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CHARACTERIZATION OF NEUTRAL SPHINGOMYELINASE IN PLASMA AND PBMCS
AND THE ROLE OF SPHINGOLIPID ENZYMES IN PATHOLOGY

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ABSTRACT

The balance of sphingolipid enzymes is critical for normal physiological mechanisms. Specifically acid and neutral sphingomyelinase play important roles in maintaining the balance for cell proliferation and apoptosis. Acid sphingomyelinase and Neutral sphingomyelinase hydrolyze sphingomyelin to ceramide and play key roles in regulating sphingolipid metabolism and determining cell fate. A number of research studies show their role in neuropathology. Specifically increased levels of ASM are serum were recorded in dementia, Alzheimer’s disease, major depressive disorder, and multiple sclerosis. Since then, it has been of further interest to determine the activity of other sphingolipid enzymes in neuropathology and also measure acid sphingomyelinase in cerebrospinal because of its close proximity to brain regions.

ASM was characterized in cerebrospinal fluid in 2013 but clinical samples have not yet been tested. In this research, an analysis of ASM activity in cerebrospinal fluid was conducted in a clinical study comparing patients with multiple sclerosis to healthy controls.

Additionally before this research, the sphingolipid enzymes, neutral sphingomyelinase had not been characterized in human blood samples. An enzyme assay was developed and optimized to measure neutral sphingomyelinase activity in PBMCs and serum and was then tested in patients with alcohol dependency. The results of these studies provide insights into the roles of sphingolipid enzymes in pathology.
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Chapter 1

Background

The field of neuroimmunology is one of the main spheres of neurology that has yet to be explored to its depths (Minghetti, 2005). Progress in this field begins on many levels, one being dissecting the details of biomolecules at a mechanistic level. This thesis explores the role of sphingolipids in autoimmune and neuropsychiatric disorders through the development of an assay for sphingolipid enzymes and the use of this new assay to test for aberrant activity in disease patients. The two main enzymes of focus are neutral sphingomyelinase (NSM) and acid sphingomyelinase (ASM). While ASM has been characterized in many different bodily fluids (Quintern, 1987), NSM has not (Hannun, 2005). This difference has provided a gap in knowledge that is important to fill. NSM is actually more concentrated in the brain than any other sphingolipid enzyme. For this reason, NSM characterization should be prioritized to aid in the understanding of neuropathology. In the following pages, I will present a new analytic method for NSM then apply this to test patients with alcoholism and multiple sclerosis for the activity of NSM. Overall, these experiments provide evidence to a closer understanding of sphingolipid enzymes within pathology and allow us to further elucidate the difference between ASM and NSM.

Role of Sphingolipids

For many years, sphingolipids were thought to be inert lipids that were part of cell membranes (Hannun, 2008). The discovery that protein kinase C was inhibited by the sphingolipid sphingosine led to a new interest in these lipids in the regulation of cell biology and signal transduction and opened an entirely new realm of research (Hannun, 1986, Hannun, 1996).
Today, it is understood that a number sphingolipids have bioactive and second messenger molecular roles (Rhein, 2012). Additionally, sphingolipids are ubiquitous components of all eukaryotic cells membranes and they are particularly abundant in the nervous system (Hannun, 2001). Sphingolipids, structurally, are a class of lipids, which are characterized by their 18 carbon amino-alcohol backbones, which are created in the smooth endoplasmic reticulum from non-sphingolipid molecule precursors (Hannun, 2001). Sphingolipids have a long change sphingoid base, an amide linked fatty acid and a polar head group at position 1 which determines the specificity of the sphingolipid (Muehle, 2013). While most people understand sphingolipids to be a core part of cell membranes, sphingolipids also play a key role in signal transduction, specifically in cell proliferation and apoptosis (Muehle, 2013). In the last 20 years, scientists have found that sphingolipids play a key role in physiology, which leads them to want to understand their role in pathophysiology (Marchesini, 2004).

Modification of the basic 18 carbon amino alcohol structure is what creates the large variety of sphingolipids that play important roles in the cell membranes, as well as acting as significant bioactive metabolites that regulate cell function (Gault, 2010).

Sphingolipids’ variety of active roles serve to regulate diverse processes which include inflammatory signaling, transduction, cell death, proliferation, and pain sensing. (Hannun, 2001) Their uncovered key roles in physiology make them of particular interest when studying molecular neurobiology and neurodegenerative diseases (Haughey, 2015).

Further, specifically in the nervous system, evidence has shown that sphingolipids play a role in synaptic neuro-transmission, central nervous system development, immune and inflammation mechanisms within the brain, and oligodendrocyte and myelin function (Muehle, 2013). Given this initial insight on the role of sphingolipids within the brain, it is of interest to further uncover their biomechanisms. To accomplish this, new methods must be developed to understand sphingolipids and sphingolipid enzymes. Currently, it is not well understood how these sphingolipids are regulated or what the role of specific sphingolipids are in pathology. For this reason, an analysis of specific sphingolipid
roles and a study of sphingolipid enzymes are critical to better understand how the molecules are regulated and function.

