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A bioinformatics approach to cuprizone model of multiple sclerosis: Focus on glial cells

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Abstract

Multiple sclerosis is a multifaceted demyelinating autoimmune disease primarily affecting myeline sheath in the central nervous system. There is currently no definitive treatment for this disease. Neuroinflammation is thought to be the main cause underlying the disease. Cuprizone is a copper-chelating chemical compound, and commonly used in experimental model of multiple sclerosis. Therefore, in this study it was aimed to investigate the changes in molecular pathways in glial cells induced by cuprizone demyelination model by using different methods.

Cuprizone-gene interaction analysis was done with The Comparative Toxicogenomics Database. The Gene Expression Omnibus database was used to access bioinformatics datasets. The gene expression data were analyzed to compare cuprizone fed and normal diet animal brain samples. Differentially expressed genes were determined by using bioinformatics tools. Pathway analysis was studied with Enrichr. The String Database was used to show protein-protein interactions network.

In this study, it has shown that the cuprizone model of multiple sclerosis mainly targets oligodendrocytes. However, microglia and astrocyte related signaling pathways are also affected by multiple sclerosis. Thus, combined therapeutic approaches are needed to multiple sclerosis treatment.

Keywords: Glial cells, Cuprizone, Demyelination, Multiple sclerosis, Bioinformatics databases

Introduction

Glial cells are the main cell types of central and peripheral nervous system after neurons. Mostly classified into three groups, including astrocytes, oligodendrocytes and microglia for the central nervous system [1]. Astrocytes predominantly function as critical regulators of cerebral homeostasis and glutamate catabolism. They engage in the reparative mechanisms of damaged neural tissues and inhibit the propagation of neuroinflammatory responses [2,3]. Oligodendrocytes mainly play another important role in covering the axons of neurons. They produce myelin proteins and make an insulator function to speed up synaptic transmission [4]. Microglia are resident cells of the immune system that are mainly located in the central nervous system [5]. Activation of microglia and astrocytes is triggered after cuprizone administration, a copper-chelating agent used to mimic demyelination in animal models of multiple sclerosis (MS) [6]. In this model, cuprizone causes oligodendrocyte cell death, and demyelination occurs in different regions of the animal brain [7]. For this model, researchers generally use 0.2-0.25% cuprizone mixed rodent chows and feed the experimental animals with this for at least three weeks to observe the acute demyelination effects of cuprizone [8]. When previous literature searched in detail, it seemed that the cuprizone model of MS is generally applied to animals for at least 3 weeks, and in some of the studies, 5 weeks of cuprizone administration is accepted to observe demyelination processes in different regions of brains. Until 5 weeks of feeding is accepted as the acute demyelination phase, when the feeding is stopped at this week and replaced with normal rodent chow remyelination processes start and if the feeding is followed until 12 weeks, the chronic demyelination phase continues after the fifth week [9]. The main cause of MS is not fully understood yet, and several disease models are needed to understand the pathological processes of the disease for animal studies. However, recent reports are focused on neuroinflammation, because it's a chronic inflammatory autoimmune disease, and oxidative damage may also be related with MS progression and by targeting these pathways could be a therapeutic approach for MS treatment [10–12]. Recently, a well-known oxidative stress-related transient receptor potential ankyrin subtype 1 (TRPA1) was also found to contribute to demyelination processes [13,14]. The cuprizone model of demyelination is also not fully elucidated yet and needs to be enlightened with novel studies [15]. Hence, with this study it was aimed to investigate how cuprizone administration affects differentially expressed genes those related to different signaling pathways in glial cells in the experimental cuprizone model of MS. To fulfil this objective, three distinct datasets were selected to represent astrocytes, microglia, and oligodendrocytes within the central nervous system. Subsequently, bioinformatics methodologies were employed to elucidate the target pathways implicated in the pathogenesis of multiple demyelination.

Methods

Chemical-Gene Interactions

For chemical-gene interactions of cuprizone molecule and copper, The Comparative Toxicogenomics Database (CTD, https://ctdbase.org/) was used [16]. After the CTD query, the top ten genes that interacted with cuprizone from previously published literature were given in a bar graph in **Figure 1**.

