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Metabolomic Profiling of Chiari Malformation Type I: Comparison of Bioinformatic Programs for Untargeted Analysis

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Introduction

Chiari Malformation Type I (CM) is a neurological disorder that results from herniation of the cerebellum through the foramen magnum into the upper spinal column. Symptoms of CM include neck pain, abnormal gait, declined motor skills, bodily numbness, vertigo, aphasia, headaches or migraines, and trouble with vision.[1,2] Currently, treatments for this disorder are limited to surgical decompression or symptom management with analgesics.[3–5]

Herniation of the cerebellum may cause symptoms through multiple mechanisms including damage to brain tissue or altered cerebral spinal fluid (CSF) flow.[6] We hypothesized that a block in CSF circulation would change the levels of endogenous metabolites in the CSF. From this hypothesis, we have sought to understand how metabolic alterations contribute to Chiari pathology by utilizing mass spectrometry-based (MS-based) metabolomics. Metabolomics is a method that focuses on the comparative analysis of endogenous small molecules (less than 1000 Da), including glycolytic intermediates, nucleic acids, neurotransmitters, and other related metabolites.[7] A comprehensive technology platform combines profiling polar metabolites by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) coupled with shotgun lipidomics to detect membrane and signaling lipids.[8,9] The MS-based profiling requires the use of bioinformatics to identify metabolites based on statistical significance (FDR adjusted p-value) and regulation (fold change) in Chiari CSF versus controls.

A typical LC-MS/MS metabolomic run identifies thousands of changes in biological samples. This leads to the problem of multiple comparisons testing and necessitates the use of methods such as false discovery rate (FDR). In addition, statistical testing can use either parametric or non-parametric methods depending on the sample size and population distribution. Parametric tests compare group means whether the groups have the same or differing variance

but require an assumption of normally distributed data. However, larger sample sizes these tests can still be utilized. Analysis of variance (ANOVA) is a collection of statistical models that analyzes variation between means of two or more groups.[10] Volcano plots use the determined p-values and fold changes from initial data and plot respective spectra data with log based parameters.[11] Non-parametric tests compare group medians if medians better represent the data than the mean for smaller sample sizes or data that is ranked.[12] An additional consideration is that more than one variable is being collected in a sample for each profiling experiment and this necessitates the use of multivariate techniques. Principle Component Analysis (PCA) shows variance between groups by identifying a smaller group of uncorrelated variables from a large dataset. Mapping of this variance facilitates exploratory data analysis of metabolite levels by visualizing the differences between two or more experimental groups.[13] Additional data visualization techniques such as heatmaps which express fold change as changes in color, are also used throughout this report.

This study compared CSF metabolite levels in CM with samples isolated patients with another disorder of CSF flow, normal pressure hydrocephalus (NPH). We then compared three bioinformatic programs to determine which pipeline gave the most robust dataset. In evaluating these programs, we focused on the following parameters: the qualitative identification of the number of endogenous compared to exogenous metabolites, strictness or flexibility in statistical testing, user-interface accessibility, experimental data matching database data, and preciseness between each software.

Methods and Materials

CSF Sample Collection

The human subjects protocol for this study was approved by the institutional review board at the Chiari Institute (Great Neck, NY). CSF was collected from 22 CM patients and 5 NPH patients during surgical intervention. CSF samples were stored at -80°C before metabolite extraction.

Chemicals

HPLC grade reagents of water, methanol, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, US), chloroform was purchased from Alfa Aesar (Ward Hill, MA, US), and ammonium acetate and ammonium hydroxide were purchased from MilliporeSigma (St. Louis, MO, US).

Metabolomic Analysis

Samples were thawed and proteins were separated and removed by adding 4 X methanol to CSF samples. Proteins were then precipitated by setting in -20 °C for 2 hours and then centrifuged at an RCF of 18,000 x g at 4 °C for 20 minutes. Supernatant was collected in separated tube and concentrated in a CentriVap (LACONCO, Kansas, MO, US). Concentrates were then stored at -80 °C until LC-MS analysis. Prior to LC-MS, samples were suspended in 200 μL of 35:65 (v/v) acetonitrile: water solution. Suspended concentrates were separated by HILIC with column (Luna 3 μ NH2 100Å, 150mm×1.0mm, Phenomenex, Torrance, CA, US) using a Micro200 LC system (Eksigent, Redwood, CA, US). Two mobile phases were used to construct the gradient, mobile phase A was 5 mM NH_4OAc, 5 mM NH_4OH, and water, and mobile phase B (pH 8.4) was 5 mM NH_4OAc, 5 mM NH_4OH, and acetonitrile. To separate metabolites, a linear gradient was used as follows: 0 min at 98%, 0.5 min at 98%, 1 min at 95%,

5 min at 80%, 6 min at 46%, 13 min 14.7%, 17 min at 0%, 17.1 min at 100%, and 23 min at 100%.

Samples were analyzed on the 5600^{+} TripleTOF Mass Spectrometer (SCIEX, Framingham, MA, US) and detected in negative and positive mode using Information Dependent Acquisition (IDA). Parameters were optimized for ionization and set to the following conditions: 15 psi nebulizer gas (GS1), 20 psi heater gas (GS2), and 25 psi curtain gas (CUR). The specific mass range selected for observation was 60 to 1,000 Da for TOF MS. Precursor ion acquisition was performed with a 250 ms accumulation time. The elimination of spectra with low signal noise (S/N) was done at 10 count/s background threshold for targeted ion selection. The ionization voltage for positive mode was +5000 V ionspray voltage (ISV) and +100 V declustering potential (DP). Collision energy spread (CES) was +(25-40) for MS/MS fragmentation and negative mode, -4500 V ISV, -100 DP, and -(25-40) V CES were selected.

