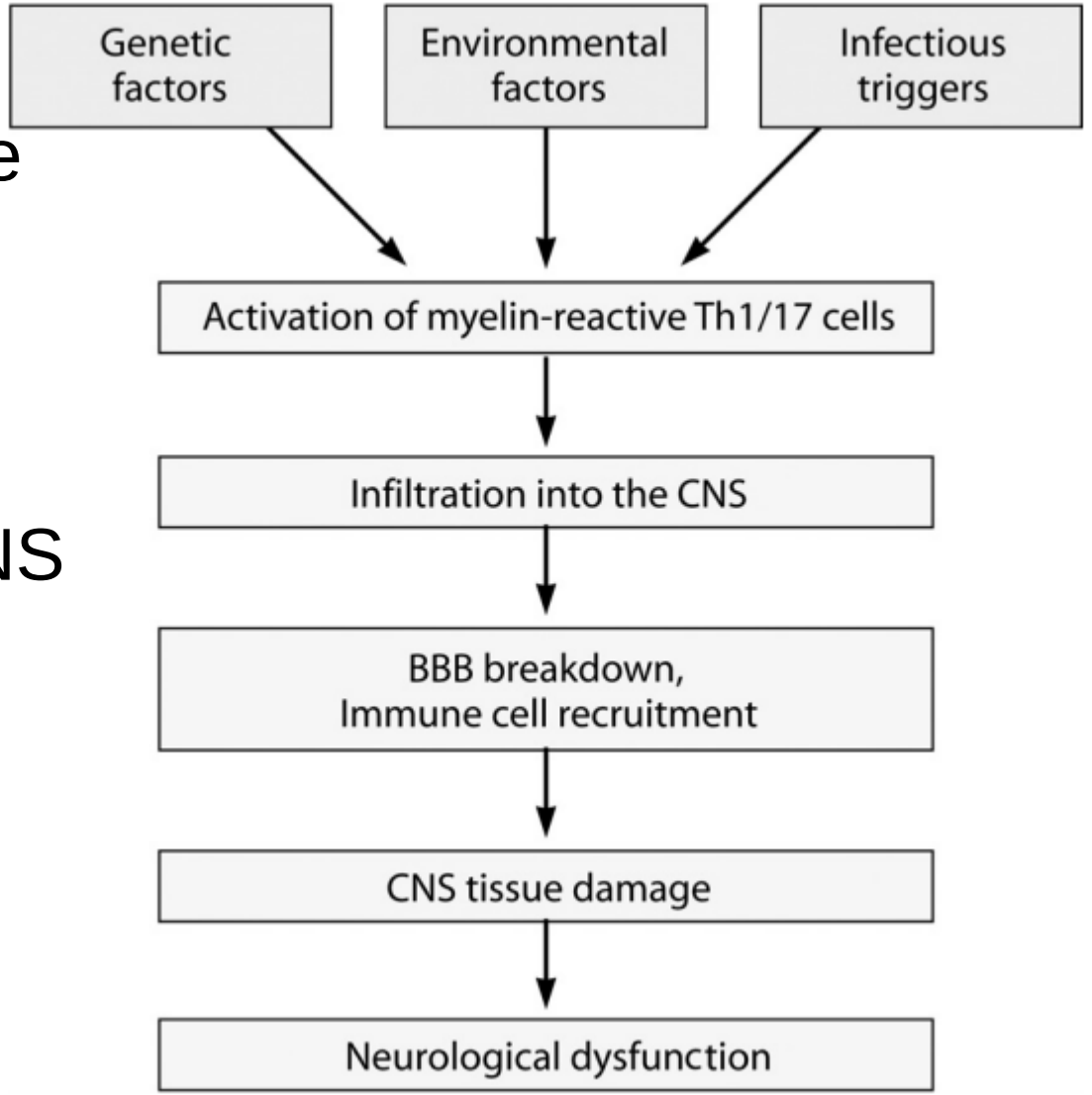
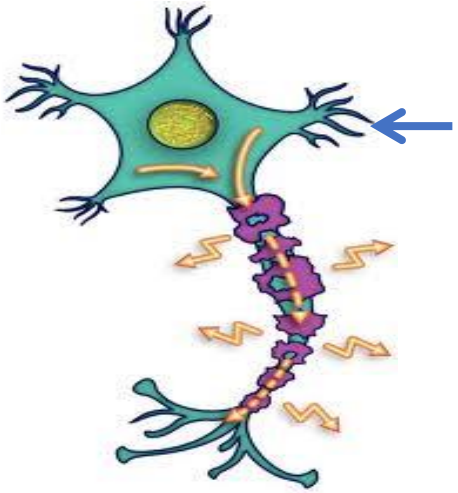


FIG : Pathophysiology of multiple sclerosis;

Krementsov et al. DOI:10.1128/MCB.00688-13

1. Introduction



>Affected 1 million per year

About 30% of them become paralyzed

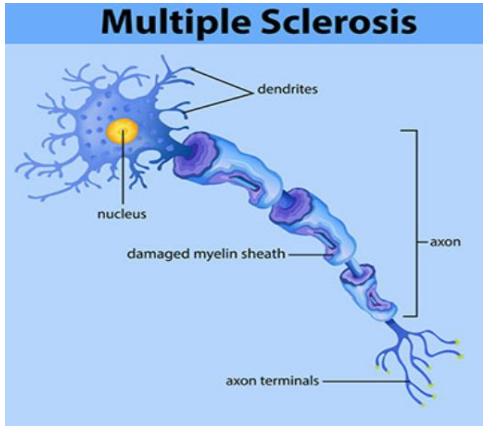
- Multiple sclerosis (MS) can occur at any age but usually occurs around 20 and 40 years of age.
- The highest rate of MS founding in new York, USA
- The average risk of developing MS in the USA is roughly 3.5 in 1000.

1. Introduction

- Experimental autoimmune encephalomyelitis (EAE) is an inflammatory autoimmune disease of the central nervous system resulting from degeneration of the myelin sheath. This is most commonly used as an animal model of MS.
- EAE is an autoimmune disease mediated by T cells. Th1 cells recognized as the main effector T cells for autoimmune inflammation.
- CD4 + Th17 cells secrete IL-17 in chronic inflammatory pathological condition including EAE.

1. Introduction

Moderately obese individuals are 1.28 times and extremely obese are 2.10 times susceptible to developing MS than the normal individual.



- Study found the association of brain inflammation in a rodent model of diet-induced obesity.
- Diet- induced obese mice were found to have exacerbated colitis and EAE disease compared with normal diet (ND) mice.
- Genomic factors related to MS as monozygotic twins display higher concordance compared with dizygotic twins.
- The relatives of MS patients have a 15% chance of being affected.