Table 1. *List of selected datasets for this study.*

Exp. Animal	GEO ID	Administration	Sample	Platform	Citation
Mus musculus	GSE134372	Cuprizone (0.2%) diet – 3 w	Astrocytes	GPL23038 [Clariom_S_Mouse] Affymetrix Clariom S Assay, Mouse (Includes Pico Assay)	[17]
Mus musculus	GSE84113	Cuprizone (0.2%) diet – 4 w	Microglia	GPL6246 [MoGene-1_0-st] Affymetrix Mouse Gene 1.0 ST Array [transcript (gene) version]	GSE84113
Mus musculus	GSE48872	Cuprizone (0.2%) diet – 5 w	Oligodendrocyte Progenitor Cells (OPCs)	GPL11202 Agilent-026655 Whole Mouse Genome Microarray 4x44K v2 (Probe Name version)	[18]

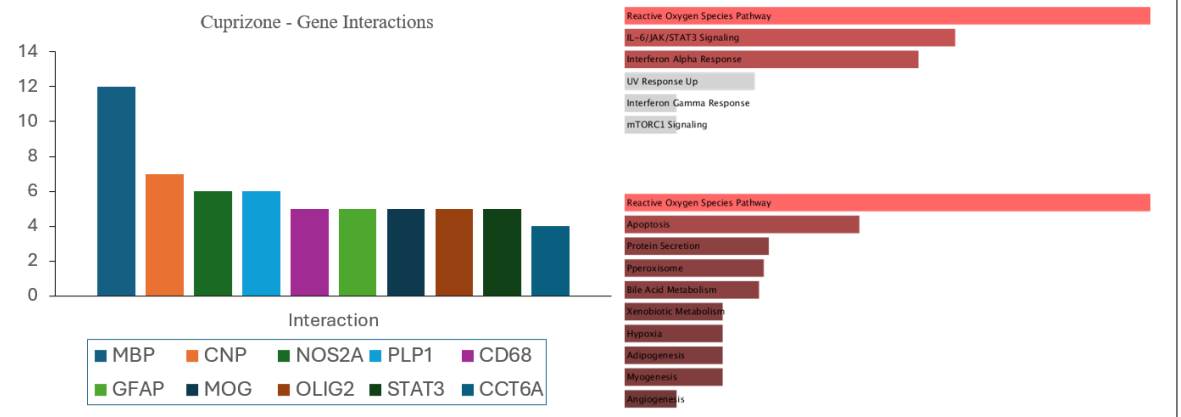

***Figure 1**. The CTD query results of cuprizone-interacted genes. The bar graph shows interaction numbers and of top ten gene names (left), right upper graph shows cuprizone-related main pathways, right lower graph shows cupper-related main pathways after Enrichr analysis (both data taken from MSigDB Hallmark 2020).*

Selection of Microarray Datasets

The Gene Expression Omnibus (GEO) DataSets web page (https://www.ncbi.nlm.nih.gov/gds) was used to search microarray datasets. For searching, 'cuprizone', 'microarray', 'astrocytes', 'microglia' and 'oligodendrocytes' terms were used to make a combination such as 'cuprizone and astrocytes and microarray', 'cuprizone and microglia and microarray', 'cuprizone and oligodendrocytes and microarray'. After the search, three different datasets were selected for this study, as detailed in **Table 1**.

Differentially Expressed Genes (DEGs)

Analyses of microarray datasets were done with web-based R programming language using the online tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). For each dataset, Cup administered animal samples compared to WT control values. Data were restricted with an adj-p value below 0.05, and for Log2(FC) above 0.5 and listed differentially expressed genes (DEGs) were used for further analysis. Probes without any gene symbols were filtered, and upregulated/ downregulated gene numbers were given in **Table 2** [19–21].

