

Chemically Induced Brain Cancer in Sprague-Dawley Rats: Changed Lipidomics Mimics the Human Conditions

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Abstract: Malignant gliomas are one of the most treatment-refractory cancers. Development of resistance to chemo- and radiotherapies contributes to these tumors' aggressive phenotypes. Elevated lipid levels in gliomas have been reported for the last 50 years. However, the molecular mechanisms of how tumor tissues obtain lipids and utilize them are not well understood. In our study, 48.6% of phosphatidylcholines were significantly changed during an early stage of brain cancer in females, and 66.2% in males. As for lysophosphatidylcholines 57.1% metabolites were significantly changed in female, and 64.3% in male rats. We observed the most interesting results in the group of sphingomyelins, where 85.8% metabolites were significantly elevated during brain cancer. According to VIP projection, the most important metabolites were: PC ae C40:3, PC ae C38:1, PC ae C30:1, PC ae C38:3, PC ae C44:3, PC aa C40:2, PC aa C42:0, PC ae C30:2, SM C20:2, PC aa C42:1 in females, and PC ae C38:1, PC ae C40:3, PC ae C30:1, PC ae C42:1, SM C20:2, PC aa C34:4, PC ae C38:4, PC aa C32:2, PC aa C38:5, lysoPC a C14:0. The identification of lipid biomarkers during the early stage of cancer could improve patient prognosis.

Keywords: Brain cancer, metabolomics, lipid metabolism, phosphatidylcholines, lysophosphatidylcholines, sphingomyelins.

INTRODUCTION

Brain and other central nervous system (CNS) tumors are among the most fatal cancers and account for substantial morbidity and mortality [1]. Although primary malignant brain and other CNS tumors are rare, they account for a disproportionate burden of cancer mortality because of their high fatality rate; only one-third of individuals survive at least 5 years after diagnosis [2]. The most malignant glioma form of brain cancer is glioblastoma multiform (GBM). It is based on astrocyte cells, is usually located in the cerebral hemispheres, and mainly affects adults. It arises either *de novo* or less often by malignancy of lower grades of astrocytomas (WHO grade II - astrocytoma with a lower degree of malignancy, WHO grade III - anaplastic astrocytoma). GBM therapy is a palliative surgical, radiotherapeutic and chemotherapeutic solution [3]. GBM is characterized by poor prognosis, low survival rates, and extremely limited opportunities for therapy. Due to the absence of effective surgical and medical treatments currently available for GBM, an early diagnosis coupled with an accurate tumor classification is of key importance to select a personalized treatment [4, 5]. An important aspect that should be considered in the diagnosis of brain tumor is its high internal

heterogeneity, which is characteristic of both newly detected and recurrent tumors [6]. For example, morphologically different brain tumor cells exhibit different *in vitro* invasion, as well as cell migration abilities, depending on the nature of the surrounding microenvironment [7, 8]. The identification of changes in concentrations of small molecules or low-molecular compounds in cancer cells, compared with normal cells, can be used as diagnostic and prognostic markers, as well as for a correct classification of brain cancer type [9, 10]. The category of small molecules includes cell lipids, metabolites, organic compound, and monomers able to rapidly diffuse across cell membranes, thus reaching intracellular and extracellular spaces [11].

Animal modeling for primary brain tumors has undergone constant development over the last 60 years, and significant improvements have been made recently with the establishment of highly invasive glioblastoma models [12]. Numerous animal models have been developed to study brain tumor initiation and development. The models can be divided into 3 categories: (1) chemically induced models, (2) genetically engineered mouse (GEM) models, including virally induced models, and (3) xenograft models [12]. Such models have made contributions to the understanding of the mechanisms related to tumor

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initiation and progression. However, many of them (such as GEM models or xenografts) have only to a limited extent been translated into more effective treatment principles. These types of models failed in a clinical setting predominantly due to several factors, such as: (1) The tumor models do not reflect the biological properties of the patient tumors, (2) the animals used do not display the same pharmacokinetics as humans [13], and (3) the tumors established do not reflect the cellular heterogeneity of human tumors [12]. Chemically induced brain cancer allows for *in vivo* modeling of brain tumors with similar histopathology, etiology, and biology as in humans. The aim of our study was to monitor the changes in lipidomics during chemically induced brain cancer in Sprague-Dawley rats, in respect with sex-differences and also sex-independently.

MATERIALS AND METHODS

Animals and Conditions

Parental generation of Sprague Dawley rats (Velaz, Praha, Czech Republic) (10 females, 5 males) was used. Animals were adapted to standard vivarium conditions with a temperature of 21 – 24°C, relative humidity of 50 – 65% and to a 12:12 hour light:dark regimen. Animals were fed standard rat pellets Altromin 1328 (Velaz, Praha, Czech Republic) according to EU animal feed legislation and guidance and had free access to tap water. Parental females have mated with parental males. For further experiments, the progeny was used. The animals were handled by the guidelines established by Law No. 377 and 436/2012 of Slovak Republic for the Care and Use of Laboratory Animals and approved by the State Veterinary and Food Administration of the Slovak Republic (Approval Number: Ro-2219/19-221/3).

Experimental Design

Pregnant females were divided into two groups. One group served as a control, and one was described as GBM group (tumor-bearing animals). Ethyl-Nitroso-Urea (ENU) was administered as one intraperitoneal dose (100mg/kg b.w.) to pregnant GBM females at 15th day of pregnancy as described before [14-16]. After the birth, the progeny was kept with the mothers. At the age of 30 postnatal days, the progeny was divided according to sex. At the age of 4 months, the rats were euthanized, the brains were excised and also the blood was taken to see the differences in lipidomics.

Blood Collection and Metabolomics Measurement

The blood from all experimental animals was collected at one-time point from *vena caudalis* in a total volume of 100 µL into microtubes. The place of collection was treated with a disinfectant. After isolating, blood serum was stored at –80°C. Frozen serum was thawed on ice and used for further analysis. The samples were measured by AbsoluteIDQ p180 kit (BIOCRATES Life Sciences AG, Innsbruck (Austria). Flow injection analysis (FIA) and liquid chromatography-tandem mass spectrometry-based (LCMS/MS) targeted metabolomics measurement of a selected group of lysoPCs, PCs and SMs was performed on serum samples. The fully automated assay was based on PITC (phenylisothiocyanate) derivatization in the presence of internal standards followed by FIA-MS/MS and LC-MS/MS using a SCIEX 4000 QTRAP® (SCIEX, Darmstadt, Germany) or a Waters XEVO™ TQMS (Waters, Vienna, Austria) instrument with electrospray ionization. The assay was based on the principle described in the study of Pena et al. [17]. Determined values were log2-transformed to obtain normally distributed data and to stabilize the variance.

