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Investigating Iron Metabolism in Subarachnoid Hemorrhage Patients

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Investigating Iron Metabolism in Subarachnoid Hemorrhage Patients

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Abstract:

Subarachnoid hemorrhage (SAH) is a stroke characterized by bleeding into the subarachnoid space of the brain, typically resulting in high mortality rate.⁸ Delayed cerebral ischemia (DCI), characterized by vasospasms induced arterial constriction, occurs in roughly one third of the surviving patients.²⁰ The development of DCI and neurodegeneration could be linked to metabolic pathology that occurs after SAH, specifically iron induced changes in redox status. The oxidized environment induced by iron has the potential to functionally affect the ferroxidase ceruloplasmin (Cp), which is linked to neurodegeneration.¹⁵ Global LC-MS based metabolomics data revealed alterations in metabolism in the CSF at early and late timepoints after SAH as compared to controls. Early phase SAH and late phase SAH patients showed metabolic differences, however there was limited statistical significance. Specifically, the heme degradation product bilirubin (BR) and the glycolysis intermediate 3-phosphoglyceric acid were tentatively found to be down regulated in SAH. Iron toxicity was analyzed in a hemin induced model using differentiated SHSY5Y human neuroblastomas, revealing concentration-dependent cell killing and morphological changes. This data will be used as a baseline in future studies attempting to reverse iron toxicity via Cp treatment.

Introduction:

Subarachnoid hemorrhage (SAH) is a type of stroke affecting approximately 30,000 people in the United States annually with 15% mortality.⁸ SAH is commonly caused by aneurysm and characterized by bleeding into subarachnoid spaces of the brain.⁸ Patients who survive the initial hemorrhage are at risk of developing future complications such as delayed cerebral ischemia (DCI) up to 2 weeks after the initial bleed.²⁰ DCI occurs in approximately one third of the individuals who survive the initial attack and is most commonly associated with

cerebral vasospasm, an arterial spasm, resulting in constriction of the vessel.²⁰ However, the biochemical mechanisms that lead to the development of DCI are incompletely characterized.

The goal of this project is to define the pathological alterations that are involved in the development of DCI and neurodegeneration that characterize SAH. Bleeding into the brain results in the induction of heme oxygenase 1 (HO1), a heat shock protein which responds to stress stimuli.⁷ HO1 participates in heme degradation.⁷ The oxyhemoglobin (oxyhb) released from the blood is further degraded into carbon monoxide, ferrous iron, and biliverdin (BV), and subsequently bilirubin (BR).² The heme degradation product bilirubin can become oxidized forming oxidized bilirubin (BOXes), which has been suggested to induce DCI via vasospasm.¹⁷ Free iron is also neurotoxic due to its participation in Fenton chemistry.¹⁵ Therefore, iron handling and storage in the CNS is essential for neuronal protection. Within the blood and CNS the major iron handling protein is ceruloplasmin (Cp), a ferroxidase that facilitates sequestration of free iron.¹⁵ A decrease in Cp activity has been linked to other neurodegenerative conditions and is thought to promote mishandling of iron, leading to tissue degeneration.¹⁵ A structural change in Cp, inducible by oxidative stress, was found to negatively affect ferroxidase activity.¹⁵ We hypothesize that the iron handling abilities and antioxidant properties of Cp, are compromised during SAH, resulting in a high oxidative stress environment. Hemin will be used to imitate SAH heme release in *in vitro* studies (**Figure 1**).

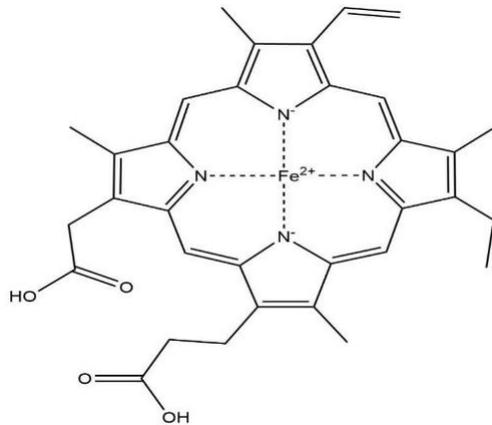


Figure 1. Chemical structure of heme.

A better understanding of the mechanism leading to DCI could aid in the development of better options for SAH and thus increase survival rate. Determining which patients are at risk of DCI presents a unique opportunity to initiate treatment prior to the onset of the condition thus avoiding any additional damage.³ Additionally, this project will facilitate a better understanding of antioxidant responses required for limiting neuronal damage induced by SAH.

Materials and Methods:

Cell culture

The SH-SY5Y semi adherent human neuroblastoma cell line was used for all in vitro experiments. Cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) 1X, with 10% Fetal Bovine Serum (FBS), and 1% penicillin streptomycin. Cells were stored in an incubator maintaining an environment of 37 C° and 5% carbon dioxide with 95% air.

When cells reached a confluence of 70% in T-75 flasks, they were split into a new flask to keep the cell line alive. The media was pipetted out into a 50 mL falcon tube. The flask was

washed with 4 mL of phosphate buffer solution (PBS) to remove any remaining media, which was also placed in the 50 mL falcon tube. The adherent cells were subjected to 3 mL of 0.25% trypsin and 2.21 mM EDTA 1X. The flask with the trypsin was placed back into the incubator for 2 minutes. Upon removing the flask from the incubator, the flask was agitated slightly to knock cells into suspension. Trypsin was washed out with 3 mL media, pipetted out of the flask, and placed in the 50 mL falcon tube. The solution was centrifuged for 10 minutes at 10,000 rpm. The supernatant was removed using aspiration. The pellet was resuspended in 3 mL of media. A new T-75 flask was filled with 14.5 mL of media. 0.5 mL of cell suspension was added to the new T-75 flask, which was then placed back into the incubator.