***Table 2.** List of DEGs of glial cell datasets.*

Dataset	Upregulated genes	Downregulated genes
GSE134372	288	297
GSE84113	6	-
GSE48872	269	75

Protein-Protein Interactions

For the protein-protein interaction network, The String Database (https://string-db.org/) was used for the top 20 DEGs of each dataset [19,22]. For the analysis, the False Discovery Rate (FDR) value was adjusted to less than 0.05, the confidence level was medium, and all interaction sources were active.

Pathway Analyses

The gene set enrichment analyses were done with The Enrichr web tool (https://maayanlab.cloud/Enrichr/) and with all upregulated genes of each dataset, target pathways of cuprizone intoxication were shown in **Figure 2, 4, 5 and 6** [23].

***Table 3.** List of top 20 upregulated and downregulated genes of datasets.*

GSE134372		GSE84113	GSE48872	
Up	Down	Up	Up	Down
Erdr1	Fzd2	Mmp12	Cdkn1a	Jph4
Bcl2a1a	Gdf10	Fam20c	Trem2	Col2a1
Bcl2a1b	Kirrel2	Lgals3	Eif4ebp1	Smtnl2
Mmp12	Rpe65	Csf1	Vgf	2610002 M06Rik
Ccl3	Kcnk1	Cxcr4	Tagln2	Kif6
Mpeg1	Msmo1	Gas2l3	Grik3	Carns1
Bcl2a1d	Lama3		Asns	Ppp1cc
Cdkn1a	Cml5		C1qc	Rasl12
Ly86	Chrdl1		Trio	Expi
C3ar1	Scara3		Col5a3	Rasal1
Syt4	Dhcr24		Slc7a1	Itih3
ENSMUST 00000178789	Prex2		Nupr1	1700063 D05Rik
Il1b	Hapln1		S100a10	Fn3k
Slc15a3	Grin2c		Atf3	Ninj2
Tyrobp	Ucp3		Dpysl4	Hhatl
Serpina3n	Lfng		Ecel1	Sgk2
Cybb	Carns1		Emp1	Wnt5b
C1qa	Entpd2		Ccnd1	Rhox1
Plek	Smim3		Lgals3	Synj2
Cd14	Slco1c1		Vim	Bcorl1

Statistical Analysis

The gene lists obtained from GEO2R, genes showing adj-p<0.05, Log2(FC)>0.5 were included in the study. In the signal pathway analysis performed with the STRING database, the false discovery rate (FDR) value was taken as the basis to be less than 0.05.

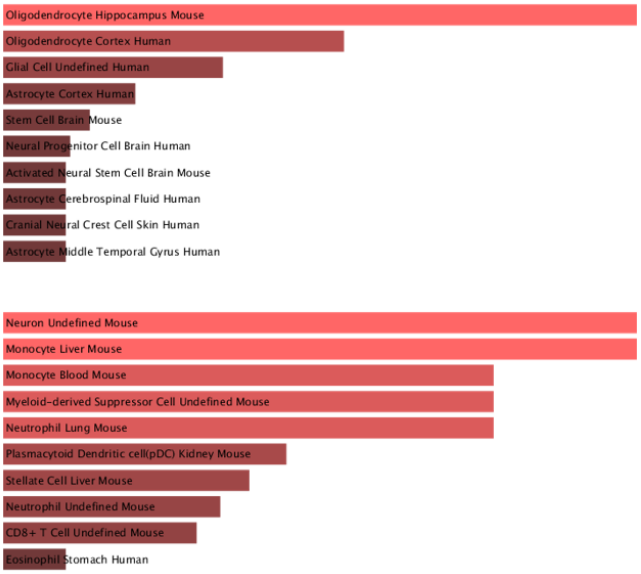

Figure 2. *Enrichr query of cuprizone and cupper-related cell types, both data taken from CellMarker 2024.*

Results

Chemical-Gene Interactions

The cuprizone-interacted genes were listed as MBP, CNP, NOS2A, PLP1, CD68, GFAP, MOG, OLIG2, STAT3 and CCT6A after CTD query. Because cuprizone is a copper chelator, copper-related genes were also searched in the same database, and according to these results, SLC31A1, PRNP, ATP7A, ATP7B, CAT, CP, APP, SOD1, GSR and MT2 were found to top copper-interacted genes, respectively. When these genes were taken into gene set enrichment analysis, the most common pathways were found to be related to the 'reactive oxygen species pathway'. After Enrichr cell type analysis, cuprizone was found mainly overlapped with 'oligodendrocytes', and when cupper-interacted top ten genes taken into Enrichr, it was found that cupper-interacting genes were mainly overlapped with 'neurons'.