Gianfrancesco2016 doi:10.29245/2572.942x/2016/7.1064

2. Objectives

- The aim of this study is-
  - To identify differentially expressed genes (DEGs) in the brain of EAE-induced normal diet (ND) mice and high-fat diet (HFD)-induced obese mice, and
  - To identify novel genes responsible for elucidating the mechanism of the disease.

3. Materials

1. High-fat diets (HFDs) (60% calories from fat)
2. Immunization peptide: MOG35–55
3. Complete Freund’s adjuvant;
4. Trizol RNA extraction reagent;
5. RNase-free DNase set.
6. RNeasy mini kit.
7. SYBR Green supermix kit.
8. Omniscript RT kit.

3. Materials

9. NanoDrop ND-1000 spectrophotometer.
10. Experion automated electrophoresis system.
11. Illumina Total-Prep RNA amplification kit.
12. Illumina mouse WG-6v2 expression bead array chip;
13. GenePix 4000B scanner
14. Sonic dismembrator;
15. GenomeStudio 3.0
16. Real-time RT-PCR

4. Experimental Procedure

4. 1 Animals

- Male C57BL/6J mice (four weeks of age) randomly divided and kept either on a ND or on a HFD group for ten weeks providing ND or HFD respectively.
- All mice were kept in a temperature-controlled facility (25 °C) with a 12-h light/dark cycle.
- After ten weeks, ND and HFD mice were divided into two subgroups as control and EAE.
- EAE was induced by subcutaneously injecting 150 µg MOG33–35 (MEVGWYRSPFSRVVHLYRNGK) emulsified in complete Freund's adjuvant containing 400 µg of H37Ra *Mycobacterium tuberculosis*. However, the control mice were only treated with complete Freund's adjuvant.
- Immediately after subcutaneous injection, both the control and EAE mice were injected intraperitoneally with 200 ng of pertussis toxin and again two days later.

4. Experimental Procedure

4. 1 Animals

- Mice were monitored daily for body weight and clinical scores.
- Clinical scores were recorded as the following grade:
  - 0, no clinical signs;
  - 1, flaccid tail;
  - 2, hind limb weakness;
  - 3, hind limb paralysis;
  - 4, severe paraparesis;
  - 5, tetraplegia; and
  - 6, moribund or death
- Brain samples from both ND and HFD groups were collected at day 30 post-immunization after the peak disease clinical scores had been confirmed.
- DEGs were revealed in both ND- and HFD- EAE mice compared in respective of controls.
- At day 30, mice were sacrificed after ether anesthetization,
- Samples were collected after transcardial perfusion with phosphate-buffered saline.

4. Experimental Procedure

4. 1 RNA isolation, purification, and quality check

- Frontal cortices were immediately separated from brains and stored at -70 C.
- Total RNA was extracted using the Trizol extraction method.
- Each tissue sample was dissolved in 800 µl of Trizol reagent and homogenized with sonic dismembrator.
- The extracts were centrifuged at 14,000 xg for 15 min after addition of 200 µl of chloroform.
- The clear supernatants (500–600 µl) were then collected and mixed with 500 µl of isopropanol, inverted ten times, and centrifuged at 14,000g for 10 min.
- The resulting pellets were then reconstituted with 1 ml of 75% ethanol, inverted five times, and centrifuged at 11,000 xg for 5 min.

4. Experimental Procedure

4. 1 RNA isolation, purification, and quality check (Cont..)

- The aqueous phase was removed entirely, and the tubes were put in a desiccator for around 10–15 min to dry.
- The dried samples were then reconstituted with 50 µl of RNase-free DEPC water and kept in a water bath at 55 C for 10 min.
- The samples were maintained at -70 C until the next purification steps. The obtained RNA was purified with the RNeasy mini kit.
- The genomic DNA was removed using the RNase-free DNase set.
- The concentrations of RNA were measured with the Nano Drop ND-1000 spectrophotometer.

4. Experimental Procedure

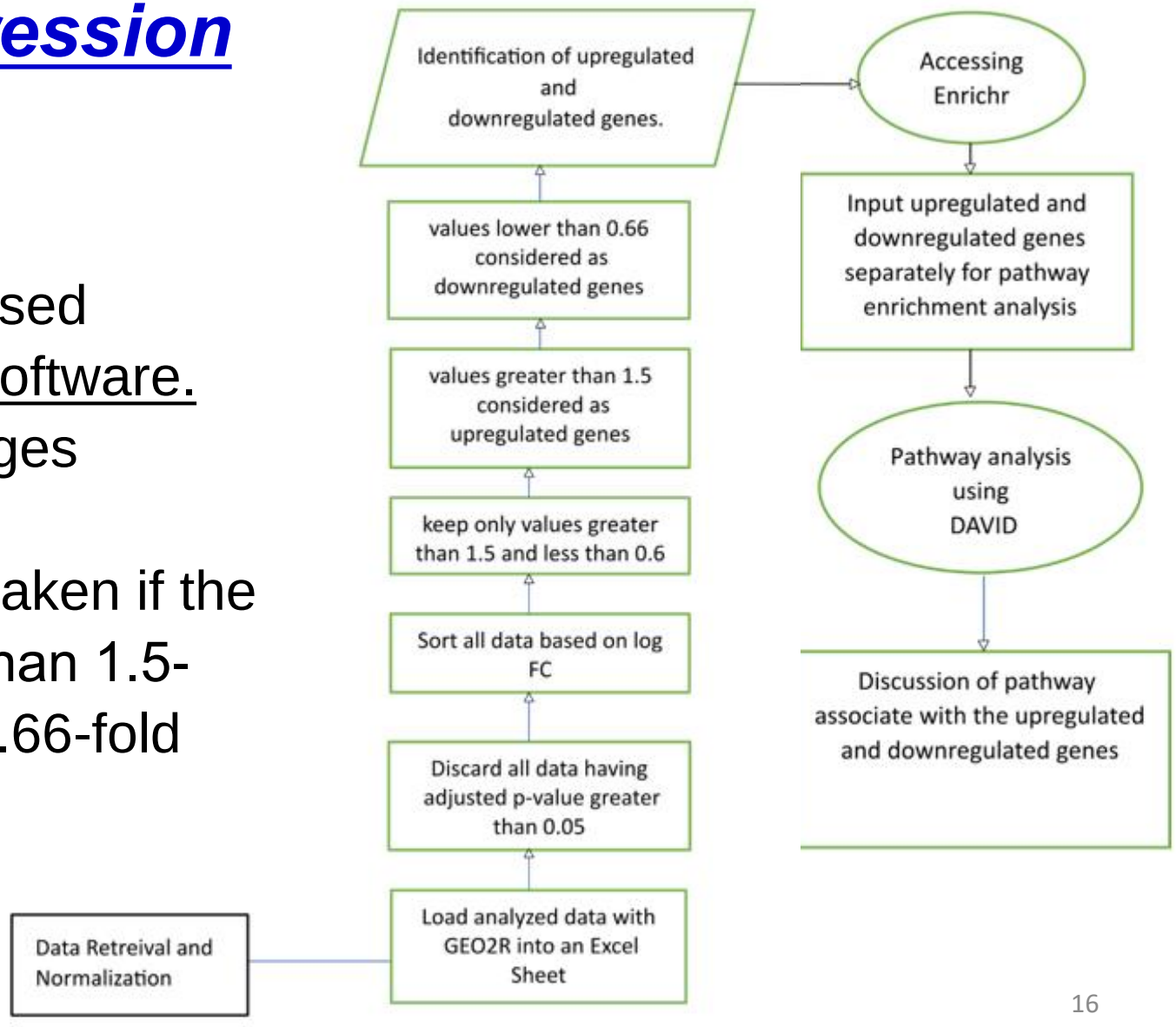
4.2 Differential gene expression analysis