Cell plating was done by first coating the intended wells with poly-L lysine to help with adhesion. 1 mL of 1 mg/mL poly-L lysine was added to 9 mL of deionized (DI) water. This solution was added to the intended well to sufficiently cover the surface. After 5 minutes the poly-L lysine was aspirated out. The well was washed 4 times with DI water. Cells were isolated in pellet and resuspended into 3 mL of media as described above. A portion of this solution was added to trypan blue in a 1:4 ratio of cells to trypan blue. 50 μ L of this solution was then placed onto a hemocytometer. Cells in each of the 4 quadrants of the hemocytometer were counted under a light microscope and averaged to calculate total cell count.¹ The original cell solution was diluted as necessary with media to give 30,000 cells in each well.

Differentiation of SH-SY5Y Cells

Cells were plated on a 96 well plate as described above in 50 μ L of growth media and incubated overnight. 50 μ L of growth media and retinoic acid diluted from the stock of 100 mM in DMSO, was added to give a final concentration of 10 μ M retinoic acid. Due to the

photosensitive nature of retinoic acid, retinoic acid addition was done in the dark, and after the addition of retinoic acid plates were kept in aluminum foil. This media was refreshed every 2-3 days for a total of 5 days of treatment. The cells were observed under a light microscope to check for differentiation of long, extended processes and a flattened body. The media was then replaced with serum free growth media, DMEM 1X (1% p/s), containing 50 ng/mL brain derived neurotrophic factor (BDNF).⁵

Resazurin based toxicity assay

Cells were plated on a 96 well plate and differentiated. The cells were then exposed to varying concentrations of hemin dissolved in DMSO for 24 hours. A Blank, untreated control, and vehicle control were also measured. Resazurin was added at a volume giving 10% of total solution. Cells were incubated under exposure of resazurin for 2 hours. A plate reader was used to measure the absorbance at a wavelength of 600 nm.¹⁴

Immunocytochemistry

Three coverslips were placed into a 6 well plate. The coverslips were coated with poly-L lysine as described above. Cover slips were plated with 10^5 cells, and cells were incubated in 2 mL of growth media for a day at 37 C° and 5% carbon dioxide. Cells were differentiated using retinoic acid. After the differentiation process completed, the growth media was removed and 1 mL of 4% paraformaldehyde in PBS was added for 10 minutes. Cells were washed 2 times with PBS. Cells were incubated in 0.2% triton x-100 for 10 minutes. Coverslips were washed 3 times with PBS. A 1 mL aliquot of 10% FBS in PBS was added to each well to block all nonspecific binding sites for 1 hour in humidity chamber. All remaining steps including the addition of

antibody were done in the dark. After blocking, FBS solution was removed and 200 μ L of primary antibody at a 1:500 dilution in 10% FBS was added. The plate was covered in aluminum foil and placed in humidity chamber for 2 hours. Antibody solution was removed, and the coverslips were washed 2 times with PBS for 5 minutes each. A 1 μ L aliquot of 5 mg/mL DAPI was added to 10 mL of PBS. To each of the wells, 1 mL of DAPI solution was added for 5 minutes. DAPI was aspirated out, and coverslips were washed 2 times with PBS for 5 minutes each. PBS was removed, and cells were washed with 1 mL of DI water for 10 minutes. Coverslips were mounted onto slides as follows. A small amount of mounting media was applied to the slide. Cover slips were placed cell side down onto the mounting media, making sure to avoid any bubbles. After slides are dry, clear nail polish was applied to the edge of the cover slip to seal. Slides were stored in a refrigerator while covered in aluminum foil. Images were taken on a Nikon A1+ confocal microscope.

Metabolomic Analysis of CSF from SAH Patients

Samples were collected by and received from The Cleveland Clinic as part of the ICEFISH study. All information were deidentified besides the grouping that the sample belonged to; control, early SAH, or late SAH. The study was approved by the IRB of Cleveland Clinic. Prior to LC-MS analysis of human samples, the MS was tuned with the following parameters: GS1: 18 psi, GS2: 18 psi, Cur: 25 psi, Ion Spray voltage: 5500 V, Temperature: 300 C°, Scan Type: TOF, Polarity: positive, Scan window (Min to Max): 100-1000 Da. Parameters for SCIEX TripleTOF MS were set as previously described.⁹ The samples were run through a Micro200 LC containing a hydrophilic interaction liquid chromatography column. The first mobile phase was conducted with water, and the second mobile phase was conducted with acetonitrile. Both phases were performed with the addition of 5 mM ammonium acetate and 5 mM ammonium hydroxide.

Flow rate was set to 30 $\mu\text{L}/\text{min}$. The following gradient was used: 0 min 98%, 0.5 min 98%, 1 min 95%, 5 min 80%, 6 min 46%, 13 min 14.7%, 17 min 0%, 17.1 min 100%, 23 min 100%.