Findings of DEGs

The GSE134372 was selected as an example of how white matter astrocytes react to cuprizone intoxication, 3 WT and 3 cuprizone-fed mice data were analyzed. The GSE84113 was used to evaluate sorted microglia reaction to cuprizone administration, 2 WT and 3 cuprizone-treated mice data added to analyze. The GSE48872 dataset was defined to understand how cuprizone-feeding changes gene expressions in isolated oligodendrocyte progenitor cells from mouse brain, 3 WT and 4 cuprizone-treated adult mice progenitor of oligodendrocytes data were analyzed. The list of DEGs was identified in control samples compared to cuprizone-intoxicated samples through statistical analysis given in **Table 2**; lists of the top 20 upregulated and downregulated genes were also provided in

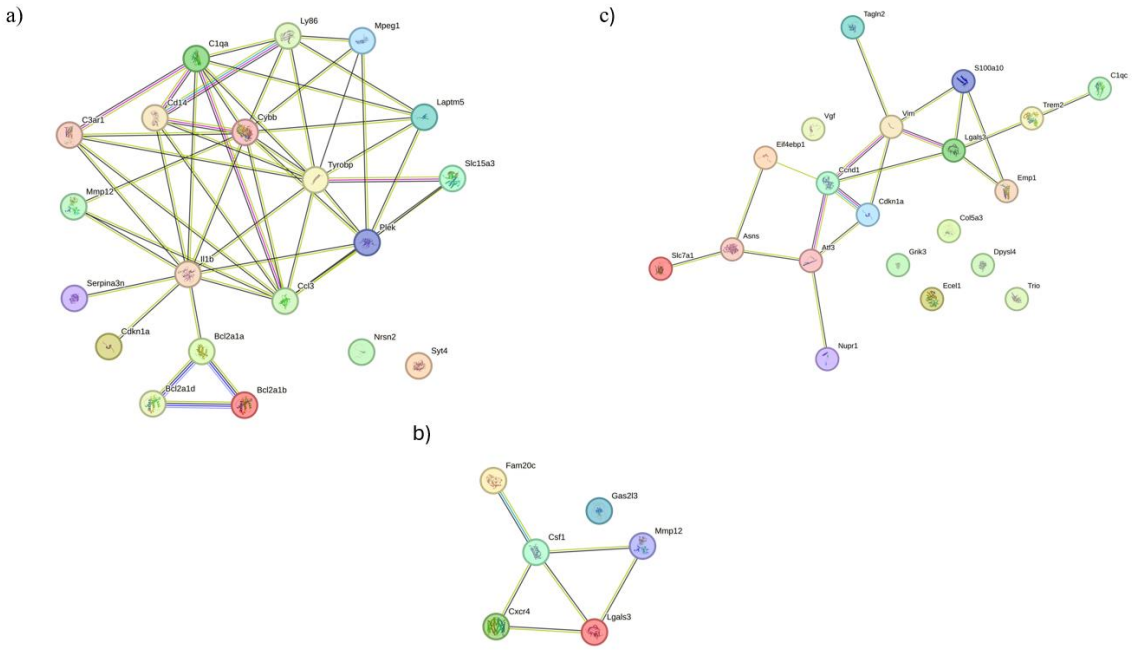

Figure 3. *The STRING Database query of top 20 upregulated genes in each dataset. Top 20 genes are included in the interaction network for GSE134372 (a), GSE84113 (b) and GSE48872 (c).*

Table 3. After analysis of datasets and filtration of unnamed or duplicated expression levels, for GSE134372, 585 DEGs (adj-p value<0.05) were determined while 288 genes were upregulated, and 297 genes were downregulated. In the GSE84113 (adj-p value<0.05), only 6 genes were found as DEGs, and all these genes were upregulated in the cuprizone-intoxicated group. For GSE48872, 344 DEGs (adj-p value<0.05) were founded and among these genes, 269 upregulated and 75 downregulated.