Statistical Analysis

Quantification of metabolite concentrations and quality assessment was performed using the MetIQ software package (BIOCRATES Life Sciences AG, Innsbruck, Austria). Internal standards served as the reference for the metabolite concentration calculations. Univariate (t-test) and multivariate statistics (partial least squares-discrimination analysis PLS-DA) as well as the variable importance in projection (VIP) plot, were performed using Metabo Analyst 3.0 (24). Cross validation of PLS-DA classification applied 5 number of components for selection optimal number of components. LOOCV cross validation method was used. It has been considered performance of measures as Accuracy, R², Q². As a part of PLS-DA method, a VIP score was measured. VIP score is a measure of a feature's importance in the PLS-DA model. It summarizes the contribution a feature makes to the model. The VIP score of a feature is calculated as a weighted sum of the PLS weight. PLS weight is the squared correlations between the PLS-DA components and the original feature. Tables, heat map and box plots were performed using Graph Pad 8.0 (Graph Pad Software, Inc., San Diego, CA, USA) and programming language R (version 3.6.0) with standard library and libraries ggplot2 (version 3.1.1), ggpvr (version

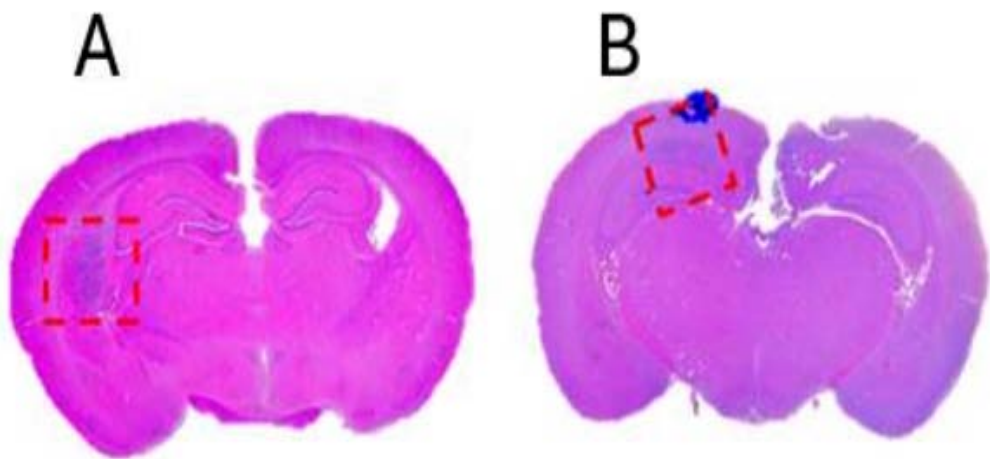


Figure 1: ENU-induced solid tumors. Staining with HE. A: Brain tissue with a solid tumor in the perihippocampal region of the brain in the deep white matter of the brain; 40x magnification, solid tumor indicated by the red box. B: Brain tissue with two solid tumors in the corpus callosum region in the deep white matter; 40x magnification, two solid tumors indicated by the red box.

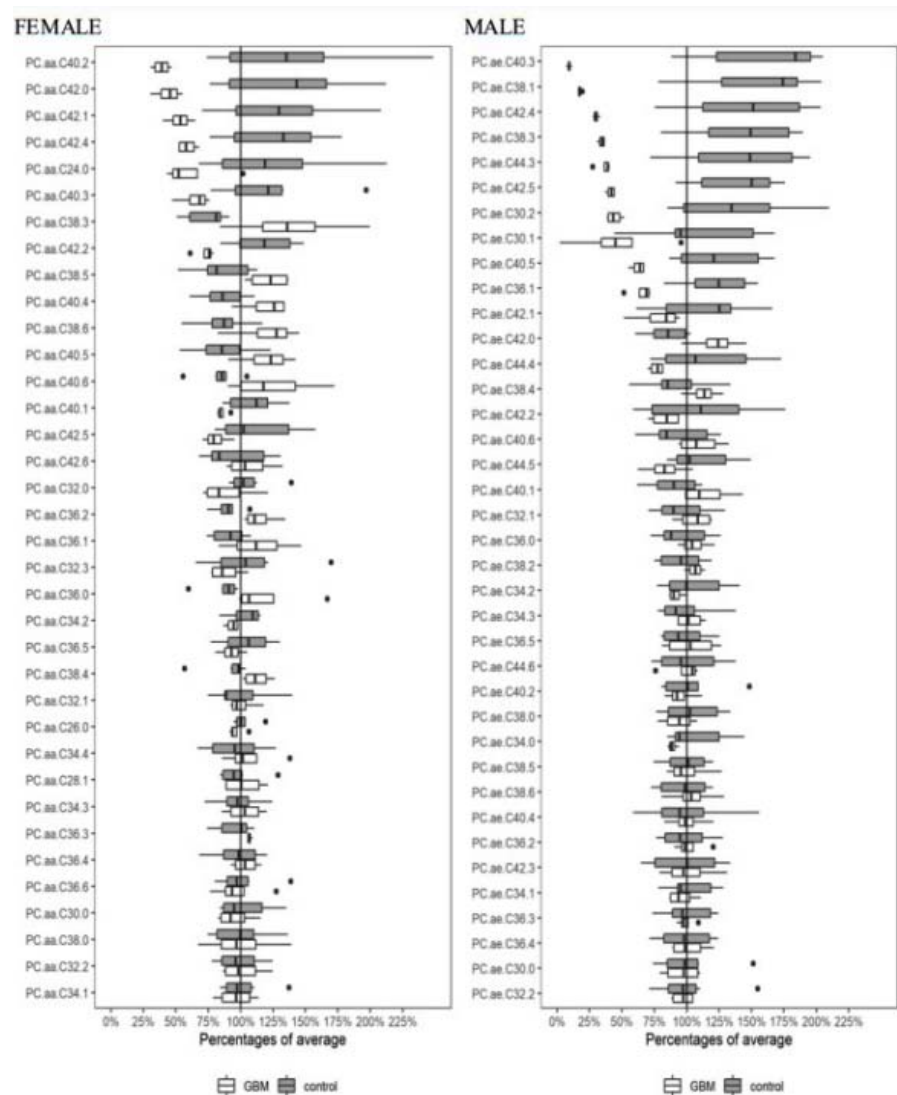


Figure 2: Polar plots of differences in phosphatidylcholines averages of glioblastoma female and male animals to averages of control female and male animals. Values are normalized; the inner circle represents the value of average. Figure was generated from "R statistics" software.