- Quality control and integrity of RNA samples were confirmed with the Experion automated electrophoresis system.
- Total RNA was reverse transcribed to cDNA.
- Obtained cDNA was then converted to biotinylated cRNA in an in vitro transcription reaction using Illumina Total-Prep RNA amplification kit.
- Obtained cRNA was then hybridized on Illumina Mouse WG-6 v2 Expression BeadArray Chip.
- The arrays were then scanned using GenePix 4000B scanner and data were extracted using GenomeStudio 3.0.

4. Experimental Procedure

4.2 Differential gene expression analysis (Cont..)

- The pre-processing and unsupervised clustering were done in GenPlex software.
- Data are given as target fold changes compared with references.
- Variations in the expression were taken if the significant changes were greater than 1.5-fold (upregulation) and less than 0.66-fold (downregulation).



4. Experimental Procedure

4.3 Functional annotation

- Functional annotation of DEGs was conducted by DAVID (Database for annotation, visualization, and integrated discovery) software version 6.7 (http://david. abcc.ncifcrf.gov/home.jsp)
- Calculated modified Fisher's exact p-values less than 0.05 from DAVID demonstrated gene ontology (GO) or molecular pathway enrichment.
- The functional partnership and interactions among proteins were exhibited using the string online database tool (http://string-db.org/)

4. Experimental Procedure

4.4 Quantitative real-time RT-PCR

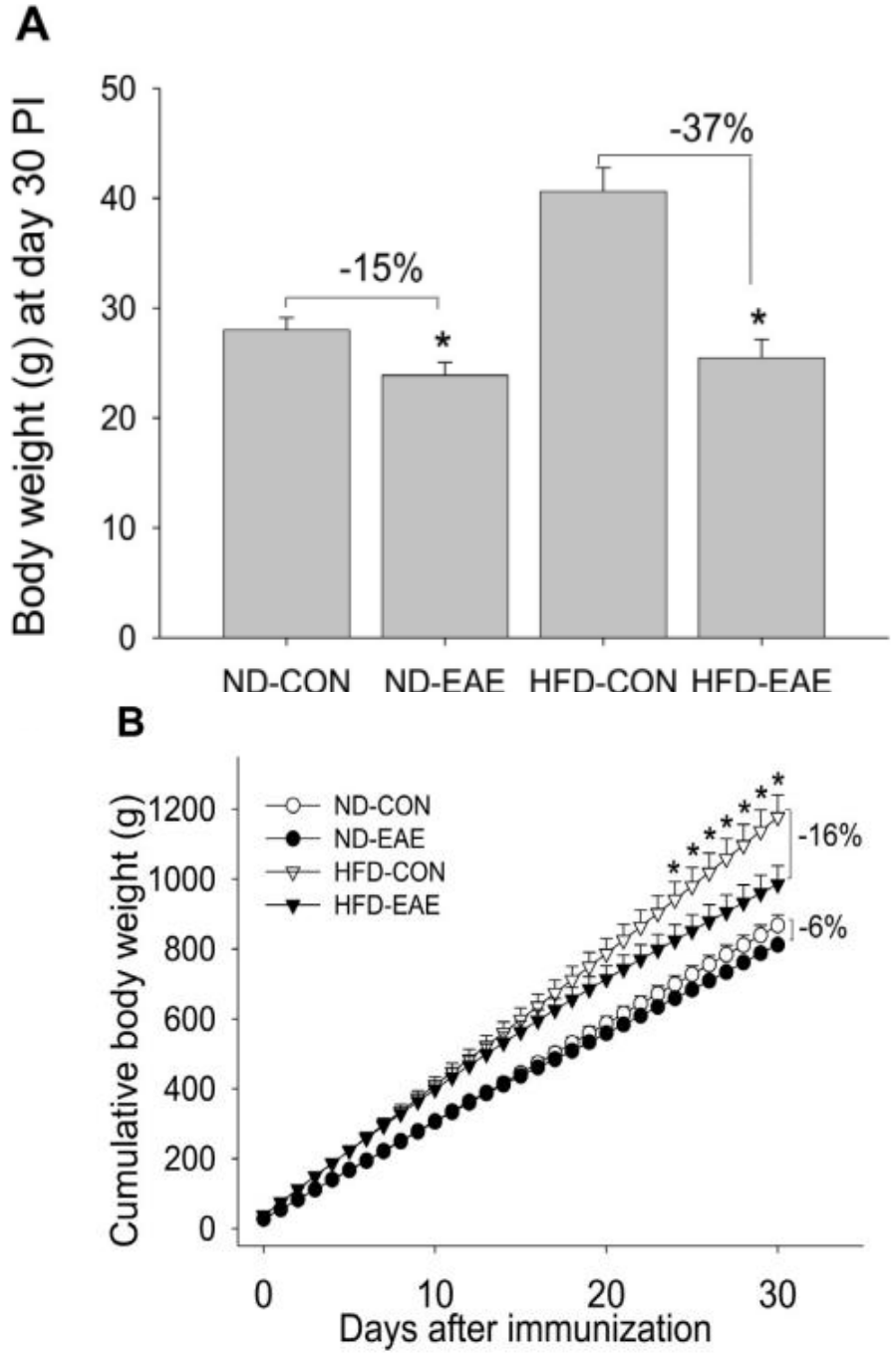
Real time RT-PCR was used for validating the selected genes. This process included-

- RNA samples were used from a cohort of 4 groups (ND-CON, ND-EAE, HFD-CON HED-EAE).
- The mRNAs were first transcribed into cDNA.
- Running mode included 45 cycles at 95°C for 20s and 60°C for 1 min - The specificity of PCR was confirmed using melting curve data.
- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ,a housekeeping gene, was used for normalization of selected sample genes.
- The relative gene expression was presented as fold changes of gene's mRNa/ GAPDH abundance compared to that of the respective reference control.