Bioinformatics

Metabolomic datasets were assessed with three informatics programs: MetaboAnalyst, MSStats, and Elements. MetaboAnalyst is a free webserver developed to profile cells, tissues, organisms and pathologies from nuclear magnetic resonance (NMR), mass spectrometry (MS), and chromatography. MetaboAnalyst is capable of versatile data processing program that provides several data normalization and processing procedures that analyze and visualize data in various parametric and non-parametric forms.[14–17] MSStats is a free R package that is available from Bioconductor, a toolbox for 'omic' data analysis that analyzes MS data. MSStats can analyze label and unlabeled independent and dependent targeted spectral acquisition. Relying on a family of linear mixed models, MSStats identifies *m/z* features that are significantly different

from the assumption that groups that share qualities, yet are different, in the case of this experiment.[18] Elements for Metabolomics software (version 1.2.1, Proteome Software, Inc.) is used for peak alignment, statistical analysis creating volcano plots, PCA, heatmaps, and putatively identifying compounds from MS/MS spectra available in public databases.

Collection of MS/MS Data for Glucuronic Acid

Glucuronic acid was obtained from MilliporeSigma (St. Louis, MO, US) for MS/MS characterization. Parameters for MS/MS fragmentation under positive mode was +5000 V ionspray voltage ISV, +100 V DP and +(25-40) CES. For MS/MS fragmentation under negative mode instrument parameters were set to the following: -4500 V ISV, -100 DP, and -(25-40) V CES.

Results

Metabolomics offers a global view of changes in cell physiology that arise due to tissue pathology. While this type of analysis holds great promise for uncovering new pathways that that could be targeted therapeutically, these experiments also generate large datasets that are difficult to deconvolute. The purpose of this study is to compare a single dataset using the following bioinformatic programs: MetaboAnalyst, MSStats, Elements for Metabolomics, and Cytoscape. CSF was collected from CM and NPH patients and metabolites were extracted and analyzed by LC-MS/MS. Peaks were subsequently aligned in MarkerView software to correct any retention time deviation and then analyzed with bioinformatic software. We first performed a multivariate statistical analysis, PCA, in MetaboAnalyst and Elements to determine whether metabolic differences between groups were identified similarly. PCA computes maximum

variance and applies vector analysis in this region of maximum variance creates values associated with vectors that give groups a common characteristic to perform statistical analyses on.[19] Therefore, PCA determines the greatest variances between groups with known similarities. With regard to CM and NPH MS aligned spectra, PCA eliminates similarities between groups within given data to reason variances between groups.[20] The PCA was performed on data collected in both positive and negative mode for CM versus NPH (**Fig. 1 and 2**).

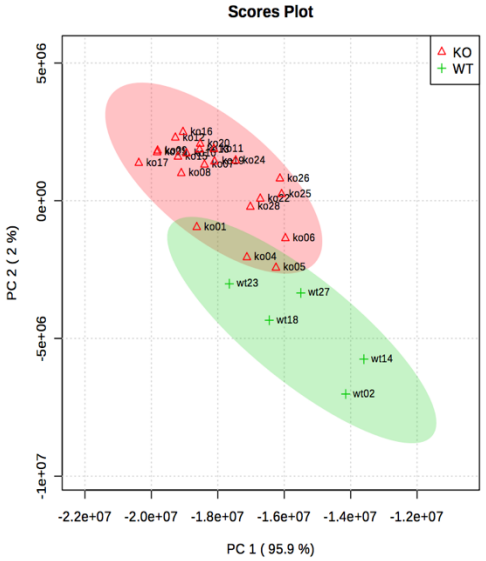

Figure 1. Principal component analysis of metabolites collected in positive mode from CM vs. NPH. PCA was performed with MetaboAnalyst.

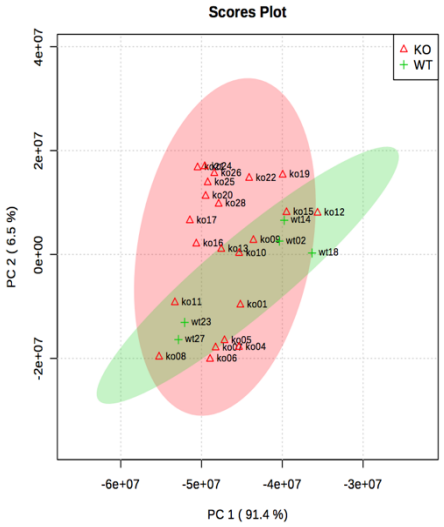

Figure 2. Principal component analysis of metabolites collected in negative mode from CM vs. NPH. PCA was performed with MetaboAnalyst.
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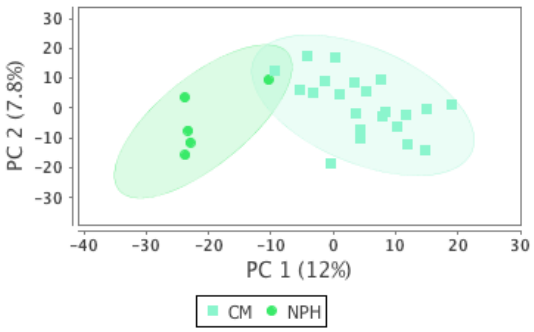


Figure 3. Principal component analysis of metabolites collected in positive mode from CM vs. NPH. PCA was performed with Elements.