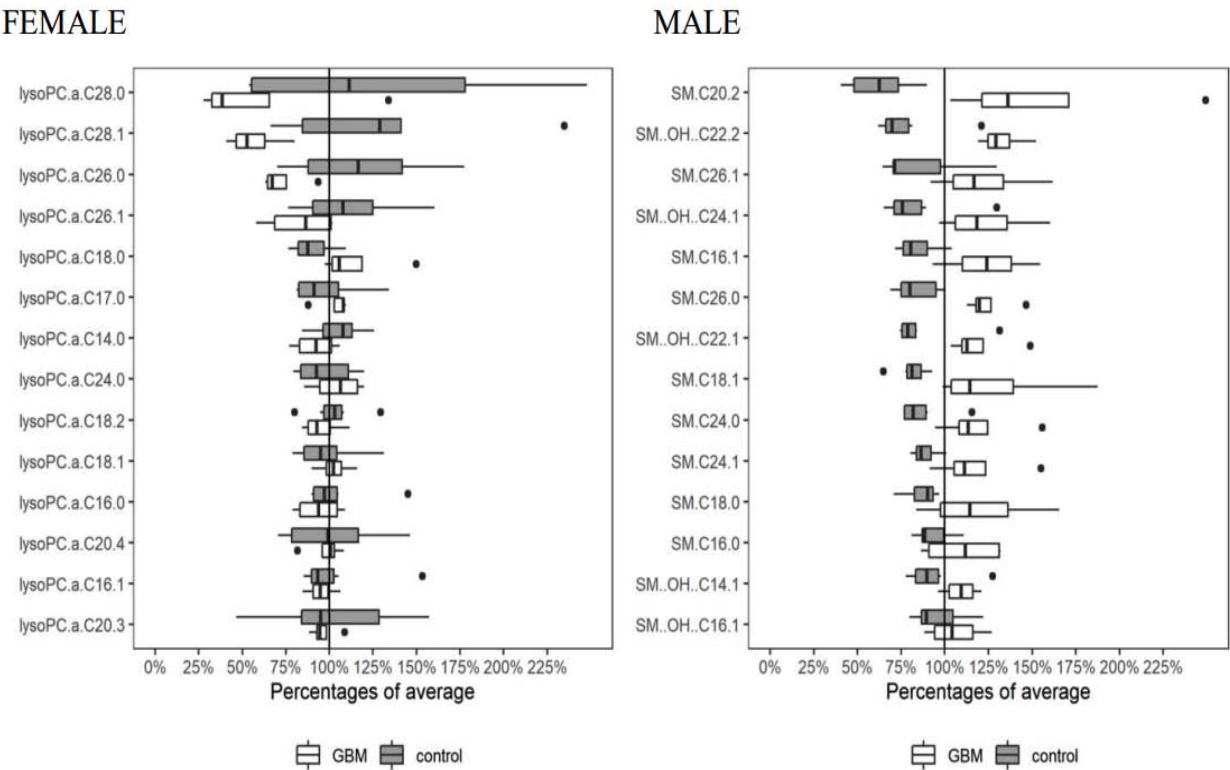


Figure 3: Polar plots of differences in lysophosphatidylcholines and sphingomyelins averages of glioblastoma female and male animals to averages of control female and male animals. Values are normalized; the inner circle represents the value of average. Figure was generated from “R statistics” software.

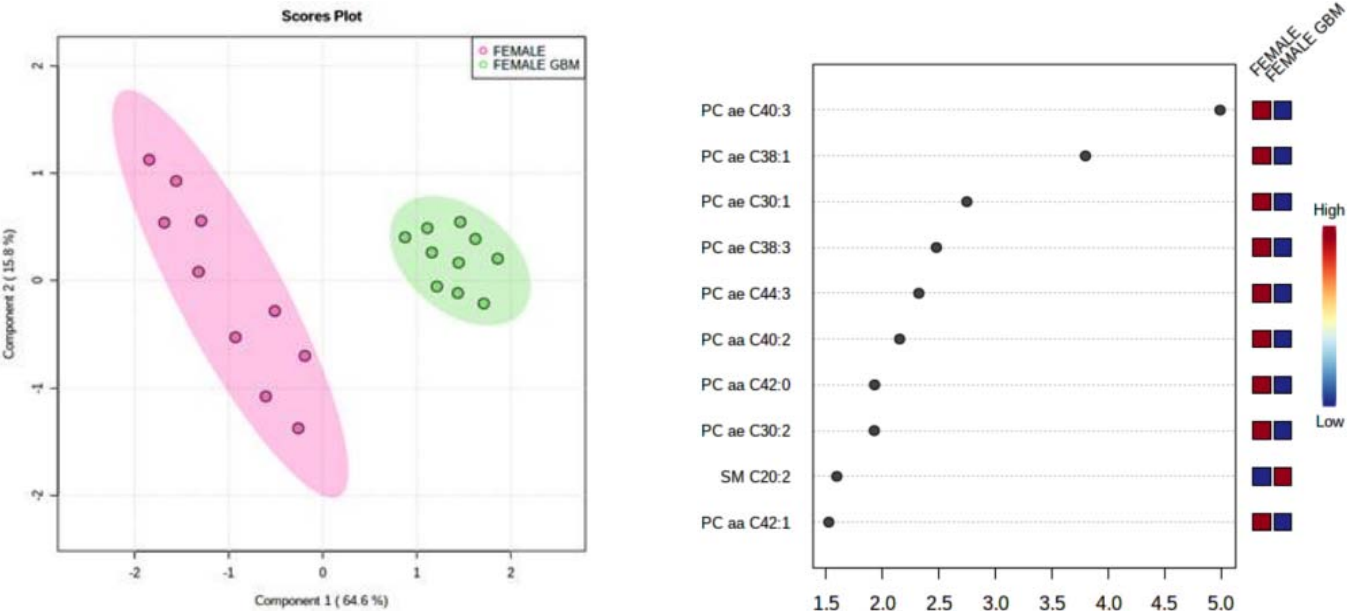


Figure 4: Partial least squares-discrimination analysis (PLS-DA) of selected metabolites in GBM and intact female. In the graphical output, 95% confidence ellipses for specific groups are included.