After defining DEGs in each dataset, the top-upregulated 20 protein-protein interactions were evaluated in The String database and given in **Figure 3**.

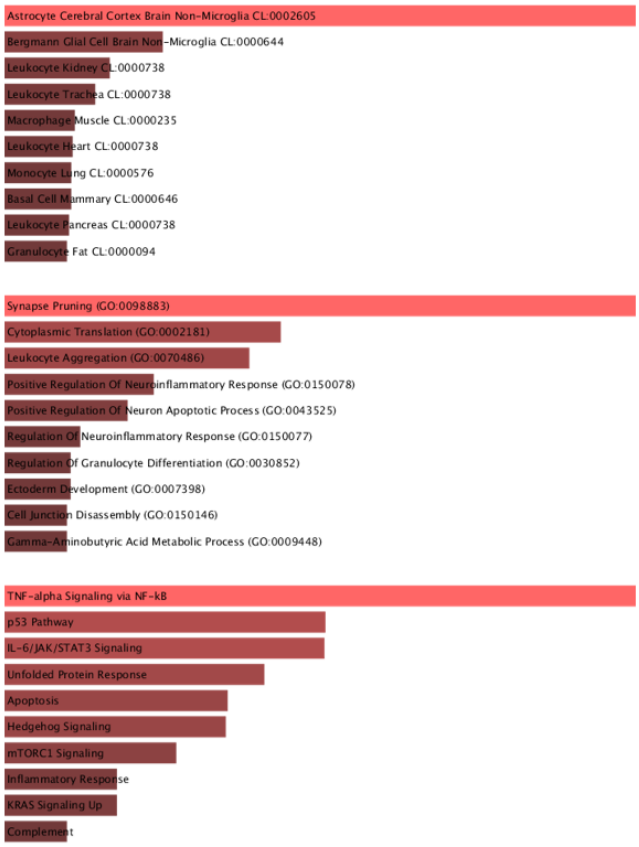

Figure 4. *The bar graphs show Tabula Muris, GO Biological Process 2023 and MSigDB Hallmark 2020 pathway results of DEGs or upregulated genes in GSE134372 dataset.*

The DEGs included for all the datasets to Cell Types (Tabula Muris or CellMarker 2024), only upregulated genes were used to GO Biological Process 2023 and MSigDB Hallmark 2020 pathway queries. For GSE134372 dataset, DEGs were related to astrocytes, however, upregulated genes were also related to different biological processes including Synapse Pruning (GO:0098883), Cytoplasmic Translation (GO:0002181) and Leukocyte Aggregation (GO:0070486), Positive Regulation of Neuroinflammatory Response (GO:0150078) and Positive Regulation of Neuron Apoptotic Process (GO:0043525). Upregulated genes also overlapped with TNF-alpha Signaling via NF-kB, p53 Pathway, IL-6/JAK/STAT3 Signaling, Unfolded Protein Response and Apoptosis pathways given in **Figure 4**.

Figure 5. *The bar graphs show CellMarker 2024, GO Biological Process 2023 and MSigDB Hallmark 2020 pathway results of DEGs or upregulated genes in GSE84113 dataset*

For GSE84113 dataset, DEGs were related to microglia, however, upregulated genes were also related to different biological processes including Chemokine (C-X-C Motif) Ligand 12 Signaling Pathway (GO:0038146), Regulation of T Cell Activation via T Cell Receptor Contact with Antigen Bound to MHC Molecule on Antigen Presenting Cell (GO:2001188), Regulation of Endothelial Cell-Matrix Adhesion via Fibronectin (GO:1904904), Positive Regulation of Microglial Cell Migration (GO:1904141) and Regulation of Mononuclear Cell Migration (GO:0071675). Upregulated genes also overlapped with Complement, Cholesterol Homeostasis, IL-6/JAK/STAT3 Signaling, Interferon Alpha Response and PI3K/AKT/mTOR Signaling pathways given in **Figure 5**.