5. Results

5.1 Aggravated EAE in obese mice

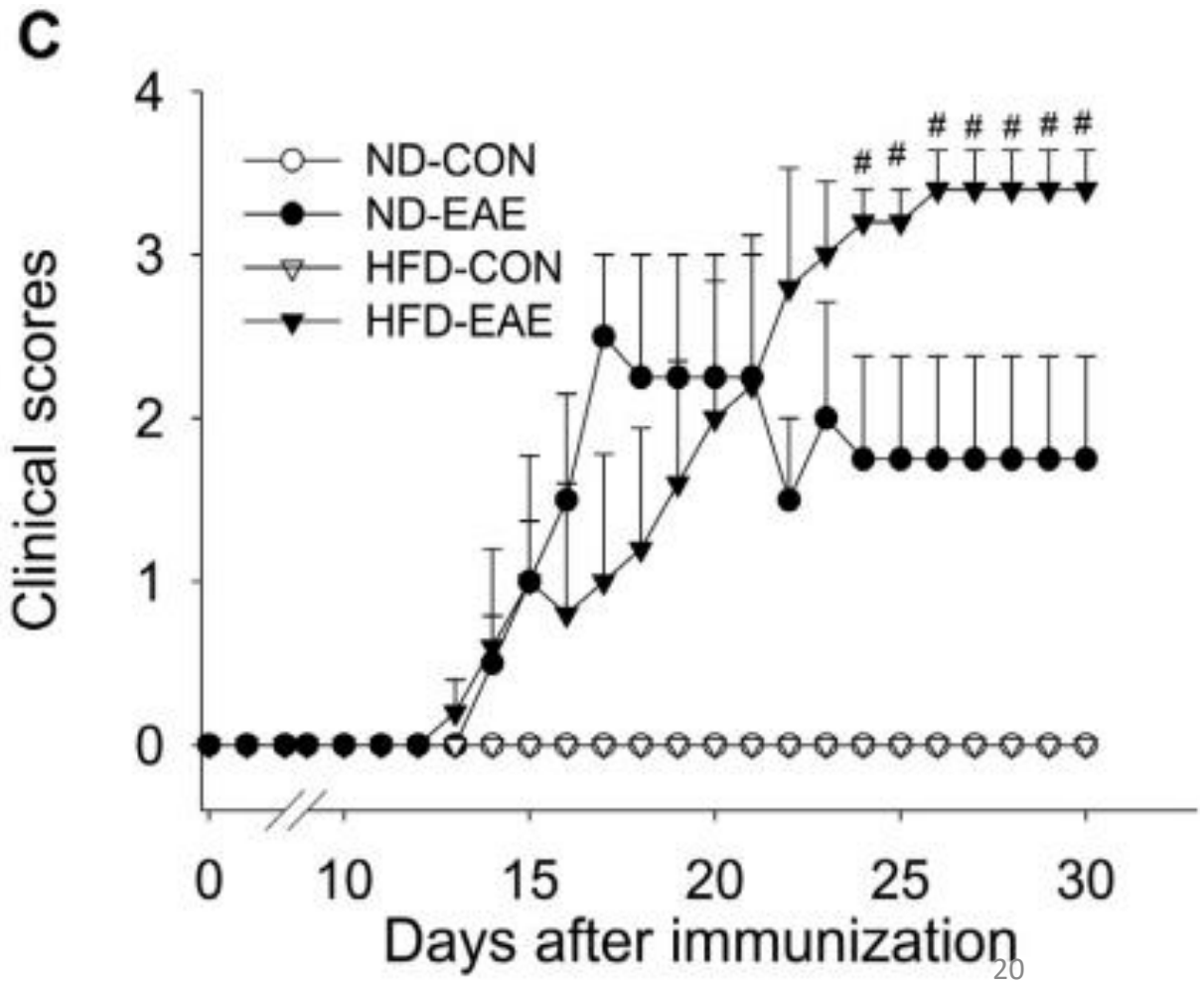
- The HFD-induced body weight of mice increased by 40% over the weight of ND mice (37.6 g vs. 27.8 g) after ten weeks.
- EAE was induced in both ND mice and HFD-induced obese mice by immunization with the MOG33-35 peptide.
- The changes in body weight at day 30 and the cumulative body weight changes were significant in EAE mice but not in control mice.