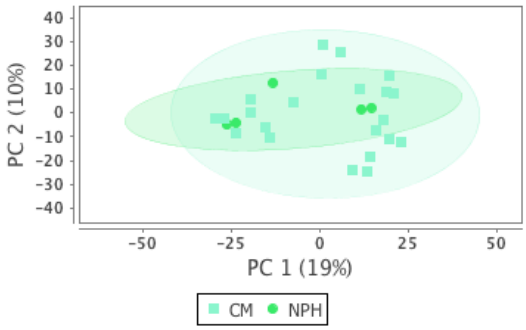


Figure 4. Principal component analysis of metabolites collected in negative mode from CM vs. NPH. PCA was performed with Elements.

For metabolites collected in positive mode, typically neurotransmitters, amino acids, and some TCA cycle intermediates, PCA shows differentiation of the two patient populations (**Fig. 1**). However, there were fewer differentiating features in the two populations in negative mode as PCA shows increased overlap between the experimental groups. We next performed the same analysis in Elements software. The statistical processing for this multivariate test was similar in this program as the amount of overlap between groups in both positive and negative mode were comparable to the result obtained from MetaboAnalyst. Again, positive mode showed well-separated groups indicating metabolic differences are detected metabolites in this ionization mode (**Fig. 3**). We continued further with the bioinformatic analysis in positive mode as negative mode did not determine sufficient variance between groups.

Volcano plots were created to visualize the number of significantly changed *m/z* values in

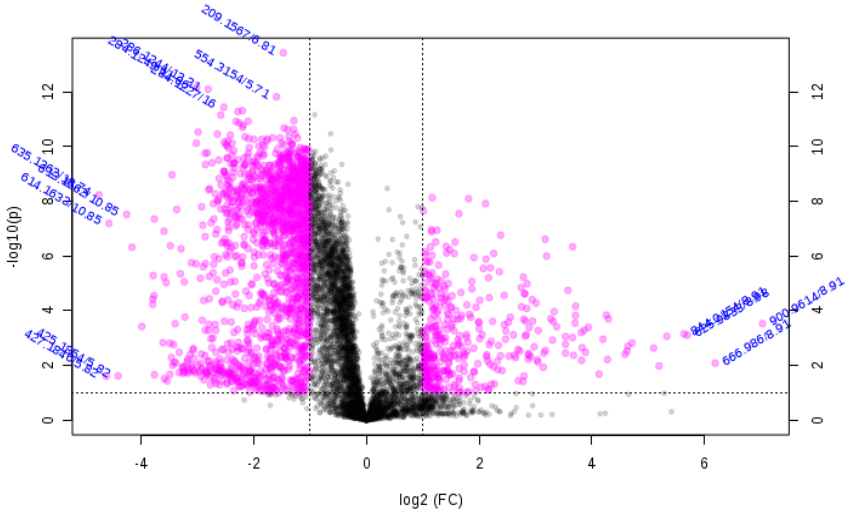


Figure 5. Volcano plot created in MetaboAnalyst under positive mode.

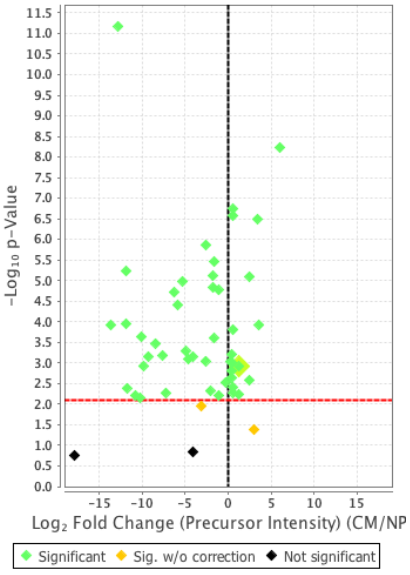


Figure 6. Volcano plot created in Elements under positive mode.

MetaboAnalyst and Elements based on p-value 0.05 and fold change less than -2 or greater than 2 (**Fig. 5 and 6**). MetaboAnalyst came up with more than 700 results under positive mode, whereas Elements came up with 30 results under positive mode after more stringent statistical

methods were used. Elements is able to apply a second correction for multiple comparisons, family wise error rate (FWER), in addition to the Benjamini-Hochberg procedure. MetaboAnalyst does not allow changes in parameters. However, within the two programs similar features (defined by a unique *m/z* and retention time) were identified by both programs in the greater *m/z* range. Most metabolites in this mass range are lipids. Between all three bioinformatics programs, some metabolites had different p-values, yet still remain significant between both programs. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) was determined to be significant by MSStats (putative *m/z* identified IPP/DMAPP in databases) with a fold change of 5.1 and a p-value of 1.69×10^{-7}, whereas in Elements the fold change was 0.5 and p-value of 1.82×10^{-7}. For some other features, p-value differed by 2 factors of 10; however, the feature remained significant.