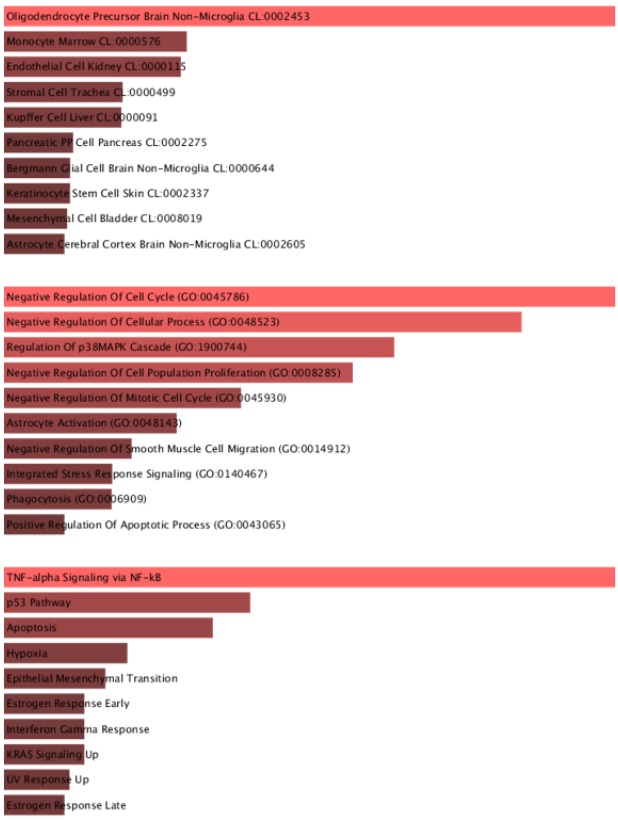

Figure 6. *The bar graphs show Tabula Muris, GO Biological Process 2023 and MSigDB Hallmark 2020 pathway results of DEGs or upregulated genes in GSE48872 dataset.*

For GSE48872 dataset, DEGs were related to oligodendrocytes, however, upregulated genes were also related to different biological processes, including Negative Regulation of Cell Cycle (GO:0045786), Negative Regulation of Cellular Process (GO:0048523), Regulation of p38MAPK Cascade (GO:1900744), Negative Regulation of Cell Population Proliferation (GO:0008285) and Negative Regulation of Mitotic Cell Cycle (GO:0045930). Upregulated genes also overlapped with TNF-alpha Signaling via NF-kB, p53 Pathway, Apoptosis, Hypoxia and Epithelial Mesenchymal Transition pathways given in **Figure 6**.

Discussion

MS is one of the most common neurological autoimmune diseases affecting the central nervous system. The most common symptoms are primarily motor and sensory disorders. The disease affects generally white matter of the brain, brainstem and spinal cord of the central nervous system (CNS) and can be seen in more than one region, with different clinical findings in each region. Although the etiopathogenesis of the disease has not been fully elucidated, it is thought that neuroinflammation and demyelination of the myelin sheath covering neurons, increased inflammatory response, and genetic mechanisms triggered by environmental factors trigger the disease on an immunological basis. Hence, most of the MS studies focused on neurodegeneration and demyelination mechanisms. There are current methods to induce demyelination in animal models such as cuprizone-containing chow feeding for several weeks or experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) immunization and pertussis toxin injection. While cuprizone is a useful model of demyelination the pathophysiology of EAE is based on the immune system's reaction to brain-specific antigens, which initiates inflammation and destruction of antigen-bearing structures, resulting in neurological and pathological features comparable to those observed in MS patients [24]. In the cuprizone-induced MS model, the copper-chelating agent cuprizone is fed to adult male mice for a period of 1-5 weeks (acute) or up to 12 weeks (chronic) and leads to a selective loss of oligodendrocytes, demyelination, and both microgliosis and astrocytosis. As a result of these intrinsic pathological processes of the brain, axons are damaged [25].