Figure 5: Variable importance in projection (VIP) plot, calculated from PLS-DA method, displays the top 10 most important metabolite features identified by PLS-DA. Boxes on right indicate relative concentration of corresponding metabolite in blood in descending order of importance. VIP is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension. The most important features have the VIP values of >1.5.

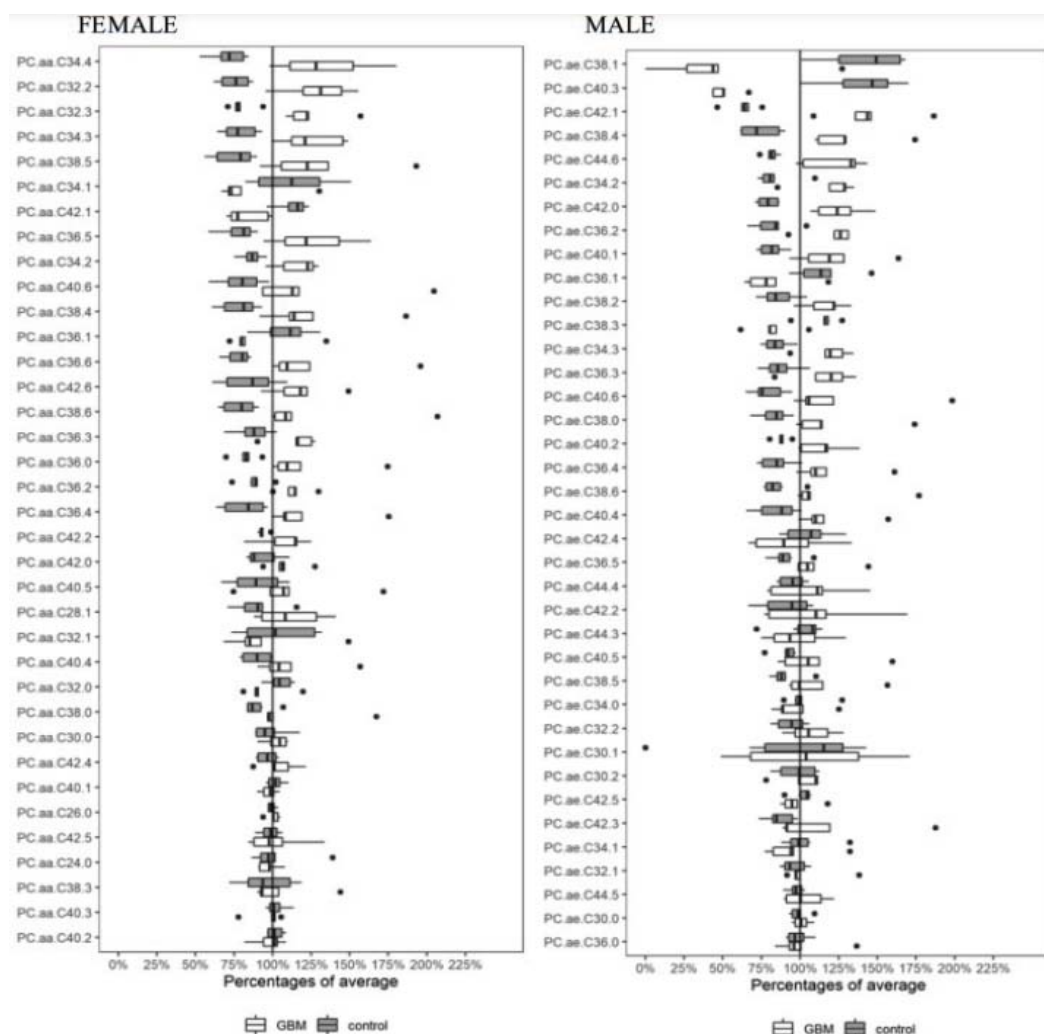


Figure 6: Polar plots of differences in phosphatidylcholines averages of glioblastoma female and male animals to averages of control female and male animals. Values are normalized; the inner circle represents the value of average. Figure was generated from “R statistics” software.

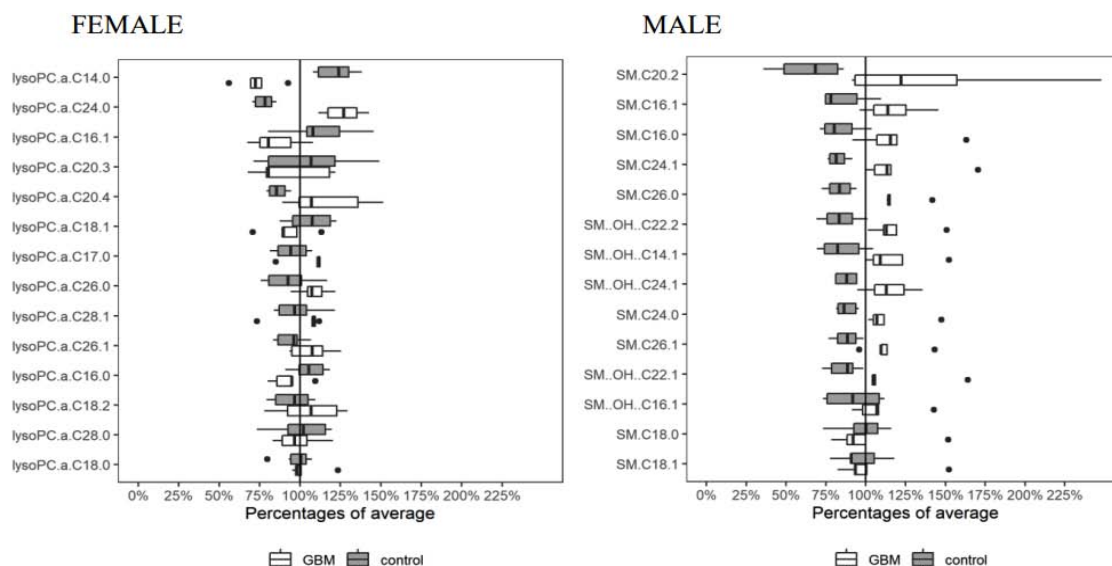


Figure 7: Polar plots of differences in lysophosphatidylcholines averages of glioblastoma female and male animals to averages of control female and male animals. Values are normalized; the inner circle represents the value of average. Figure was generated from “R statistics” software.