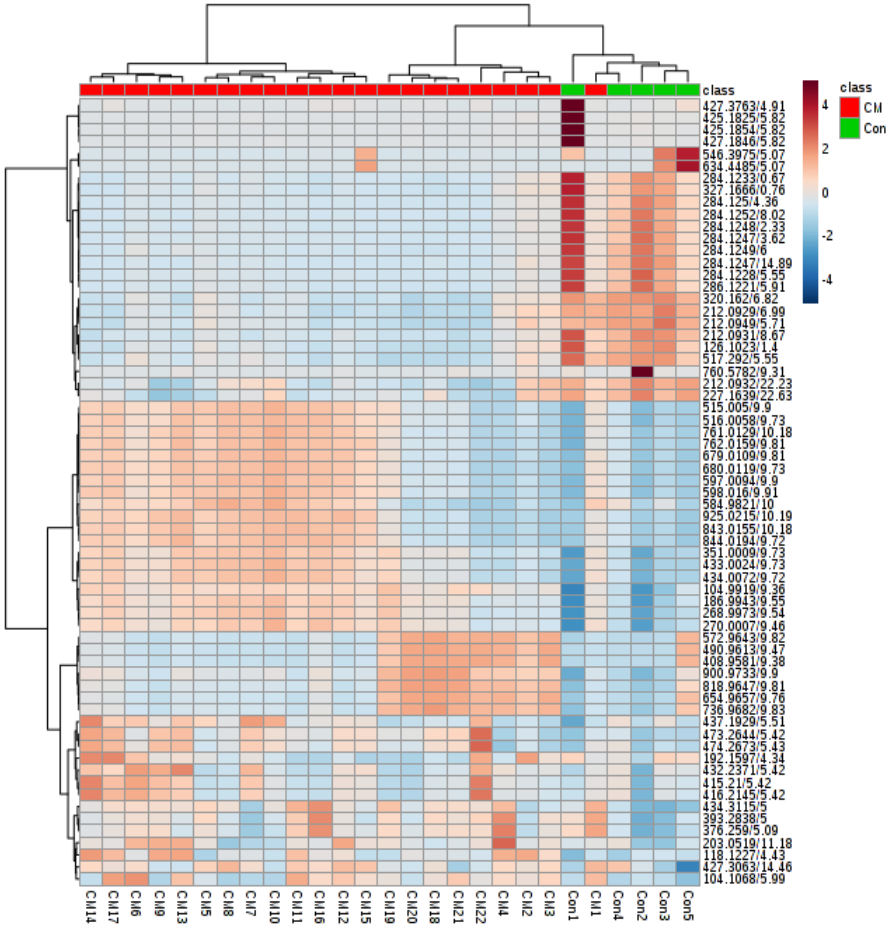


Figure 7. Heatmap of metabolic differences under positive mode in CSF of CM patients versus NPH. Heat map of significantly changing features

MetaboAnalyst and Elements were subsequently used to generate heatmaps that depict features that are statistically significant between groups. MetaboAnalyst confirms which *m/z* feature is dysregulated by PLS-DA data in Euclidian format, whereas Elements uses a two-way ANOVA to create a heatmap in Euclidian format and identifies the corresponding putative metabolite. **Figures 7** and **8** are heatmaps for metabolite dysregulated in CM vs. NPH and