Since almost all processes of cuprizone-induced acute or chronic demyelination are associated with glial cells, three different datasets representing three cell types of glia in the cuprizone-induced demyelination model in mice were included in this study. Within the scope of the study, it was shown that the expressions of the target genes or pathways of cuprizone-induced demyelination model in three glial cell types with bioinformatic analyses. With the CTD query, cuprizone or cupper interacted genes collected, when cell type and pathway analysis was done to most interacted genes, it was observed that cuprizone-interacted genes mainly located in oligodendrocytes, and interacted genes are mainly related to reactive oxygen species pathway and secondly related to IL-6/JAK/STAT3 signaling pathway. It is known that this pathway is active in other inflammatory diseases such as fibromyalgia [26]. After query of cupper-interacted genes, it was shown that cupper-interacted genes also mainly in neurons, and interacted genes are mainly related to reactive oxygen species pathway and secondly related to apoptosis pathway. These two pathways are also claimed in the pathogenesis of several diseases [27]. It is well known that cuprizone-intoxication activated astrocytes and microglia cell numbers in lesion areas, especially in the corpus callosum [28]. The effects of cuprizone in the acute demyelination model on white matter astrocytes' gene expression levels were investigated with GSE134372 dataset values. When the dataset values were analyzed with Enrichr, it was found that upregulated and downregulated genes are mainly related to astrocyte cell types, and upregulated genes were overlapped to

TNF-alpha Signaling via NF-kB, p53 and IL-6/JAK/STAT3 Signaling pathways. Although less expression data was found in GSE84113 dataset, the pathway analysis confirmed a former study that focused on the role of the cholesterol homeostasis pathway is important for remyelination processes in microglia [29]. When this dataset was evaluated with only p-value ($p<0.05$ and $Log2(FC)>1$) instead of adj-p-value ($p<0.05$), top ten upregulated genes were found as Spp1, Cst7, Lpl, Itgax, Apoc1, Gpnmb, Ch25h, Fn1, Mmp12 and Axl. However, to keep the similar analysis method for all these three datasets with GEO2R web tool, this was not prioritized for the current study. After the pathway analysis of the microglia dataset Complement, Cholesterol Homeostasis and IL-6/JAK/STAT3 Signaling pathways stood out. When pathway analysis repeated out these top upregulated genes, the first pathway also didn't change, and it was Complement pathway. In GSE48872 dataset, gene expression changes after the acute demyelination model evaluated in adult mice OPCs and upregulated genes also overlapped with TNF-alpha Signaling via NF-kB, p53 and Apoptosis pathways. Oligodendrocyte apoptosis is accepted as the first stage of the cuprizone-induced demyelination model, and astrocyte and microglia activation follows oligodendrocyte apoptosis in several reports [9,28]. The current study confirmed that oligodendrocyte cell death is the first cause of cuprizone-intoxication and apoptosis pathways were active, and for astrocytes and microglia with different signaling pathways it was shown that inflammatory mechanisms were pivotal role for the maintenance of activated microglia and astrocytes in cuprizone-model when these three different datasets evaluated individually. In order to prevent oligodendrocyte cell death and increase cell viability after cuprizone-intoxication, blocking reactive oxygen species pathway may be a strategic approach [30–32]. On the other hand, targeting TNF-alpha Signaling via NF-kB and/or IL-6/JAK/STAT3 Signaling pathways may also be considered to keep astrocytes and microglia away from inflammatory response signals [33].

Conclusion

To elucidate the intricate mechanisms underlying the pathophysiology of MS and to assess the specific roles of the three glial cell types in this pathological process, including an early phase of the disease, as well as to devise novel therapeutic strategies targeting cuprizone-mediated demyelination, comprehensive datasets and studies incorporating large sample sizes, particularly depicting the early onset of MS, are imperative.

Conflicts of Interest

There is no conflict of interest for the publication of this article.

Ethics Committee Approval

The study is not subject to ethics committee approval.

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Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper and that they have approved the final version.

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