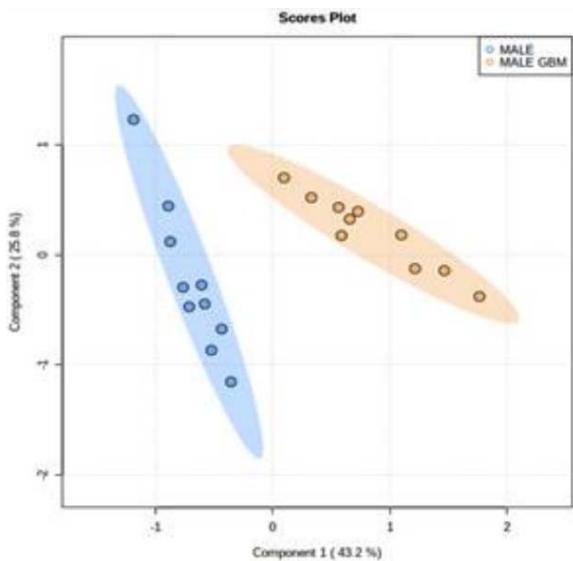


Figure 8: Partial least squares-discrimination analysis (PLS-DA) of selected metabolites in GBM and intact male. In the graphical output, 95% confidence ellipses for specific groups are included.

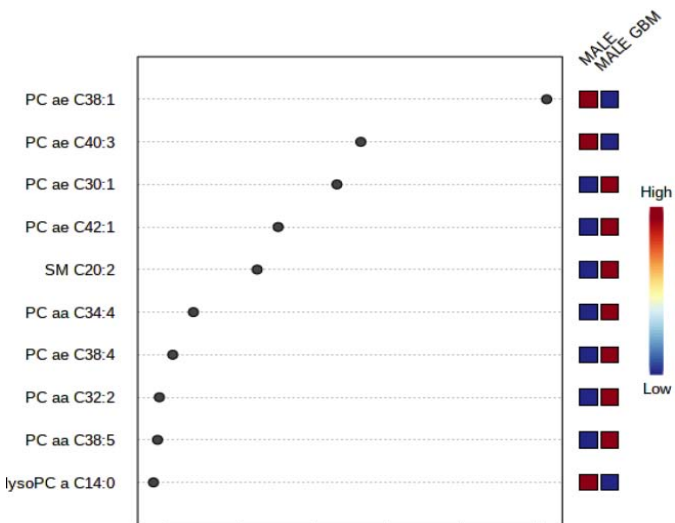


Figure 9: Variable importance in projection (VIP) plot, calculated from PLS-DA method, displays the top 10 most important metabolite features identified by PLS-DA. Boxes on right indicate relative concentration of corresponding metabolite in blood in descending order of importance. VIP is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension. The most important features have the VIP values of >1.5.

Table 1: Important Features Identified by Fold Change Analysis. Table was Created in Metaboanalyst Software

	Compounds	Fold Change	Log2(FC)
1	PC ae C40:3	0.083717	-3.5783
2	PC ae C38:1	0.16185	-2.6272
3	PC ae C42:4	0.25423	-1.9758
4	PC ae C38:3	0.31453	-1.6687
5	PC ae C44:3	0.36375	-1.459
6	PC ae C40:2	0.39181	-1.3518
7	PC ae C42:5	0.39875	-1.3265
8	SM C20:2	2.3005	-1.202
9	PC ae C42:0	0.46668	-1.0995
10	PC ae C30:2	0.47993	-1.0591
11	PC ae C42:1	0.49718	-1.0082

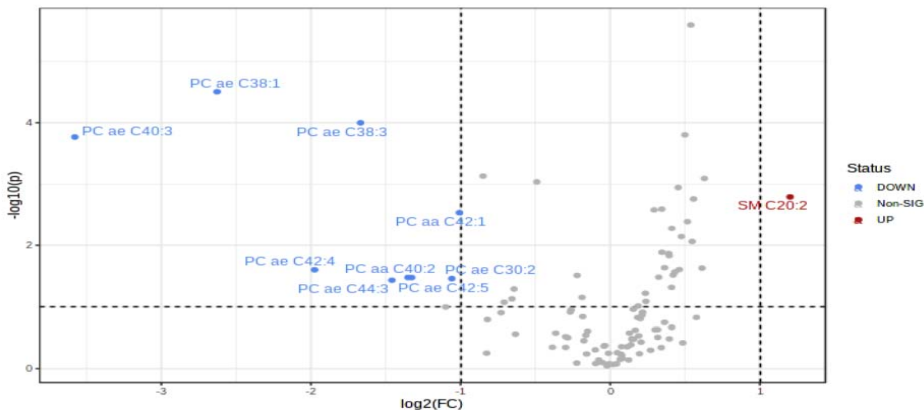


Figure 10: Important features selected by volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.1. The red circles represent features above the threshold. Note both fold changes and p values are log transformed. The further its position away from the (0,0), the more significant the feature is.