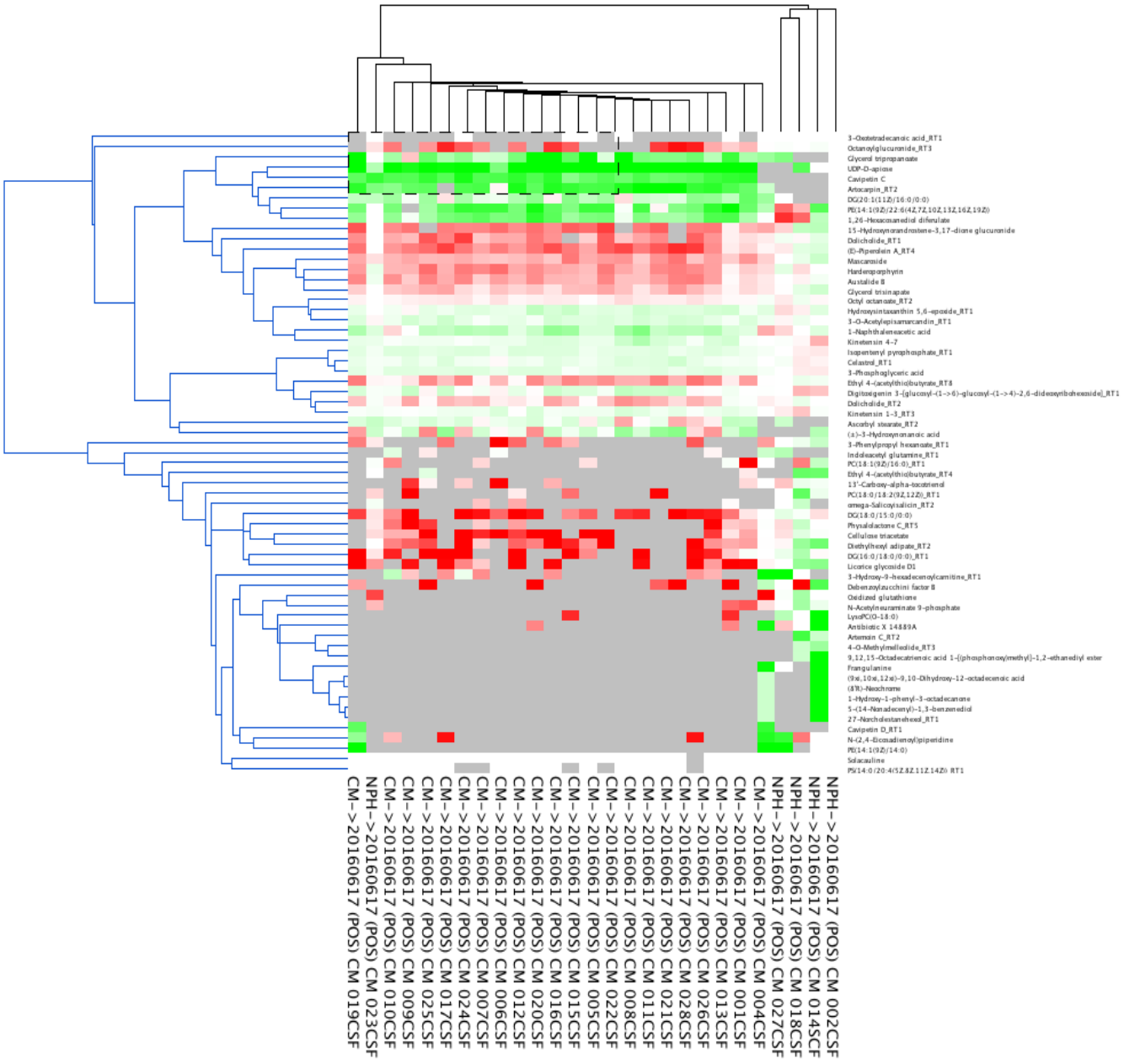


Figure 8. Heatmap of metabolic differences under positive mode in CSF of CM patients versus NPH. Heat map of significantly changing features identified by Elements.

detected in positive mode. Upregulation in MetaboAnalyst is indicated by shades of red, whereas downregulation is shown by shades of blue MetaboAnalyst does not have automated identification, so all metabolic features are expressed as a *m/z* and retention time. In positive mode, Elements identified small molecule metabolites conjugated to glucuronic acid based on accurate mass and retention time. The fatty acid (FA) octanoyl acid has a conjugated form, octanoylglucuronide, which is downregulated in CM compared to NPH (**Fig. 9**). Further, we see another steroid, an estrogen derivative estriol glucuronide downregulated across CM patients (**Fig. 10**). These metabolites are potentially products of a glucuronosyltransferase that is expressed in the brain is specific for estrogen/androgens and some medium chain fatty acids that are not eligible for energy derivation.[21,22]

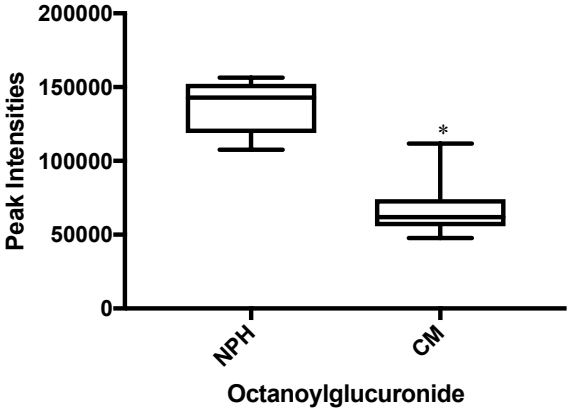


Figure 9. Box and whisker plot for measurements of the glucuronic acid conjugated octanoylglucuronide metabolite. This metabolite is significantly upregulated in NPH compared to CM ($p = 6.98 \times 10^{-4}$, fold change -4.15).

Elements found upregulation of several sugar utilizing pathways. Specifically, xylose, UDP-xylose, D-glucose, D-mannose, D-galactose, D-fructose, and myoinositol.

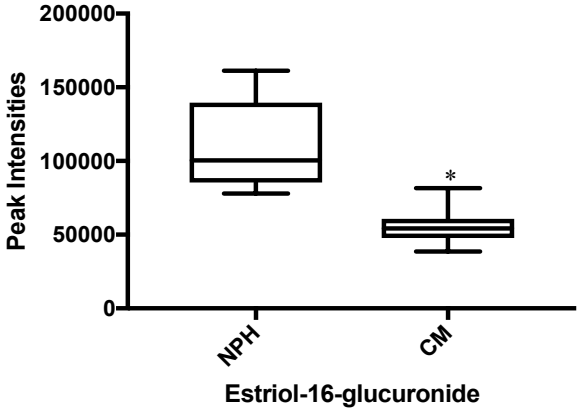


Figure 10. Box and whisker plot for measurements of the glucuronic acid conjugated estradiol metabolite. This metabolite is significantly upregulated in NPH compared to CM ($p = 8.93 \times 10^{-4}$, fold change -2.66).

Using Cytoscape, we created a comprehensive map of metabolic pathway enzyme metabolite interactions that could be useful to create additional hypotheses for the metabolic perturbations observed in the CSF of CM and NPH patients. Data was taken from Elements and mapped into enzyme-metabolite relationships with Cytoscape. **Figure 11** shows metabolites such as galactose and *myo*-inositol are linked through the activity of galactokinase (GalK), galactose 1-phosphate uridyltransferase (GALT) and *myo*-inositol oxygenase (MIOX) enzymes.[23,24]