Metabolites under the following parameters: GS1: 15 psi, GS2: 20 psi, CUR: 25 psi. +5000 V ionspray and +100 V decluster potential was used in positive mode. Background threshold was set to 10 counts/second. In negative mode -4500 V ionspray and -100 V decluster potential was used.⁹

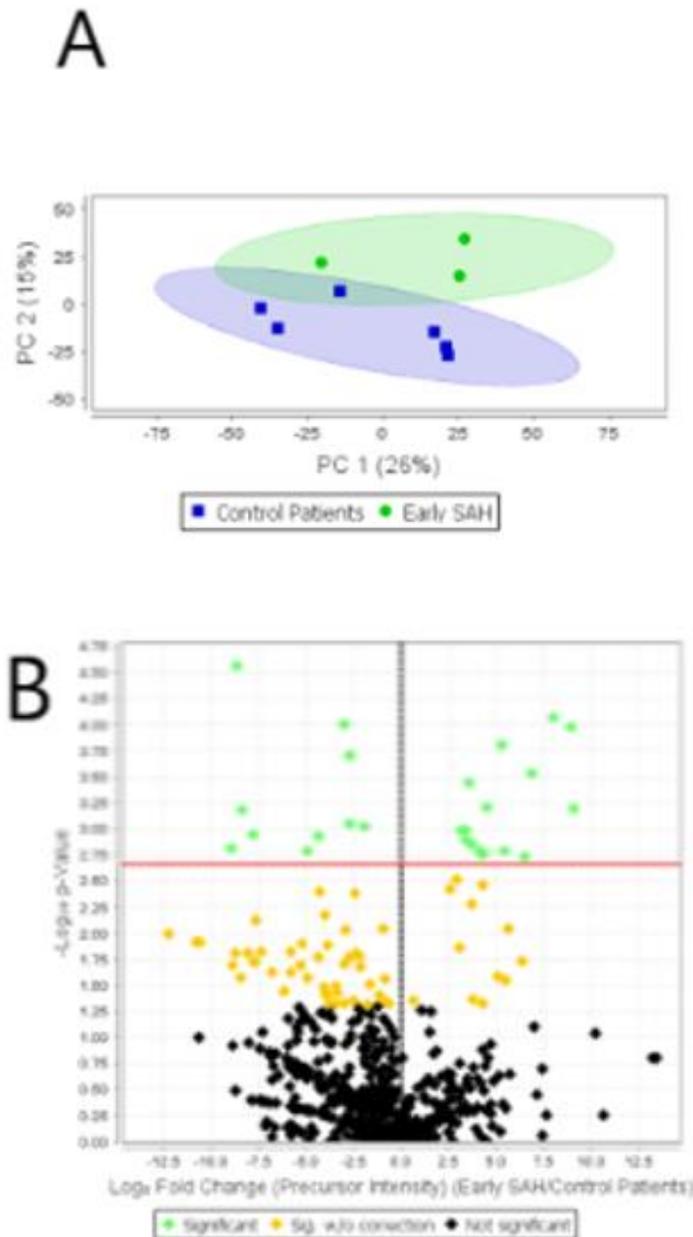
Results and Discussion:

Alterations in metabolism that were linked to redox stress in human CSF samples isolated from SAH patients were analyzed first, and the metabolic changes were compared to control CSF from patients without neurological disease.

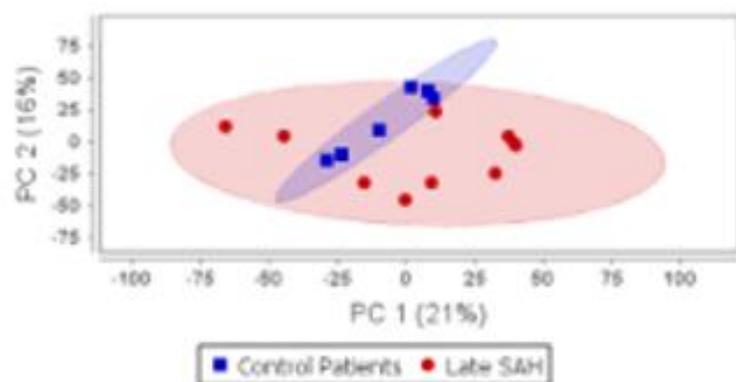
The global metabolomics data is indicative of metabolic trends observed in each group. The PCA plot comparing early and control patients indicates metabolomic shift upon initiation of SAH, showing minimal overlap between groups. The PCA plot comparing late and control patients contains a high degree of overlap. These PCA plots indicate there is more change between early and control than late and control. The PCA plot comparing late and early patients shows some overlap yet still distinctively different groups, suggesting a metabolic change as time passes from the onset of SAH.

The volcano plot comparing early and control patients shows multiple significantly different metabolites, showing a similar amount of upregulated and down regulated metabolites. The volcano plot comparing late and control patients also indicates many significantly different metabolites, with most of the metabolites being upregulated as opposed to the even distribution seen in the early vs control plot. Interestingly, the volcano plot comparing

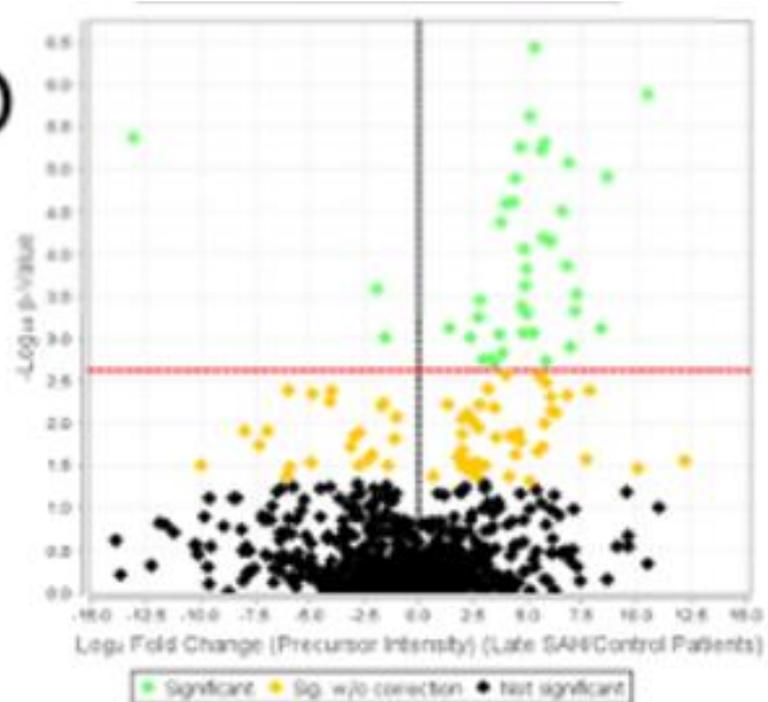
early and late patients shows limited significantly different metabolites despite the PCA plot indicating distinguishable metabolic separation between the two groups. This could be due to a low number of patient CSF samples. Global metabolomics will be run on more samples being received from The Cleveland Clinic, increasing sample size. Trends observed in the new samples will validate or invalidate **Figure 2** data. The general trends below will be further analyzed in the future using targeted LCMS on metabolites of interest.