5. Results

5.1 Aggravated EAE in obese mice (cont..)

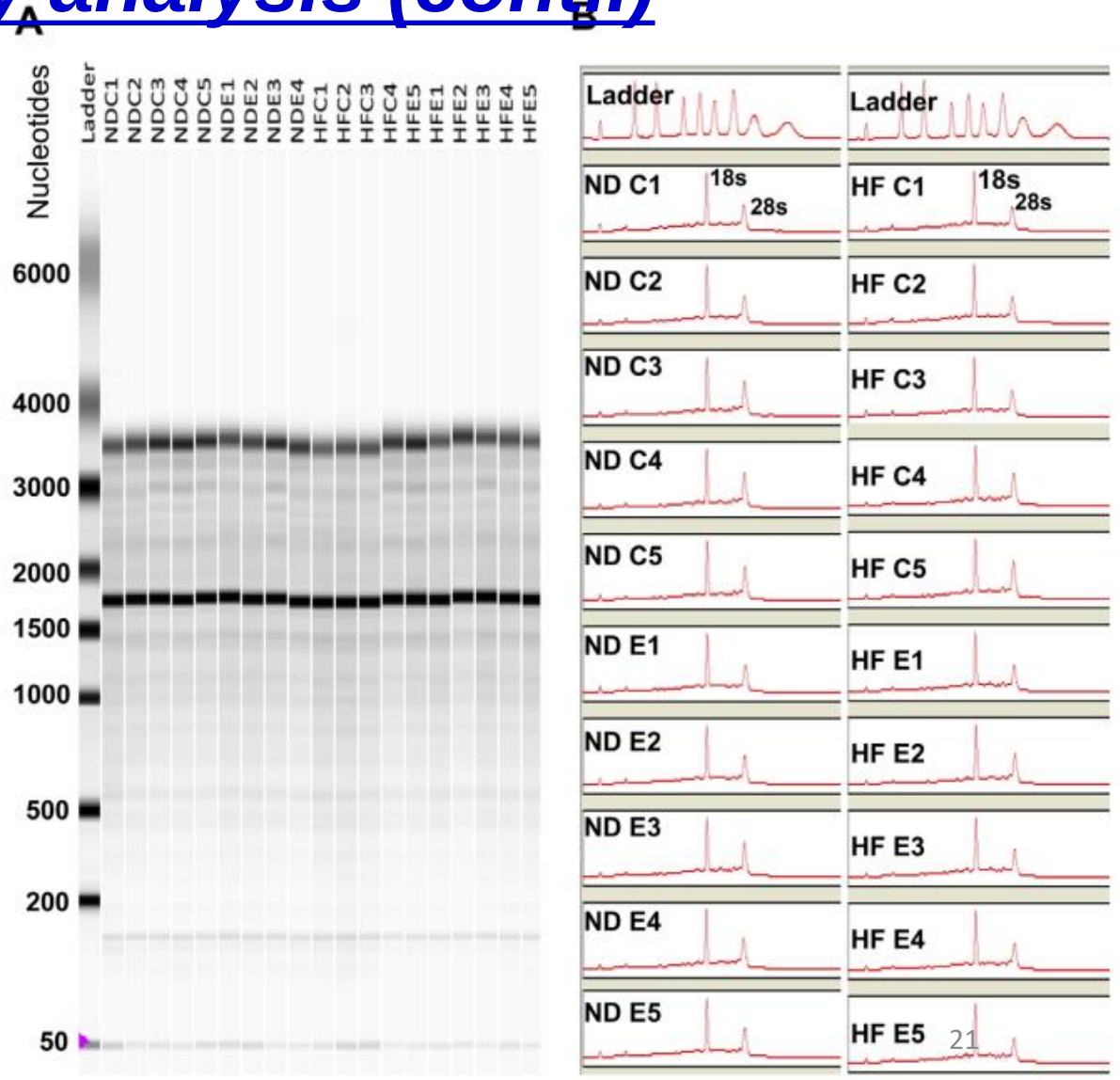
- At day 30, the body weight of ND-EAE mice was 15% lower than that of ND-CON. However, in the case of HFD-EAE, the decrement was 37% compared with HFD-CON.
- Cumulative body weight was significantly lower in HFD-EAE from day 24 and, finally, 16% lower than in HFD-CON mice.
- The average clinical score indicated exacerbated EAE in HFD-EAE vs. ND-EAE mice.
- The decreased body weight and increased clinical scores confirm the severe EAE in HFD-mice but not in ND-mice.



5. Results

5.2 RNA Quality for microarray analysis (cont..)

- The quality inspection was first performed using the Nano-Drop spectrophotometer by monitoring the ratio of absorbance at 260 and 280 nm (260/280).
- A ratio of 260/280 of more than 2.00 was identified as pure RNA and that of lower than 2.0 was regarded as containing contaminant proteins, phenol, or other components.
- Furthermore, the integrity of RNA samples was checked by gel electrophoresis and auto-electrophoresis.



5. Results

5.2 RNA Quality for microarray analysis (cont..)

- A clear separation of two distinct bands was observed in samples after electrophoresis. Figure shows an autoelectrophoresis photograph of ladder in samples, confirming a clear separation of the 28 s and 18 s ribosomal subunits.
- The sharp peaks for the 18S and 28S ribosomal units indicate the presence of pure integrated RNA.

5. Results

5.3 DEGs by microarray analysis

Gene expression analysis was compared with the control as well as the diet group (n = 5 mice/group). This design yielded four comparisons:

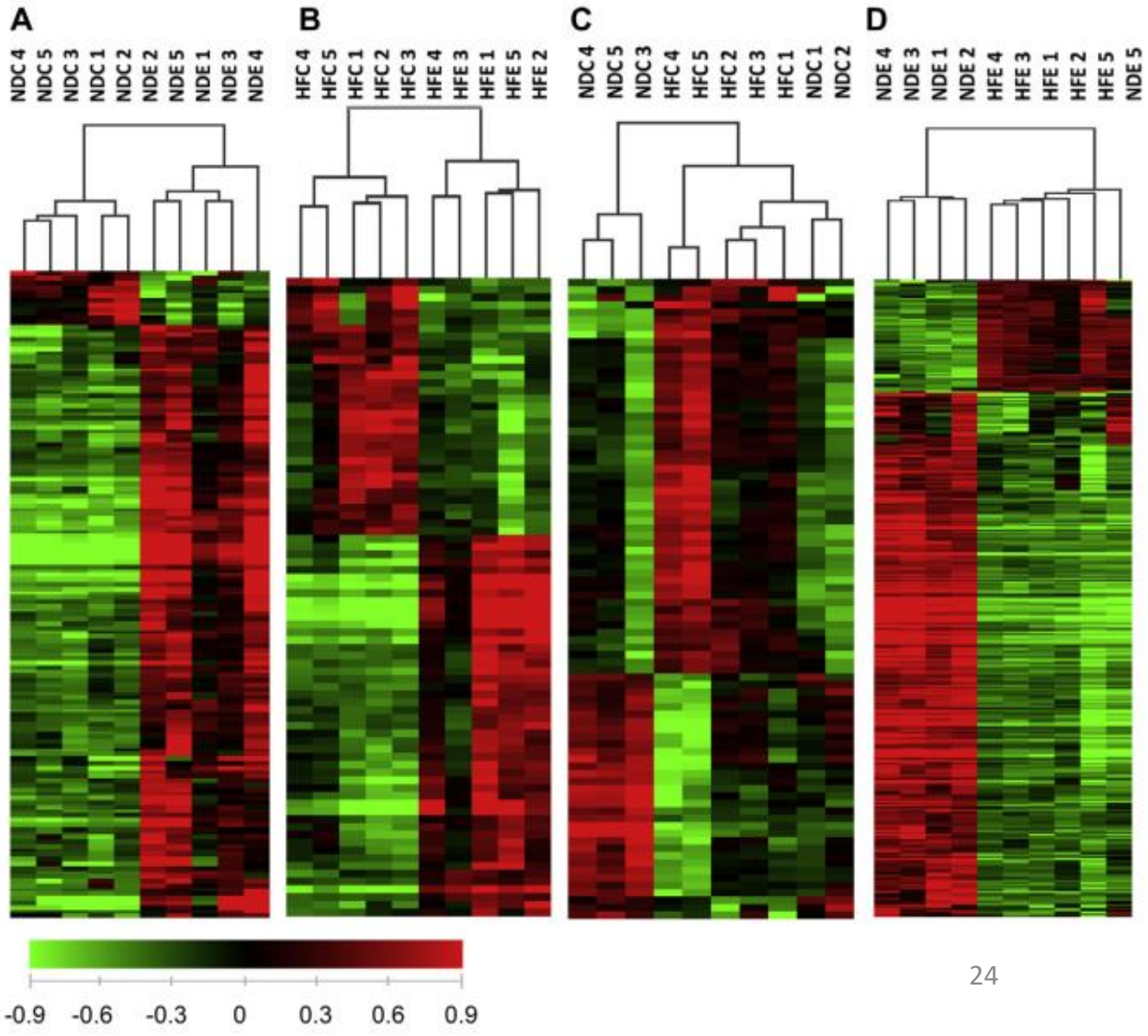
(1) EAE in normal diet mice (ND-EAE vs. ND-CON);
(2) EAE in obese mice (HFD- EAE vs. HFD-CON);
(3) obesity in control mice (HFD- CON vs. ND-CON);
(4) obesity in EAE mice (HFD-EAE vs. ND-EAE).