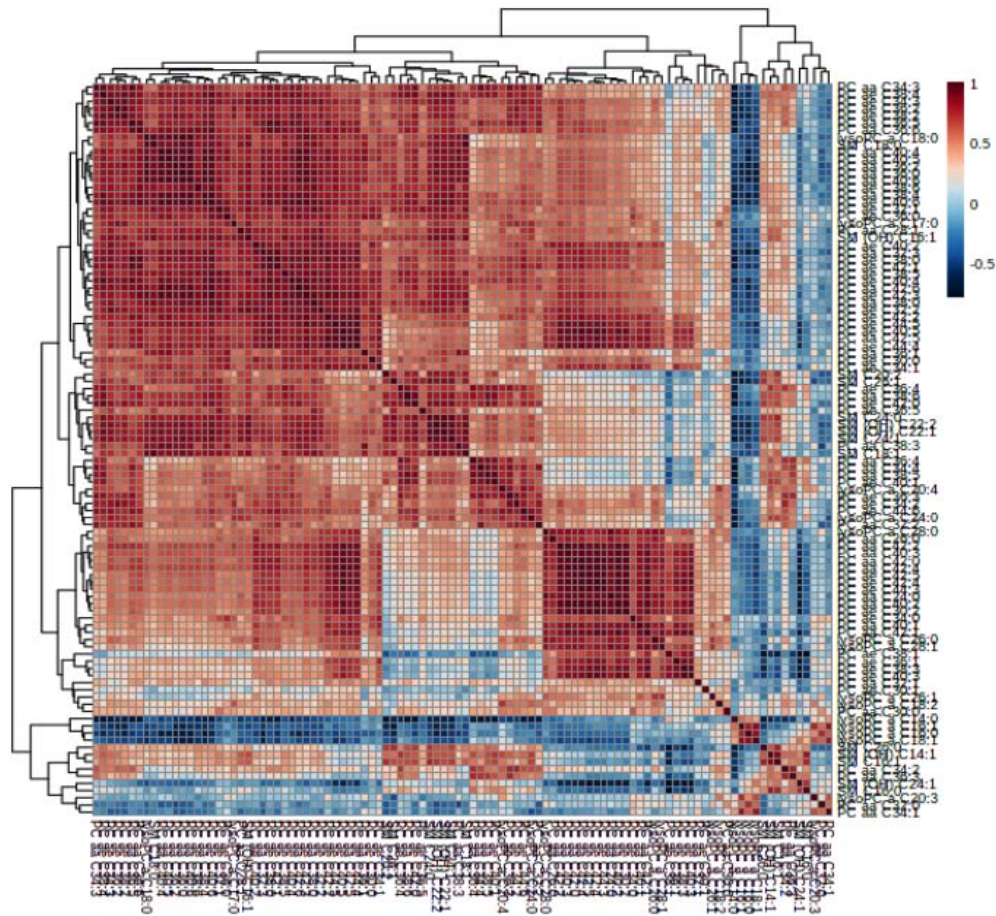


Figure 11: Correlation heatmap as a graphical representation of correlation matrix presenting the correlation between variables. R values are shown as different degree of color intensity (red, positive correlations; blue, negative correlation).

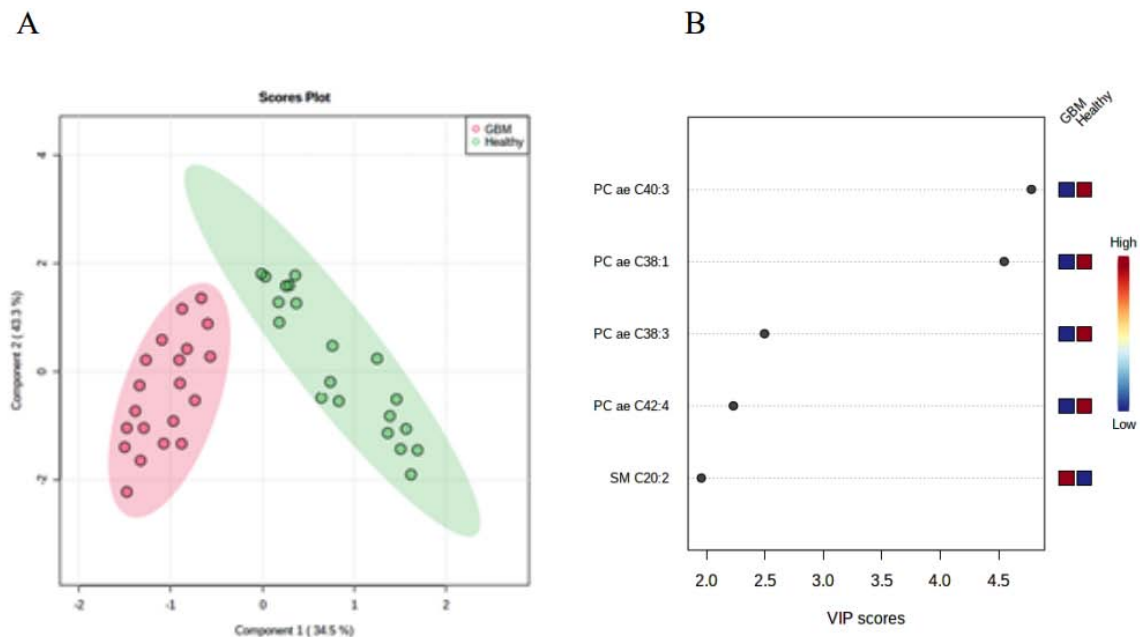


Figure 12: A) Partial least squares-discrimination analysis (PLS-DA) of selected metabolites in GBM and intact male. In the graphical output, 95% confidence ellipses for specific groups are included. **B)** Variable importance in projection (VIP) plot, calculated from PLS-DA method, displays the top 5 most important metabolite features identified by PLS-DA. Boxes on right indicate relative concentration of corresponding metabolite in blood in descending order of importance. VIP is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension. The most important features have the VIP values of >2.0.