C



D



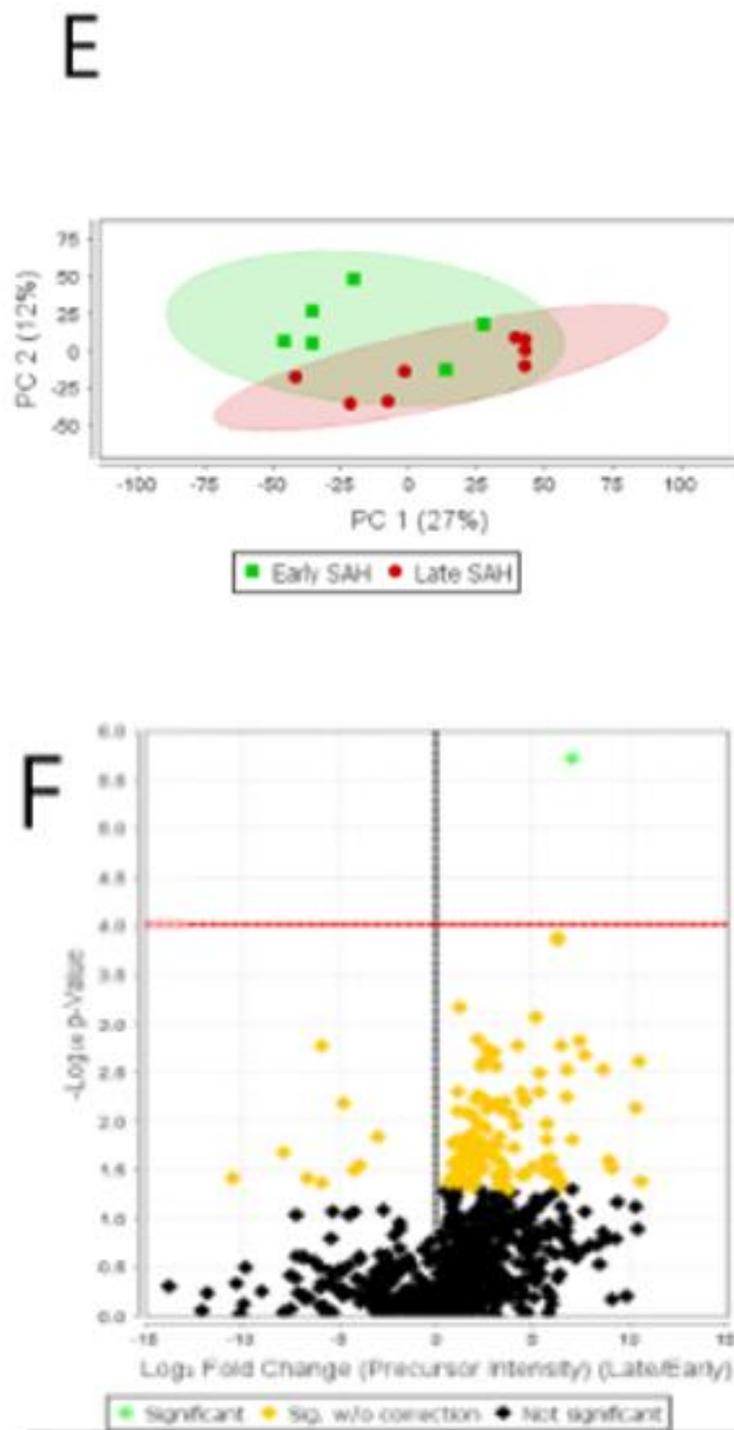


Figure 2: Metabolomic analysis of CSF from SAH patients obtained from 6 control patients and 18 SAH patients. Plots A and B compare metabolism of early SAH patients and control patients. Plots C and D compare late SAH patients and control patients. Plots E and F compare early SAH and late SAH patients.

The release of heme into the CSF causes a cascade of metabolic changes to eliminate the compound. The heat shock protein HO1, expressed in response to stressors, acts as a heme degradation enzyme. HO1 degrades heme into carbon monoxide, BV, and iron (II).² BV is then reduced to BR.² **Figure 3** indicates BR decreases with SAH and continues to decrease between early and late samples. This finding is not consistent with Pyne-Geithman et al. who showed that longer heme exposure in CSF leads to higher production of BR.¹⁷ More interestingly they found that patients who experienced secondary vasospasm after SAH had significantly more BR than those who did not.¹⁷

BR has been shown to have neurotoxic effects when present in high enough concentrations.¹³ Müllebnner et al. suggests BR may affect mitochondrial function, encourage endoplasmic reticulum stress, and may even induce apoptosis.¹³ Under highly oxidizing environment, BR will become oxidized and form BOXes, which have been shown to negatively affect neurons.¹⁰ BOXes have been suggested to cause late vasospasm.^{10, 17} Treatment with BOXes lead to high vasoconstriction.¹⁰ Patients that experience DCI were found to have more oxidative stress.¹⁷ Pyne-Geithman et al. proposed the following mechanism: heme gets degraded in the CSF, increasing BR, the increased BR along with a highly oxidative environment leads to production of BOXes, which cause vasospasm.¹⁷ BOXes were not identified in LCMS data.