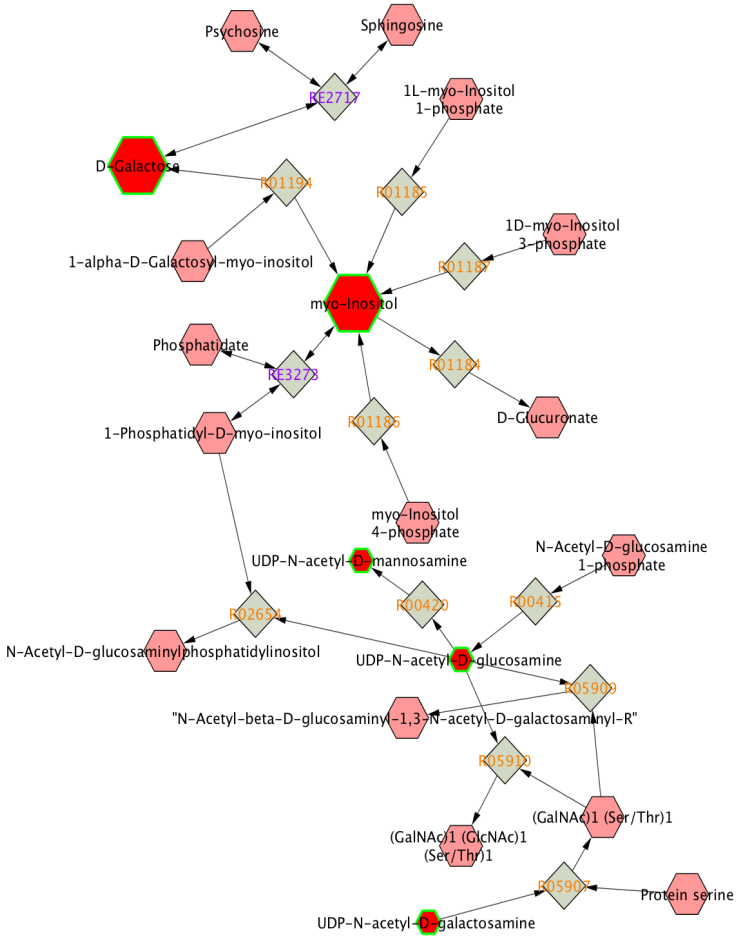


Figure 11. Inositol and glycan metabolism interactions. *Myo*-inositol is a key intermediate in the glucuronidation pathway.

Galactose metabolism also has direct effects on glucuronic acid and PPP interconversions.[25] Nucleotide metabolism would subsequently be perturbed by alterations in

sugar metabolism associated with PPP (**Figure 12.**).[26,27]

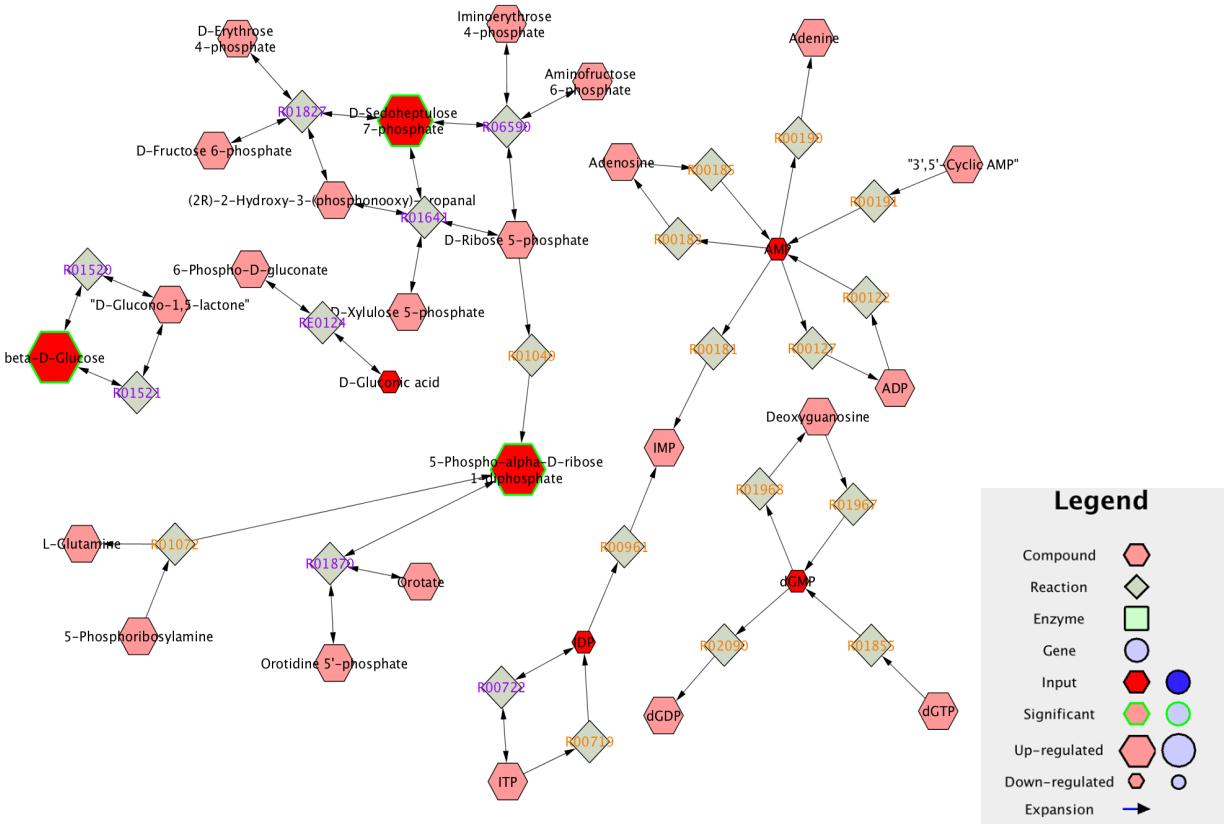
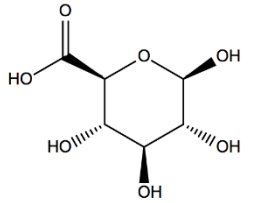


Figure 12. Overlap in PPP and purine/pyrimidine metabolism.