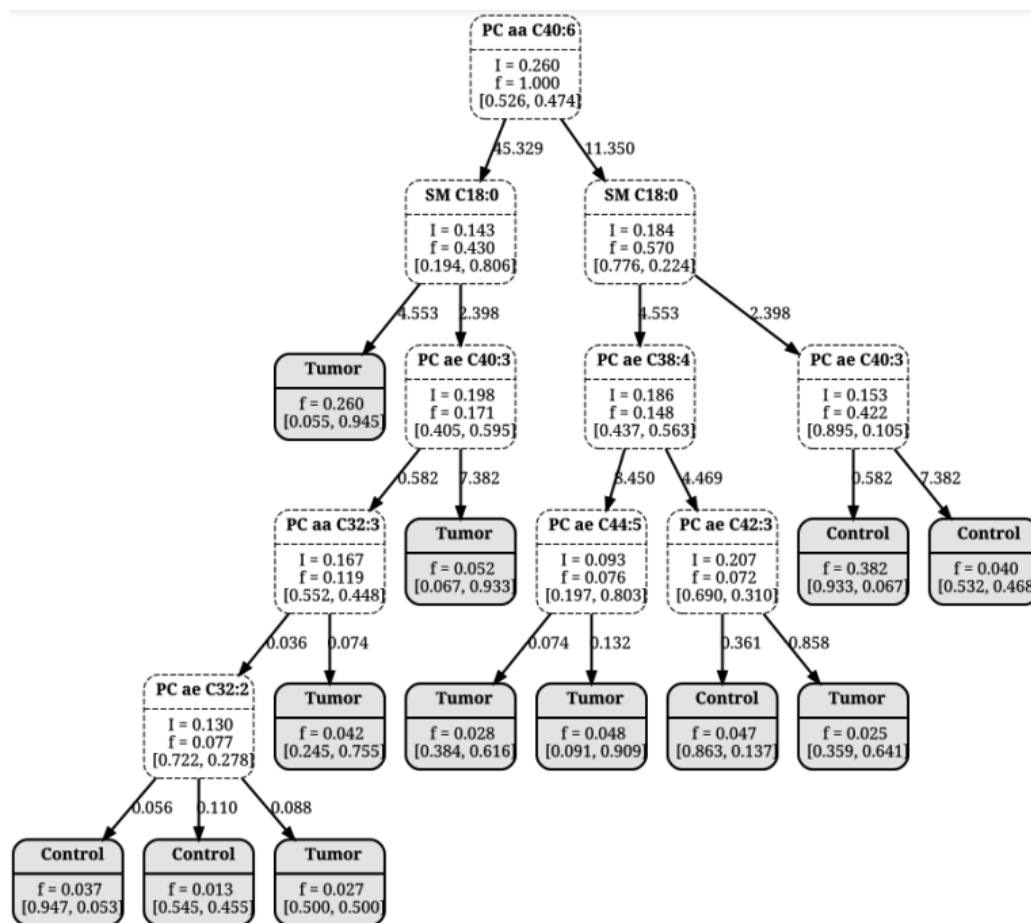


Figure 13: Fuzzy decision tree.

0.2),psych (version 1.8.12) and G Gally (version 1.4.0). Fuzzy decision tree was made to visualize the data that incorporate fuzzy logic principles. Analysis of the structure of the FDT was done to determine how frequently each attribute is used in the decision-making process. Attributes that appear higher in the tree or are used at multiple levels are generally considered more important.

RESULTS

Brain Tumors

ENU-induced tumors were predominantly solid and localized in white and grey matter. They formed early stages of tumors.

Lipidomics

A total of 102 lipid metabolites from the groups of phosphatidylcholines (PCs), lysophosphatidylcholines (lysoPCs) and sphingomyelins (SMs) were measured. The comparison of measured metabolites was done in healthy and GBM animals, both dependently and independently on the sex group.

In the group of females, a total of 36 out of 74 PCs were significantly changed during an early stage of brain cancer, which represents 48.6%. Of these, 16 (44.4%) had higher levels in GBM females while the remaining 20 (55.6%) had higher levels in intact females. In the group of lysoPCs, 8 of 14 (57.1%) metabolites were significantly changed, 2 (25%) had higher levels in GBM females and 6 (75%) had higher levels in the group of intact females. We observed the most interesting results in the group of sphingomyelins, where 12 of 14 (85.8%) metabolites were significantly elevated during brain cancer (GBM group).

In the group of males, a total of 49 out of 74 phosphatidylcholines were significantly changed, which represents 66.2%. Of these, 41 (83.6%) had higher levels in GBM males while 8 (16.4%) had higher levels in intact males. In the group of lysophosphatidylcholines 9 of 14 (64.3%) metabolites were significantly changed, 5 (55.6%) had higher levels in GBM males and 4 (44.4%) had higher levels in the group of intact males. We observed the most interesting results in the group of sphingomyelins again, where 11 of 14 (78.6%) metabolites were

significantly changed. All of them showed elevated levels in GBM males.

When handling the data sex-independently with the aim to find a metabolite that characterizes the disease during the early stages regardless of sex, we revealed that many features are common in males and females. According to fold change analysis (Table 1) and volcano plot analysis (Table 1 and Figure 10), an important up-regulated feature found during brain cancer in rats was the metabolite SM C20:2.

Other, predominantly PCs were down-regulated (PC ae C38:1; PC ae C38:3; PC ae C40:3; PC aa C42:1; PC aa C40:2; PC ae C42:4; PC ae C42:5; PC ae C30:2; PC ae C44:3).

Table 2: Attribute Importance Analysis with Corresponding Importance Value Shown as “P” Value

Attribute	Importance
PC aa C32:3	0.091
PC aa C38:5	0.034
PC aa C40:1	0.031
PC aa C40:4	0.014
PC aa C40:6	0.692
PC aa C42:0	0.145
PC ae C32:2	0.325
PC ae C38:4	0.327
PC ae C40:3	0.079
PC ae C40:4	0.070
PC ae C42:0	0.016
PC ae C42:3	0.189
PC ae C42:5	0.034
PC ae C44:4	0.620
PC ae C44:5	0.232
SM (OH) C22:2	0.014
SM C18:0	0.773

According to VIP score, calculated from PLS-DA method, displays the top 5 most important metabolite features. During early stages of brain cancer, PC ae C40:3, PC ae C38:1, PC ae C38:3 and PC ae C42:4 have been found to be decreased and are exclusive markers. On the other hand, another important feature is SM C20:2, increased significantly during brain cancer.

The Table 2 presented below shows importance of attributes calculated from the decision tree. Each row

represents a specific attribute, and the corresponding importance value is provided in the 'Importance' column. This information allows us to assess the significance of different attributes in the context of the selected task.

According to analysis of attribute importance, the most important attributes are SM C18:0, PC ae C44:4, PC ae C38:4, PC ae C32:2, PC aa C40:6.

DISCUSSION

Since 2007, the World Health Organization (WHO) has classified gliomas based on their cell type and aggressiveness, with Class I consisting of benign tumors, and Class IV comprising the most aggressive types of tumors. GBM is a Class IV brain tumor [18]. The early diagnosis of brain cancer remains a challenge, as newly proposed drugs must meet specific requirements, such as being able to cross the blood-brain barrier (BBB) and efficiently infiltrating the tumor [19]. In 2016, the WHO introduced new guidelines for the diagnosis of brain gliomas based on new genomic markers. The addition of these new markers to the pre-existing diagnostic methods provided a new level of precision for the diagnosis of glioma and the prediction of treatment effectiveness. Yet, despite this new classification tool, GBM, a grade IV glioma, continues to have one of the highest mortality rates among CNS tumors. Metabolomics is a particularly promising tool for the analysis of brain tumors and potential methods of treating them, as it is the only “omics” approach that can provide a metabolic signature of a tumor’s phenotype [20].