The down regulation of BR contradicts other studies and is difficult to explain logically. Since BR is a heme degradation product, more heme should give more BR.¹³ Targeted metabolomics still needs to be run to assure that this finding is accurate for the samples. The BR levels in the new CSF samples will also be analyzed to look for the same trend. The observed

downregulation could be the result of inaccuracy in global metabolomics or due to the low sample size. Further confirmation is needed to accept or reject this observation.

The metabolite 3-phosphoglyceric acid is a glycolysis intermediate. The early samples showed a slight decrease in this metabolite, but the late samples showed a greater decrease. This could be due to induction of DCI via vasospasm. Vasospasm is characterized by narrowing of arteries and thus decrease in blood flow.¹¹ The decreased blood flow would decrease accessibility of oxygen and blood glucose to the affected regions. The decrease in oxygen will slow the electron transport chain.⁶ This will then lead to a build of NADH, which acts as an inhibitor of isocitrate dehydrogenase and alpha ketoglutarate dehydrogenase in the tricarboxylic acid cycle(TCA).^{21, 19, 18} Slowing TCA leads to a buildup of acetyl CoA, which along with NADH, signals pyruvate dehydrogenase to be phosphorylated inactivating it, slowing down glycolysis.¹⁶ NADH also regulates the function of glyceraldehyde 3 phosphate dehydrogenase runs in glycolysis, with more NADH down regulating glycolysis.¹² The decrease in available blood glucose will decrease input also down regulating glycolysis. This is being suggested only circumstantially. In order to confirm this pathway, analysis of electron transport chain function would need to be directly tested as well as quantification of NADH. The activity of the regulated enzymes noted above would need to be measured, and isotopic labeled metabolite experiments could be performed to analyze how the pathways of interest are running. Targeted metabolomics also needs to be run to assure the observed trends are accurate to the samples.

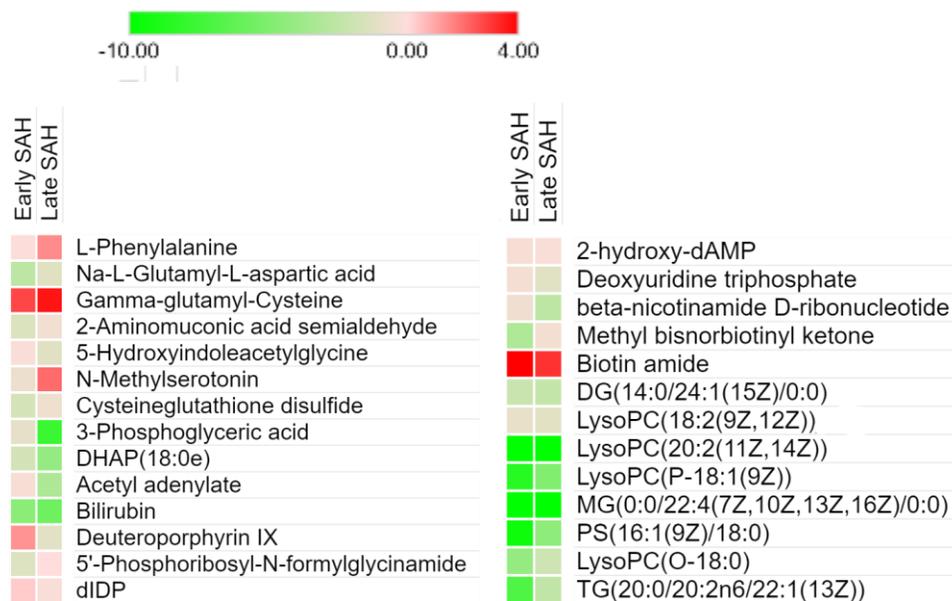


Figure 3: Heat map showing change in metabolites as determined by the global metabolomics LCMS data obtained from 6 control patients and 18 SAH patients. Red indicates increase, and green indicates decrease as compared to the control samples.

The ferroxidase Cp is responsible for converting iron (II) to iron (III). This prevents Fenton chemistry where iron (II) chemically induces reactive oxygen species.¹⁵ Decreased ferroxidase activity of Cp has been linked to other neurodegenerative disorders, including Parkinson's disease.¹⁵ Cp contains NGR motifs which are susceptible to deamidation.⁴ This process leads to a structural change in Cp converting the function of the enzyme from a ferroxidase to participation in integrin binding and intracellular signaling.⁴ Olivieri et al. showed Cp of PD patients had an increase of this structural change and thus a change in function.¹⁵ Olivieri et al. also showed that oxidative stress played a role in inducing structural change. The oxidative environment caused by the release of large amounts of heme could be changing the activity of Cp, leading to iron mishandling and further increase in oxidative stress.

Cp activity will be assessed in the CSF samples in future studies, using a ferroxidase assay. Those results will give further insight into the validity of the effects on Cp function.

To investigate changes in cell function and signaling that lead to alterations in metabolism seen in CSF, iron overload was examined in a cell culture system. SH-SY5Y cells are a neuroblastoma cell line that can be differentiated by retinoic acid via changes in transcriptional regulation.⁵ The differentiation of SH-SY5Y human neuroblastomas allows the undifferentiated stem cells to develop elongated processes similar to post-mitotic neurons.⁵ Differentiation also alters the biochemistry of the cell.²² Differentiated cells stop proliferating and express neuron specific proteins such as MAP, GAP-43, NeuN, and synaptophysin, which are common indicators of mature neurons.²²

Differentiation leads to cells that are morphologically and biochemically similar to neurons, making them a more applicable model for neuronal diseases than undifferentiated cells.²² **Figure 4** shows successful differentiation of SH-SY5Y human neuroblastomas, increasing the accuracy of our in vitro model.