- Hierarchical cluster analysis or heat mapping was performed for the genes in the four groups.
- A grid of colors indicates the gene expression values of each sample.
- Here, columns show the number of sample in each group,
- while rows represent some of the analyzed genes.

5. Results

5.3 DEGs by microarray analysis

- Genes in target vs. control altered with a fold change greater than 1.5 or less than 0.66 and a p-value less than 0.05 were considered as significantly up- and down-regulated, respectively.
- A red color indicates the highest expression, whereas green one indicates the lowest expression and black, no change in expression levels.
- The ordering of rows and columns was determined by hierarchical cluster analysis with similar gene expression patterns grouped together as shown in the dendrograms at the top of the figure.



5. Results

5.3 DEGs by microarray analysis

- In the volcano plots, the x-axis indicates the fold change on a base-2 logarithmic scale.
- The y-axis indicates the statistical significance of differential expression (p-value) on a base-10 logarithmic scale.
- Differentially up- and down-regulated genes are shown in red. The value of p ($p < 0.05$) by Welch's t-test was used for sorting significantly different genes and the criteria of fold changes >1.5 for upregulation and <0.66 for downregulation were applied.
- Differentially expressed gene (DEGs) counts were observed in ND-EAE vs. ND-CON (up = 138, down = 11), HFD-EAE vs. HFD- CON (up = 49, down = 33), HFD- CON vs. ND-CON (up = 53, down = 33), and HFD-EAE vs. ND-EAE (up = 76, down = 355).

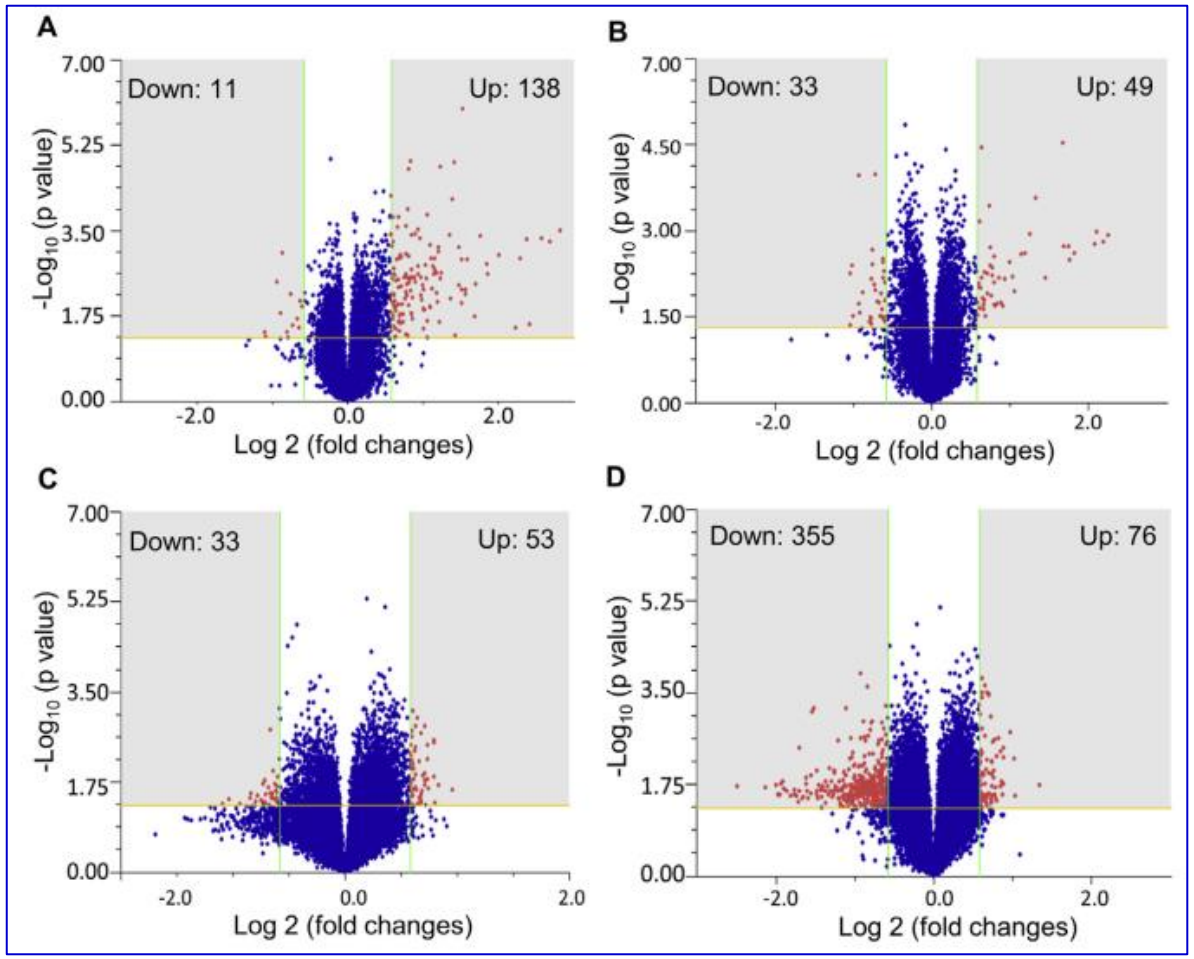


Figure: Volcano plots for comparison of gene expression. Comparison in (A) ND-EAE vs. ND-CON; (B) HFD-EAE vs. HFD-CON; (C) HFD-CON vs. ND-CON; and (D) HFD-EAE vs. ND-EAE is shown.