Seeing that estriol-16-glucuronide and octanoylglucuronide are downregulated in CM patients, we obtained a glucuronic acid standard to identify its presence in NPH patients. Glucuronic acid (**Fig. 13**.) is derived from glucose by the oxidation of C6. We obtained MS/MS on glucuronic acid by using the 5600^{+} TripleTOF Mass Spectrometer. **Figure 14** shows glucuronic acid fragmentation under negative mode and **figure 15** shows glucuronic acid fragmentation under positive mode. The parent ion is so small in intensity that the fragmentation masks the parent ion. These fragmentation patterns could help us to



(2S,3S,4S,5R,6R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-carboxylic acid
Chemical Formula: $C_6H_{10}O_7$
Molecular Weight: 194.14

Figure 13. Glucuronic acid made in ChemDraw 16.0.

subsequently identify other glucuronic acid conjugates.

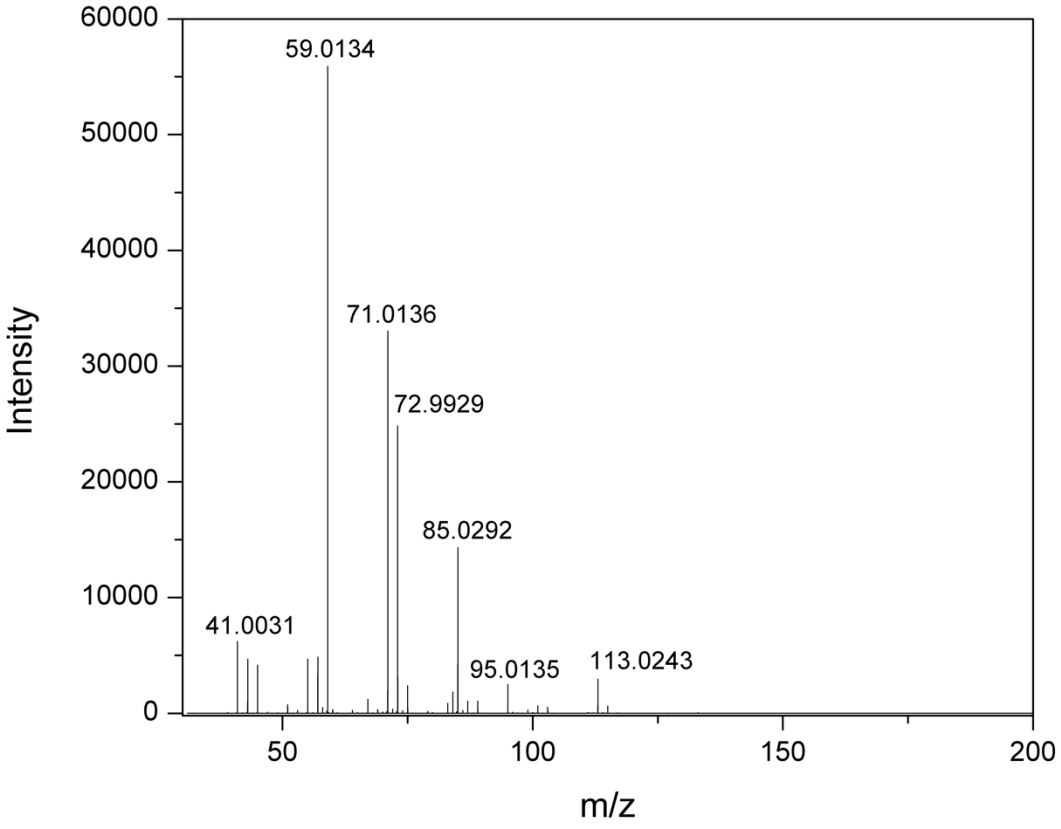


Figure 14. Glucuronic acid MS/MS fragmentation under negative mode with H^- adduct. Parent ion of 193.04.