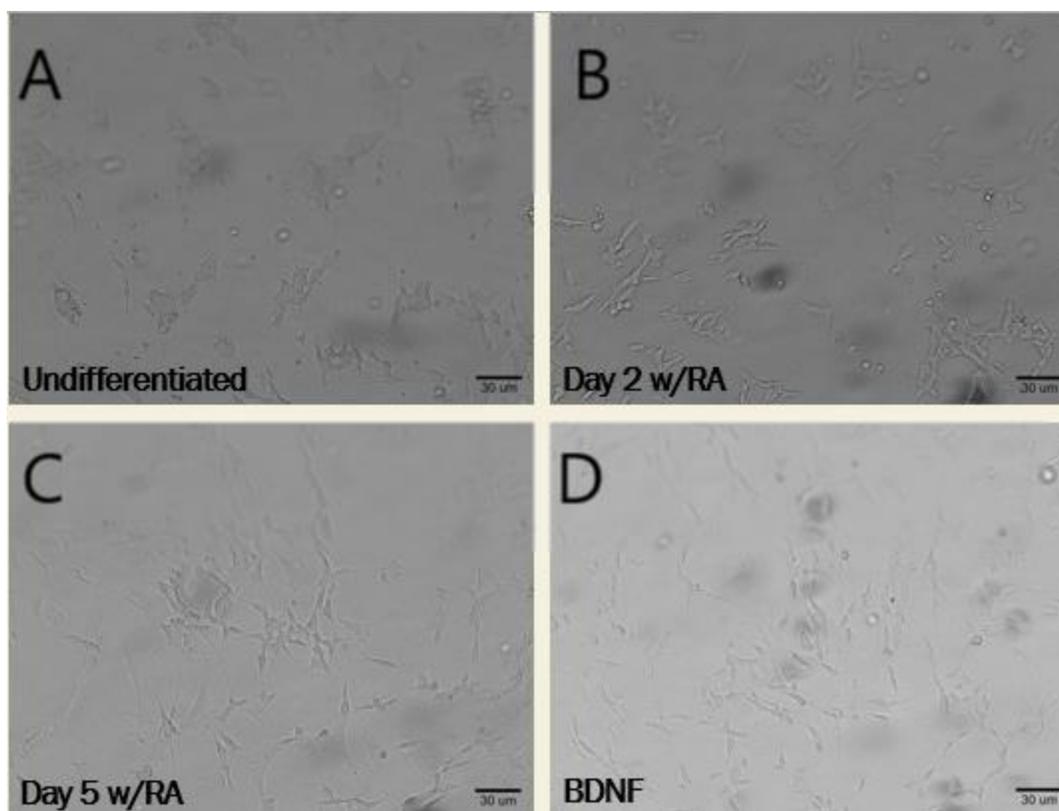


Figure 4: Pictures taken with a light microscope at varying stages of differentiation of the SH-SY5Y neuroblastoma cell line. Scale bars in lower right corner set to 30 µm. Differentiation was performed using retinoic acid treatment with a 5-day treatment period. A) Undifferentiated cells. B) Cells after 2 days of treatment. C) Cells after 5 days of treatment. D) Cells after exposure to BDNF.

Cellular response to iron overload was tested by treating cells with hemin. The cytotoxicity assay of hemin indicates that increasing concentrations of hemin correlates to increasing toxicity. Cell viability of 50% was observed between 5µM and 50 µM. Cells exposed to hydrogen peroxide showed a viability of around 150%, while complete cell death was expected. Also, of note is the large amount of toxicity shown in the vehicle control. Additional experiments need to be done to eliminate some of the failures. DMSO concentration needs to be brought down by increasing the solubility of hemin. This could be done by increasing wait time, increasing vortex and sonication time, or trying different solvents. The hemin treated wells show a general trend of increased concentration increasing toxicity. This trend is most likely accurate

despite high vehicle toxicity, but vehicle toxicity needs to be reduced to determine accurate visibility as it corresponds to specific hemin concentrations.

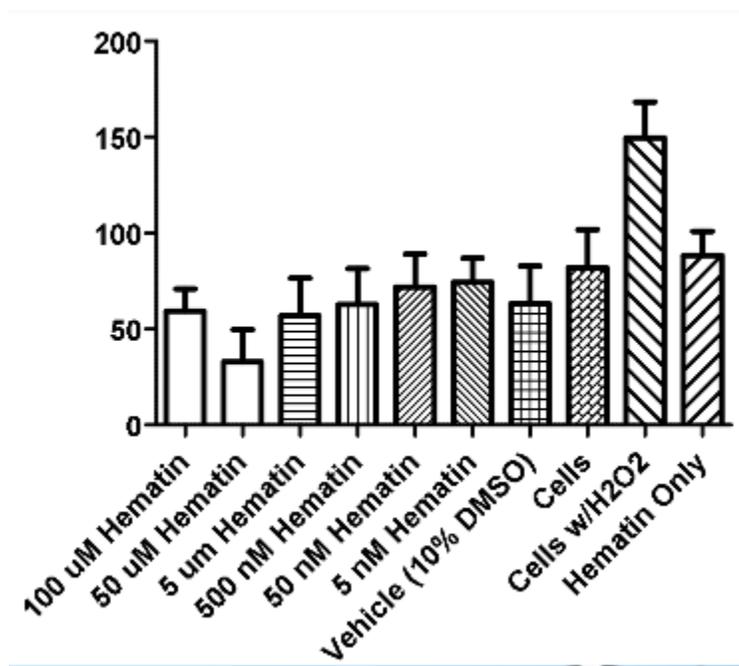


Figure 5: Resazurin based cytotoxicity assay performed on differentiated SH-SY5H neuroblastomas.