5. Results

5.4 Pathway Analysis

Table 2. GO functional analysis (biological processes) of differentially regulated genes in ND-EAE and HFD-EAE

Biological processes	ND-EAE *vs.* ND-CON				HFD-EAE *vs.* HFD-CON			
Term	Count	%	*p*-Value	Benjamini	Count	%	*p*-Value	Benjamini
Immune response	42	40	7.2E−39	4.4E−36	23	68	3.2E−28	7.9E−26
Antigen processing and presentation of peptide antigen	16	15	2.6E−25	7.9E−23	9	26	1.8E−15	1.1E−13
Antigen processing and presentation	19	18	1.3E−23	2.6E−21	11	32	5.2E−16	4.6E−14
Defense response	30	29	4.5E−23	7.0E−21	17	50	1.4E−17	1.7E−15
Antigen processing and presentation of exogenous antigen	12	12	2.8E−18	2.9E−16	6	18	2.0E−09	5.5E−08
Leukocyte-mediated immunity	15	14	9.6E−17	8.4E−15	7	21	1.8E−08	2.8E−07
Antigen processing and presentation of peptide antigen via MHC class II	10	10	5.9E−17	9.7E−15	4	12	3.9E−06	4.2E−05

- The functional categorization of DEGs in both ND-EAE vs. ND-CON and HFD-EAE vs. HFD-CON mice was performed with the web-based DAVID annotation tool.
- A GO analysis was performed regarding the biological process, molecular function, and cellular component for the upregulated DEGs in both ND- EAE and HFD-EAE with the respective controls.
- A compressive table lists the term biological processes with the gene count, percentages of total DEGs, p-value, and Benjamin score of both ND-EAE and HFD-EAE mice.
- In the case of a biological process, genes related to antigen processing and presentation, cell-mediated immunity, immune response, activation of innate and adaptive immune responses, wound-healing responses, and inflammatory response were highly focused.

5. Results

5.4 Pathway Analysis

Table 3. GO functional analysis (molecular functions) of differentially regulated genes in ND-EAE andHFD-EAE

Molecular functions	ND-EAE *vs.* ND-CON				HFD-EAE *vs.* ND-CON			
Term	Count	%	*p*-Value	Benjamini	Count	%	*p*-Value	Benjamini
Chemokine receptor binding	8	8	6.5E−10	5.5E−08	4	12	3.0E−05	2.1E−04
Chemokine activity	8	8	5.4E−10	9.1E−08	4	12	2.8E−05	2.3E−04
Cytokine activity	8	8	2.7E−05	1.1E−03	4	12	2.7E−03	1.4E−02

Table 4. GO functional analysis (cellular components) of differentially regulated genes in ND-EAE and HFD-EAE

Cellular components	ND-EAE *vs.* ND-CON				HFD-EAE *vs.* HFD-CON			
Term	Count	%	*p*-Value	Benjamini	Count	%	*p*-Value	Benjamini
MHC protein complex	12	12	1.14E−14	1.12E−12	8	24	1.2E−11	4.8E−10
MHC class II protein complex	6	6	1.9E−09	9.5E−08	3	9	2.0E−04	1.0E−03
Extracellular region part	21	20	2.7E−08	9.0E−07	8	24	1.0E−03	4.6E−03
Extracellular region	30	29	8.0E−08	2.0E−06	11	32	1.8E−03	6.6E−03
Extracellular space	16	15	4.1E−07	8.1E−06	8	24	8.0E−05	6.5E−04

For molecular function, chemokine receptor binding, major histocompatibility complex (MHC) protein binding followed by cytokine and chemokine activity were represented (Table 3). In the cellular component, the highly significant parts were MHC, followed by extracellular regions, cell surface, and plasma membranes (Table 4).

5. Results

5.4 Pathway Analysis

Table 5. GO functional analysis (biological pathways) of differentially regulated genes in ND-EAE and HFD-EAE

Biological pathways	ND-EAE *vs.* ND-CON				HFD-EAE *vs.* HFD-CON			
Term	Count	%	*p*-Value	Benjamini	Count	%	*p*-Value	Benjamini
Systemic lupus erythematosus	17	16.35	4.09E−16	1.95E−14	9	26.47	8.64E−10	2.42E−08
Antigen processing and presentation	14	13.46	1.09E−12	2.39E−11	8	23.53	1.31E−08	1.83E−07
Allograft rejection	11	10.58	8.43E−11	1. E24−09	7	20.59	2.84E−08	2.65E−07
Autoimmune thyroid disease	11	10.58	7.84E−10	8.63E−09	7	20.59	1.07E−07	7.46E−07
Viral myocarditis	11	10.58	1.13E−08	7.12E−08	7	20.59	5.30E−07	2.97E−06
Cell adhesion molecules (CAMs)	11	10.58	1.26E−06	5.53E−06	6	17.65	1.46E−04	5.77E−06
Graft−versus−host disease	10	9.62	2.16E−09	1.90E−08	6	17.65	1.22E−06	7.42E−06
Type I diabetes mellitus	10	9.62	4.64E−09	3.40E−08	6	17.65	1.86E−06	7.42E−06

The pathway studies were conducted using KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis; it compiled the DEGs in such a way as to show the predominantly altered pathways in EAE of ND and HFD mice. The most annotated pathways in both ND-EAE and HFD-EAE mice were those of disease related to autoimmunity, e.g., systemic lupus erythematosus, autoimmune thyroid disease, and Type I diabetes mellitus. Moreover, antigen processing, antigen presentation, complement activation, cell adhesions, and chemokine and cytokine signaling pathways were reported (Table 5)

5. Results

5.5 Validation of DEGs by real time RT-PCR

- The list of DEGs in both ND-EAE and HFD-EAE whose fold changes were P 2.00 and that were not previously reported for their association with EAE or MS were validated by real-time RT-PCR.
- Fcgr4, C4b, S3-12, Psmb8, Ly86, Ms4a6d, Psmb9, and Xlr4b were selected and validated using real- time RT-PCR. GAPDH, a house- keeping gene, was used as an endogenous control for gene expression analysis.
- After PCR validation, Fcgr4, C4b, S3-12, Psmb8, Ly86, and Ms4a6d were found to be upregulated in ND-EAE vs. ND-CON mice.

5. Results

5.5 Validation of DEGs by real time RT-PCR

- Moreover, C4b, Psmb8, Ly86, Xlr4B, and Ms4A6d were upregulated in HFD-EAE vs. HFD- CON mice only.
- Only C4b was found to be upregulated in HFD-EAE vs. ND-EAE mice.
- Moreover, genes related to metal ion-binding proteins upregulated in HFD-EAE vs. ND-EAE were also validated by quantitative PCR.
- Genes including Cul9, Zdhhc4, Zcchc6, Hpcal1, Gca, Cdh10, Eno1, Mast2, and Pde8a were validated using real-time PCR.
- Cul9, Gca, Zdhhc4, and Mast2 were found to be upregulated in ND-EAE vs. ND-CON. However, Cul9 and Mast2 were elevated in both HFD-EAE vs. HFD-CON and HFD-EAE vs. ND-EAE.