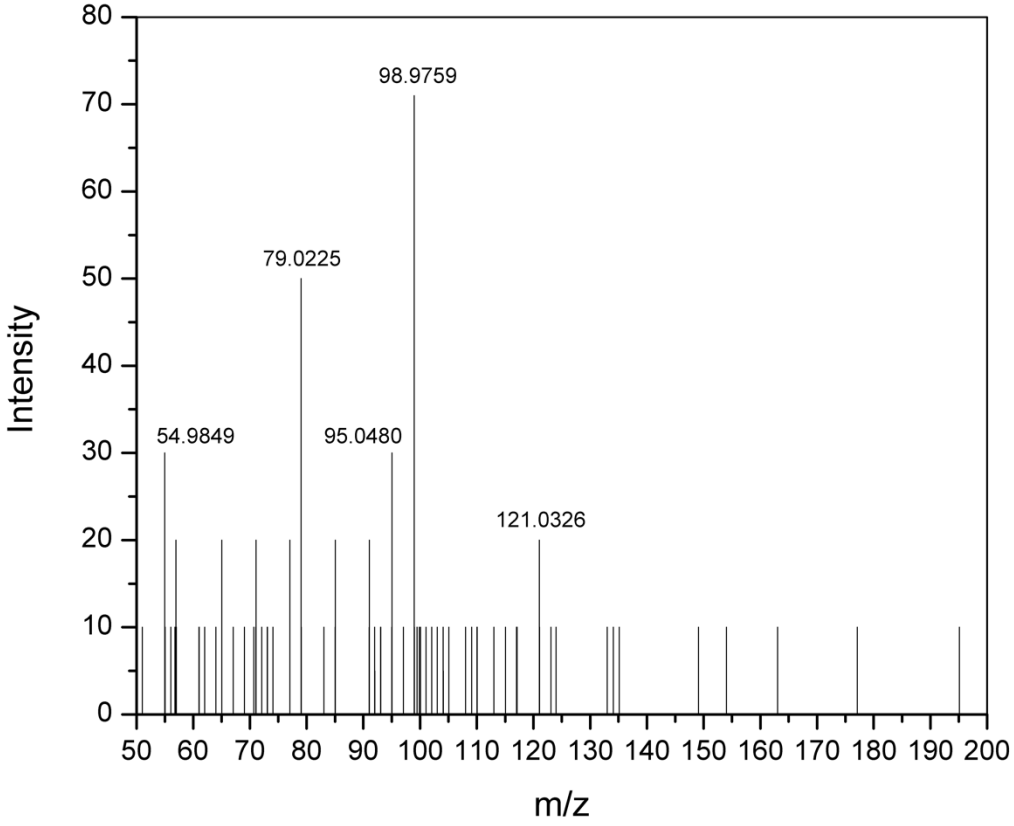


Figure 15. Glucuronic acid MS/MS fragmentation under positive mode with H^+ adduct. Parent ion of 195.15.

Discussion

After extracting experimental LC-MS data, multivariate analysis using MetaboAnalyst and Elements allows large datasets collected in positive and negative mode to be visualized with the respect to their ability to differentiate experimental groups. Statistical algorithms are completed in a similar manner, yet not identical as Elements putatively identifies metabolites, we see some differences between features that are determined to be dysregulated and subsequently identified between programs. Submitting data to both programs may allow more putative identifications to be obtained, and may be reviewed to eliminate outliers, as we had with

exogenously identified metabolites.

We examined the use of MSStats to determine dysregulated features that could be matched to endogenous metabolites. While MSStats did show more endogenous metabolites being significantly dysregulated, there were also differences between Elements and MSStats. MSStats was originally developed to process proteomics datasets. However, proteomics and metabolomics share a format for data output, namely that each analyte is represented by an *m/z* value and retention time. Similar *m/z* values were determined to be dysregulated by MSStats, Elements and MetaboAnalyst, like IPP and DMAPP; however, we see that statistical results (p-value and fold change) differ greatly between MSStats, Elements and MetaboAnalyst. After evaluation of the algorithms used by each program, MetaboAnalyst and Elements had similar stringent statistical algorithms, whereas MSStats had flexible algorithms. Thus, MSStats is a reasonable preliminary metabolomic tool to define a direction to pursue, but other metabolomic analysis is needed.

The level of stringency in the statistical analysis in Elements can be modified by the discretion of the user. In this experiment, we used a moderately strict two-way ANOVA correction that would decrease the FDR. The Benjamini-Hochberg procedure aids better for correction than that of a FWER (family-wise error rate) correction for this instance of comparing two unequal groups with small sample sizes. Further, Elements also allows for the identification of putative metabolites based on a self-curated library. We curated our own endogenous library from HMDB to cross-reference the library MS and MS/MS data with experimental data. One limiting factor in the identification of metabolites is that most databases have predicted rather than experimentally obtained spectra. When different instrumentation used for MS analysis some discontinuities may arise between expected and observed spectra which may make automated

identification using databases difficult.

Cytoscape was used to identify enzyme-metabolite interactions by using the application MetScape and MetDisease. Conversions between pathways are determined from imported, measured data and pathway interactions obtained from literature. To input compounds, KEGG IDs were needed; however, it appears not all KEGG IDs are accepted by the program. Mapping of dysregulated pathways provides information of the functional connections between metabolites. We mapped glucoronate-related metabolites and identified several glucuronated metabolites, suggesting alterations in sugar metabolism in Chiari versus NPH.

We also determined the MS/MS of glucuronic acid under both negative and positive mode. This data can be used in further experiments that identify novel glucuronic acid conjugations. This experimental data is essential for positively identifying metabolites within biological samples. Glucuronidation is an active pathway that acts to conjugate un-useful or toxic compounds to be solubilized for excretion of the body.[28] There are several transferase isoforms that are organ specific, like UGT-2B7 and further research is needed to determine the importance of this pathway in NPH.[29–31]

Conclusion

Chiari malformation is a progressive degeneration of the cerebellum that causes herniation into the spinal column, commonly forming a syrinx. After metabolomic analysis of CM CSF with comparison to a control of NPH CSF using LC-MS under positive mode. Using several bioinformatic programs, we determined that these programs provided similar results in identifying mass-to-charge values; however, p-values and fold changes can differ greatly between programs. This is due to differences in algorithms. MetaboAnalyst and Elements

showed better putative identification than MSStats. Holistically, MetaboAnalyst and Elements work well for identifying metabolites, and MSStats works well for preliminary identification, but not well for supporting metabolite identification. From these analyses, we putatively determined certain metabolites that are dysregulated and matched them to their respective pathways. Finally, we found potential alterations in glucuronic-acid metabolites suggesting changes in metabolite elimination in NPH due to altered CSF flow that is not evident in CM patients with hindbrain herniation.

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Appendix I:

Safety Considerations

Training in sterile technique was required to perform cell cultures and handling of biohazardous materials. While handling biohazardous materials, including cells for cell culture, protective eyewear, gloves, and a sterile hood were always used. Using sterile technique called for autoclaving materials used in a sterile hood as well as spraying items with 70% ethanol before use in the hood. A lab coat, long pants, close-toed shoes, protective eyewear, and gloves were always used in the laboratory. When handling and disposing of chemicals the proper disposal was taken. UA environmental health and safety was always able to be reached in any circumstance when chemicals were needed to be disposed of or if there was an emergency in the lab.