In order to develop conditions more tractable for cell studies, the solubility of hemin was tested in 3 common organic solvents; DMSO, ethanol, and acetone. In each solvent three solutions of varying concentration were attempted. The target concentrations were 15 mM, 25 mM, and 35 mM. The solutions were subjected to varying lengths of vortexing and sit times up to 30 minutes. Using just these methods, ethanol and DMSO were able to completely dissolve 15 mM, but the dissolved hemin would fall out of solution within a few minutes. Both higher concentrations never fully went into solution. Acetone never fully dissolved hemin at 15mM. The solutions were then subjected to varying times of sonication. DMSO and ethanol were able to dissolve hemin at 25mM upon sonication for 30 minutes. Hemin fell out of solution at an

observationally slower rate than with just vortexing, indicating 30 minutes of sonication should be the standard for preparing hemin solution. This allows the final DMSO concentration upon delivery to be brought down from 10% to about 0.1% depending on the treatment group. Since both ethanol and DMSO were able to hold the same concentration of hemin, it was unclear which solvent to use and additional toxicity experiments are currently being performed.

The results from the second toxicity study indicate a higher toxicity of hemin than suggested in **figure 5**. This can be explained due to an increase in solubility of hemin. More hemin is dissolving into solution, giving a higher dose to the cells compared to the less solubilized solution. This treatment being more accurate than the initial assay in terms of the calculated theoretical concentrations. The DMSO vehicle controls in **figure 6** show almost no toxicity as compared to the control wells besides the outlier point seen in the 20uM treatment. Besides that point, all vehicle controls were plotted at over 100% viability. The hemin treated groups show a clear trend in the 20 μ M, 25 μ M, and 30 μ M groups, with 30uM leading to a viability of close to 0%. The treatment groups 5 μ M, 10 μ M, and 15 μ M, show the reverse trend as expected, where an increase of hemin lead to an increase of cell viability. During the differentiation process and the toxicity assay media is removed from the wells and replaced. Since the cells are semi adherent, it is believed that cells may be getting removed from the wells unevenly, giving strange trends. This could also explain the consistently high viability in the vehicle control. Steps are being taken in future assays to minimize this possibility.

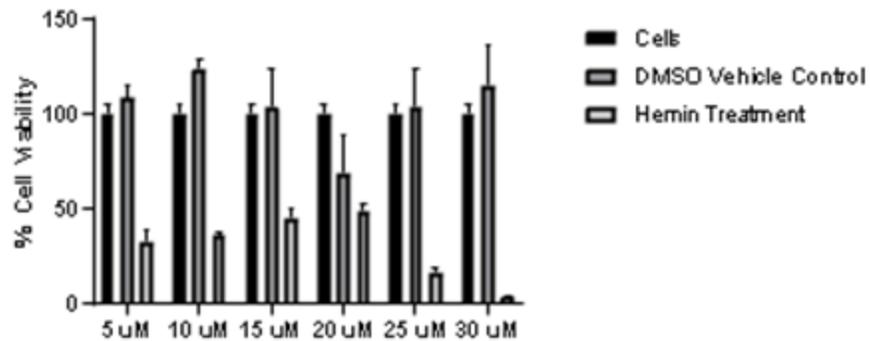


Figure 6: Resazurin toxicity study on differentiated SHSY5H cells. Study was performed after hemin solubility issues were addressed.

The differentiated cells in the control group appear healthy. They contain long branching processes and extended flat cell bodies. The cells treated with hemin show severe reduction in process length signifying stress to the cell. The DMSO treated cells appear morphologically similar to the control group. These results indicate the toxic effects of iron on neurons. The hemin treatment lead to a morphological decline in the cell's health. The appearance of the DMSO controls indicates most physical changes seen in the hemin treated cells are due to the hemin treatment and not the vehicle. It is important to note that the two cells on the hemin treated slide shown above were the only surviving cells found on the slide. The toxicity of the iron may have damaged the cells to the point that they lacked the ability to adhere to the slide.