When studying brain cancer in humans, there are many uncontrollable factors such as medication history, age of the patient or living conditions. In this regard, animal models provide an essential step for examining neural circuitry or molecular and cellular pathways in a controlled environment [21]. The aim of our study was to reveal the changes in lipidomics during early stages of chemically induced brain cancer of laboratory Wistar rats, both sex-dependently and –independently.

Rapid growth and division are among the major characteristics of malignant tumors. Given the important role of lipids in cell membrane formation and signaling transduction, identification of the differences in lipid composition between tumor and normal tissues, in order to find possible diagnostic and prognostic biomarkers for cancer patients, has been a long-term endeavor for scientists [22].

The health and function of the nervous system is intimately tied to lipid homeostasis. Thus, it is not surprising that brain is composed of nearly 60% lipid by dry weight, making it the second fattiest tissue in the body, behind adipose tissue [23, 24]. Normal cells use mainly glucose and fatty acids to generate energy and fulfil the requirements for cell growth. However, cancer cells use an altered metabolism to sustain rapid growth. This altered metabolism, where cancer cells use higher levels of glucose to generate energy by anaerobic glycolysis rather than aerobic glycolysis through the tricarboxylic acid (TCA) cycle, is called Warburg effect [25]. Decreased levels of lipids observed in the brain tumor tissue may suggest that it depends on fatty acids as a fuel source in addition to glucose from anaerobic glycolysis. Potentially, an increased level of lipolysis in the brain cancer cell generates energy for cancer cell proliferation and results in an overall decreased level of lipids detected [26, 27]. CNS has specialized pathways for lipid synthesis and degradation related to its specialized physiology and function [26]. Two scenarios may thus result in a decrease in the lipid composition: lipid rich brain tissue replacing less lipid rich tumor cells would decrease the amount of lipids overall while tumor cells would also likely exploit normal physiological pathways related to lipid metabolism in central nervous tissue. There are numerous reports in the literature that fatty acid synthetase is highly upregulated in a variety of cancers including GBM and may be a good therapeutic target [28].

In recent years, studies of the role of sphingolipid metabolism have become an integral part of cancer research. Sphingomyelins (SMs), predominant sphingophospholipids in the outer leaflet of cell membranes, and their hydrolysis by sphingomyelinases are essential to the efficacy of chemo- and radiotherapy [1,2,3,4].

Sphingomyelin is a key component of the plasma membrane that interacts with cholesterol and glycerophospholipids, thereby participating in the formation and maintenance of lipid micro domains. Lipid rafts are important signaling platforms whose structure is sensitive to membrane lipid composition [29], as are the proteins that interact with these and other membrane micro domains [30, 31]. Therefore, modifications in SM content affect lipid raft associated signaling pathways [32].

In our study we observed the most interesting results in the group of sphingolipids, where 12 of 14,

which represent 85.8%, metabolites were significantly changed during brain cancer in females, and 11 of 14, which represent 78.6% in males.

Zhaw *et al.* in their study also identified sphingomyelins as biomarkers of glioma tumors [33].

The sphingolipid pathway plays a key role in the determination of cell fate, making it an attractive drug target in processes such as inflammation, cardiovascular disease, diabetes and cancer [34, 35]. Notably, the current therapy for GBM induces multiple effects on the sphingolipid pathway. Ionizing radiation causes single and double strand breaks in DNA, but also activates acid sphingomyelinase to induce conversion of the sphingomyelin found in cell membranes to ceramide [36, 37]. This enrichment of ceramide in the plasma membrane results in the clustering of cell death receptors, promoting apoptosis [38]. TMZ also causes DNA double-strand breaks and has been shown to promote the accumulation of ceramide in GBM cells [38]. This is consistent with the known effects of many chemotherapies, which often activate ceramide formation through multiple mechanisms, including the activation of ceramide synthases, with this ceramide accumulation playing a major role in the mechanism of action of many of these agents [39-41]. Thus, the current therapies for GBM work in part by altering sphingolipid metabolism to enhance pro-apoptotic ceramide levels. Heightened ceramide metabolism via, for example, the enhanced levels of sphingosine kinases, acid ceramidase or glucosylceramide synthase, commonly observed in many cancers, may clear this elevated ceramide and overcome radio/chemotherapy [39, 40].

In our study, 48,6% of PCs were significantly changed during an early stage of brain cancer in females, and 66,2% in males. As for lysoPCs 57.1% metabolites were significantly changed in females, and 64,3% in males. According to vip projection, the most important metabolites were: PC ae C:38:1, PC ae C40:3, PC ae C30:1, PC ae C42:1, SM C20:2, PC aa C34:4, PC ae C38:4, PC aa C32:2, PC aa C38:5, lysoPC a C14:0. Most important features identified by fold change analysis were PC ae C40:3, PC ae C38:1, PC ae C42:4, PC ae C38:3, PC ae C44:3, PC aa C40:2, PC ae C42:5, SM C20:2, PC aa C42:0, PC ae C30:2, PC aa C 42:1.

We found some studies they support our results. Buentzel *et al.* in their study found significant changes in levels of metabolites PC aa C38:5, PC ae C38:3, PC aa C40:2, where metabolite PC aa C38:5 and lysoPC

C26:0 were associated with significantly shorter overall survival, thus underlining the prognostic relevance of this finding [42]. In accordance, Guo *et al.* had previously described that deviations of lysoPCs were associated with cancer progression [43]. Schmidt *et al.* after controlling for multiple testing found lysoPC a C18:0, PC aa C36:2, PC aa C36:3, PC aa C38:3, PC aa C38:5, PC aa C40:2, PC aa C40:3, PC aa C40:4, PC aa C40:5, PC aa C42:4, PC aa C42:5 and PC ae C40:1, were inversely associated with risk of prostate cancer [44]. In our brain cancer model identical occurred changes in PC aa C40:2, PC ae C30:2, PC aa C42:1 and PC aa C38:5.

CONCLUSION

Our study compares the data from preclinical model of chemically induced brain cancer with those of human. Our results indicate that this model may be used in the research of drug development.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

AUTHOR'S CONTRIBUTION

conceptualization: TK, AL, PS, MK; methodology: AL, PS, NZ, MB, AB, MK, JK, DM, TK; visualization: AL, MB, TK, JK; supervision: TK, MK, JK; writing: AL, TK; writing—review & editing: AL, PS, NZ, MB, AB, MK, JK, DM, TK

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