6. Discussion

- MS is a demyelinating disease that primarily affects the motor and sensory functions of the brain.
- In addition to genetic factors environmental factors such as- diet also modulate this autoimmune diseases
- The relationship between obesity and MS has also been reported in a study concluding that dietary intake of a low-fat diet reduces deterioration of the disease compared to a HFD.
- The validation of the microarray results by real-time RT-PCR has confirmed C4b, Psmb8, Ly86, and Ms4a6d as a new list of genes upregulated in both ND-EAE and HFD-EAE mice.

6. Discussion

- Upregulation of C4b interacts with the classical, alternative, and lectin pathways of complement activation through C1, C2, C3, and mannan-associated serine peptidase-2 proteins. Inhibition of the complement system inhibits EAE, suggesting a possible therapeutic approach for the treatment of MS.
- Psmb8 proteasome protein usually degrade proteins related to anti-inflammation, thus inhibition of Psmb8 might restore the inflammation observed in EAE.
- However, Fcgr4 and S3-12 were found to be upregulated only in ND-EAE mice, and Xlr4b was upregulated only in HFD-EAE mice.

6. Discussion

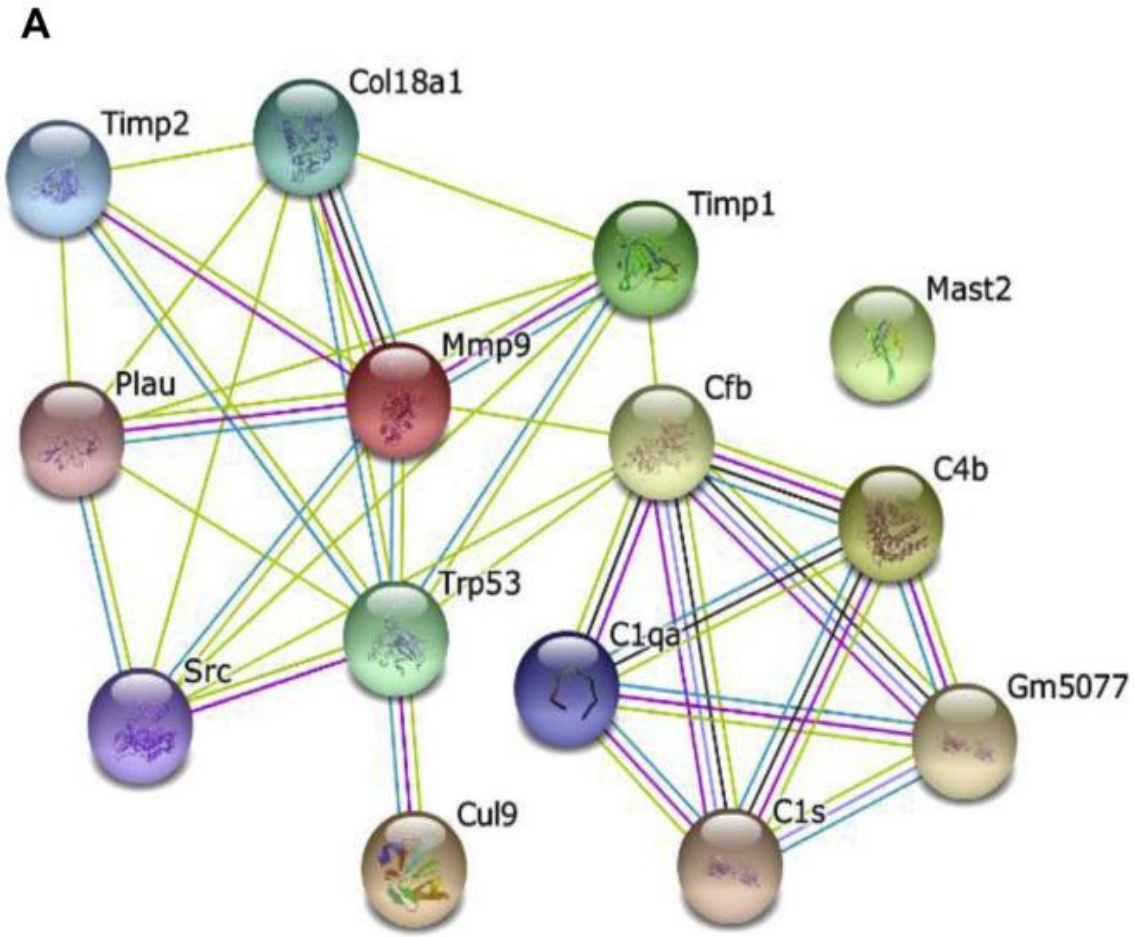
- ❖ Ly86 also interacts with the complement protein system and regulates innate immunity.
- ❖ S3-12 codes for S3-12 protein, also known as perilipin 4 (Plin4), which coats lipid droplets in adipocytes and thus protects the lipid from lipolysis.

➢ Genes related to metal ion-binding proteins upregulated in HFD-EAE vs. ND-EAE were also validated by quantitative PCR. Of which Cul9,Gca,Mast2 were upregulated in HFD-EAE.

- ❖ Gca (grancalcin) is a calcium-binding protein that may play a role in the mobilization & adhesion of neutrophils to fibronectin.
- ❖ Mast2 regulates lipopolysaccharide-induced IL-12 synthesis in macrophages by forming a complex with TRAF6.

6. Discussion

- Matrix metalloproteinase-9 (MMP-9) and IL-4 levels were shown to have an association with severe EAE in HFD-EAE mice
- Therefore, Cul9, C4b, and Mast2 were tested for the possible interaction among them using the STRING online software with ten interactions (http://string-db.org/).
- A possible interaction of MMP-9 with Cul9 and C4b was identified.
- Cul9 interacts with transformation-related protein (Trp53 or p53) and regulates MMP-9.
- On the other hand, C4b interacts with complement factor B (Cfb) and regulates MMP-9.



7. Conclusion

- This study concluded that-
- HFD-EAE mice showed more severe disease symptoms than ND-EAE mice.
- The novel upregulated genes of Fcgr4, C4b, Psmb8, Ly86, Xlr4b, S3-12, and Ms4a6d were observed in EAE mice.
- The metal ion-binding genes Cul9, Gca, Zdhhc4, and Mast2 were also upregulated in EAE mice.

Comprehensive Study of Advanced Literature

Course Code: BMB 519

Group: 2

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Md Atikur Rahman	20151216031
Palash Saha	20151216033
Bidu Pal	20151116034
Sukanto Kumar Sil	20151216035

Thank you