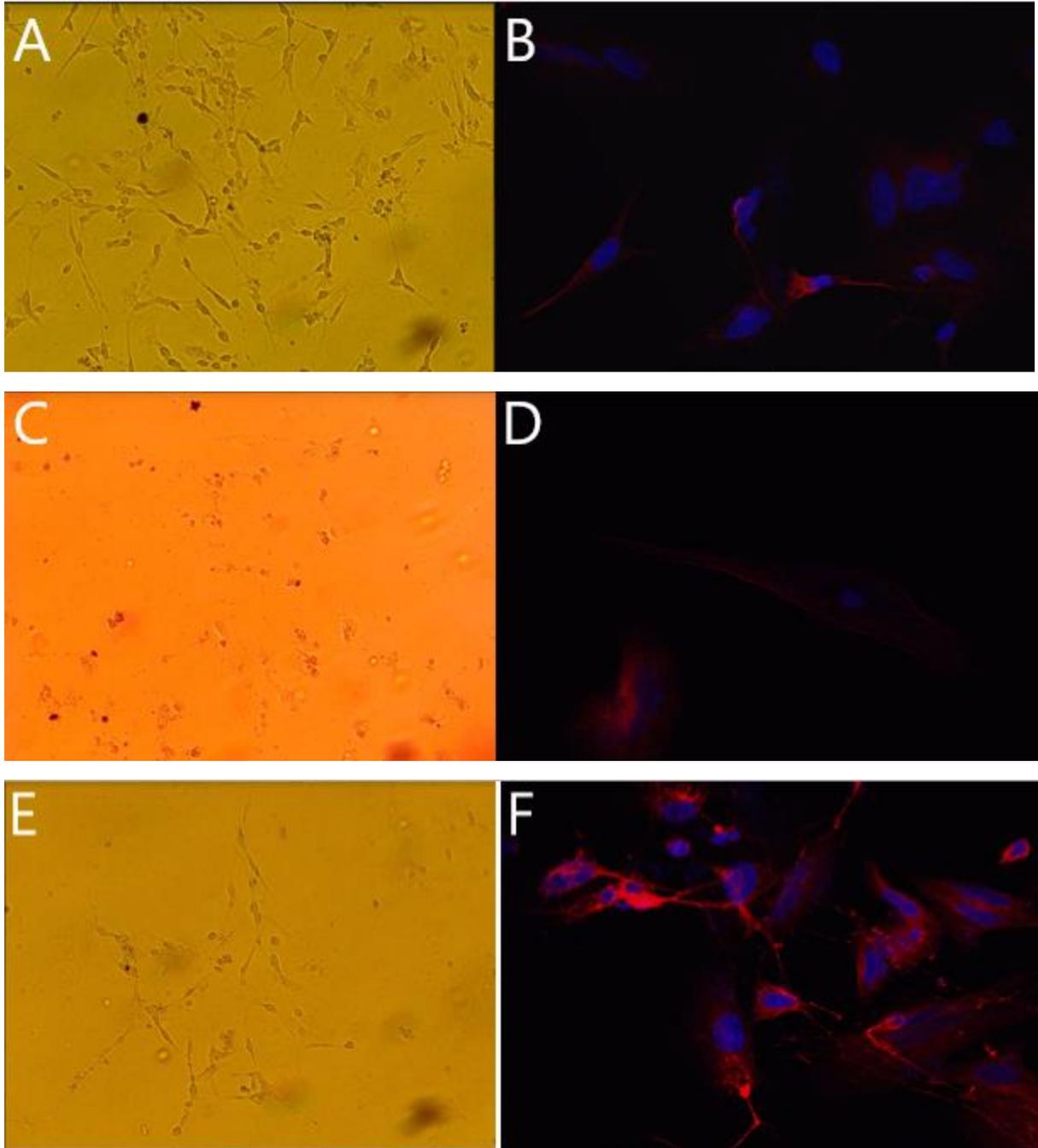


Figure 7: Images of differentiated SH-SY5Y neuroblastomas unstained and stained for beta tubulin, red, and DAPI, blue. A) Taken with a light microscope, showing untreated cells. B) Taken with a confocal microscope, showing untreated cells. C) Taken with a light microscope, showing cells treated with 25 μ M of hemin for 24 hours. D) Taken with a confocal microscope, showing cells treated with 25 μ M hemin for 24 hours. E) Taken with a light microscope, showing cells treated with DMSO. F) Taken with a confocal microscope, showing cells treated with DMSO

The toxicity data will set a baseline for future objectives of the study. A cell saving experiment will be attempted by treating cells first with hemin and then with Cp. The Cp treated cells viability and morphological appearance will be compared to the hemin toxicity data to determine if Cp treatment can counteract iron overload toxicity, presenting itself as a possible therapeutic to this condition.

Conclusion:

The global metabolomics LCMS data is suggestive that the initiation of SAH leads to a change in metabolism that may be due to iron handling. This change could give insight into possible indicators of DCI development. Specifically the LCMS data shows an decrease in BR, which continued to decrease after the bleed. Glycolysis also appears to be down regulated, but this effect appears to have somewhat of a delayed response. Further investigation into these metabolites needs to be done using targeted LCMS to assure the accuracy and validity of these findings. The 5 day differentiation process using retinoic acid was successful in inducing morphological changes in the neuroblastomas. Microscopy studies of visual changes in differentiated SHSY5H treated with 25 μ M hemin and DMSO, showed hemin affected the appearance of the cells. Hemin treated cells had limited processes and no branching. The hemin treated slide contained 2 cells total upon staining for beta tubulin. This is believed to be a result of iron induced toxicity leading to a decrease in adhesion, allowing for the cells to be washed off of the slide. The results could also be due to improper washing, however the other 2 slide contained a multitude of cells. The DMSO control showed relatively long, branching processes, leading to the conclusion that the morphological changes seen in the hemin treated cells were the result of the hemin and not the vehicle. The resazurin toxicity assay showed a general trend of increased toxicity with increased hemin concentration. The vehicle control also showed a large

amount of killing, which needs to be corrected in future assays. These results indicate that increasing iron exposure changes neurometabolism, and is damaging to in vitro cells. Future research will assess the function of Cp in relation to iron exposure and determine if the ferroxidase can participate in cell saving with exposure to hemin.

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Appendix:

Safety

In order to maintain a safe lab environment, the lab must be equipped with proper safety equipment. In case of a chemical spill onto the body or eyes of any lab personnel, the lab contained a shower and eyewash station. Another shower and eyewash station was located in the hallway. The lab also was equipped with a fire extinguisher in case of ignition of any flammable solution stored in the lab.

All cell culture work was done in a hood to protect against contamination. Living culture has the potential to cause infection through bacterial growth or other biological contaminants. Trypsin was used to break cell adhesion. Trypsin is a protease and has the potential to cause damage if it comes in contact with an individual. Trypsin was only used in the hood. Gloves were worn at all times when handling any cell culture and when using trypsin.

In order to obtain desired pH of solutions, hydrochloric acid and sodium hydroxide were used. Gloves and goggles were used when handling these solutions to avoid possible spilling onto the hands or eyes. If exposure occurs, the area should be immediately rinsed with water for 15 minutes, using the eyewash and shower station when applicable. Chloroform was used to make certain solution. The fumes from chloroform can have physiological effects on the individual exposed. The chemical was only opened in a fume hood.

CSF samples from deidentified human patients have the potential to infect an exposed individual to a number of diseases that are transmissible through body fluids. In order to avoid contraction of disease, gloves, goggles, lab coats, and face masks, were worn at all times when handling CSF samples.