Figure 1: Role of Sphingolipids (Muehle, 2007)

Sphingolipid Enzymes Overview

Sphingolipids role in signal transduction lies in its lipid metabolism pathway:
There are 3 main groups of enzymes involved in the Lipid Metabolism pathway: Sphingomyelinases, Ceramidases, and phosphorylation enzymes, kinases and phosphatase (Hannun, 2008).

Sphingomyelin is cleaved by a sphingomyelinase, acid Sphingomyelinase (ASM) or neutral sphingomyelinase (NSM) to form ceramide (Hannun, 2008). Ceramide is a bioactive lipid that mediates cell growth, differentiation, stress responses, and programmed cell death. (Muehle, 2013) Ceramide metabolism generates a cascade of bioactive lipids, all of which carry a specific signaling capacity.
(Hannun, 2008). Ceramide is then cleaved by ceramidase to form sphingosine and a fatty acid, which in turn, is phosphorylated by sphingosine kinase into sphingosine-1-phosphate, S1P. These lipids exert opposite biological effects to that of ceramide; ceramide and sphingosine are primarily anti-proliferative and pro-apoptotic, whereas S1P promotes cell growth and counteracts apoptotic stimuli. (Muehle, 2013). Overall, as a result of these mechanisms, the ratio between ceramide plus sphingosine and Sphingosine-1-phosphate levels, also referred to as the ceramide/S1P rheostat, is the true determinant of a cell’s fate, rather than the individual ceramide, sphingosine, or S1P levels (Muehle, 2013). In this research, the focus is on the first step of this mechanism the conversion of sphingomyelin to ceramide through the activity of the enzymes acid sphingomyelinase and neutral sphingomyelinase.

A substantial amount of research has been done on the enzyme, acid sphingomyelinase (ASM), however neutral sphingomyelinase (NSM) has been overlooked due of the difficulty to characterize its activity and early assumptions that NSM would simply function similarly to ASM (Norma, 2004). However, recent research has sparked interest that their functions are not identical. A summary of the current understanding of these enzymes is provided in the following pages.

**Acid and Neutral Sphingomyelinase**

As described above in the lipid biosynthesis pathway, sphingomyelinases hydrolyze sphingomyelin into ceramide. These sphingomyelinases react to external stimuli to maintain sphingolipid balances. These sphingomyelinases are split into two main group: acidic and neutral sphingomyelinase.

**Acid sphingomyelinase**

Acid sphingomyelinase is essential for the basic housekeeping of lysosomes. In Neiman’s Picks disease, a genetic disorder where no ASM is present, an accumulation of sphingomyelin occurs which leads to multi-organ abnormalities (Muehle, 2013). In light of this, ASM is essential to maintain normal function. ASM is a glycoprotein, which is mainly located in lysosomes, L-ASM, but this enzyme can also
be secreted to the extracellular compartment via the secretory pathway, S-ASM. Lysosomal ASM, L-ASM, and secretory ASM, S-ASM, differ in their molecular weight and their N-terminal amino acid sequence (Hannun, 2001). They both require zinc (Zn$^{2+}$) for catalytic activity, but L-ASM is presumed to have sufficient levels of zinc intracellularly and is thus seemingly independent of supplemented zinc in biochemical assays. For S-ASM assays, zinc must be added.

Acid sphingomyelinase, ASM, is encoded by the gene SMPD1. This gene for human acid sphingomyelinase in 5kb in length, consists of six exons and maps to chromosome 11p15.1-11p15.4 (Hannun, 2004).

**Role of ASM in Neurological and Psychiatric Disorders**

Several reports have established acid sphingomyelinases role in neuropsychiatric disorders and have connected the role of inflammation and inflammation mediators such as sphingolipid enzymes to neurodegeneration (Fakhoury, 2015, Haughey, 2010). It has been documented that there have been increased ASM activity in patients with major depression, Alzheimer’s dementia, and multiple sclerosis and alcohol disease when compared to controls (Kornhuber *et al*., 2005, Gulbins *et al*., 2013, He *et al*., 2008, Walter *et al*., 2010, Reichel *et al*., 2010). These studies were conducted using blood samples and have not been conducted in cerebrospinal fluid or for neutral sphingomyelinase. It would be of interest to measure enzyme levels in CSF because of cerebrospinal fluids direct contact with neural tissue. It is also of interest to measure NSM activity in these patients to elucidate and characterize the differences between the enzymes.
**Neutral Sphingomyelinase**

Neutral sphingomyelinase activity was first described in fibroblasts from patients with Niemann-Pick disease, who exhibit deficiencies in acid sphingomyelinase (Hannun, 2008). Since then, characterization of NSM has been attempted in the hopes of better understanding their distinct function(s) (Jana, 2007, Liu, 1997).

Characterization in mice has revealed that NSM has an optimum pH of 7.4 and is dependent on magnesium ions for activity (Hannun, 2004). Most characterizations have been in bacteria and mice models and there has been success, to our knowledge, in characterizing NSM in human blood samples.

Neutral sphingomyelinase is of particular interest because it is highly concentrated in the brain in comparison to the rest of the body, making it of particular interest in the study of neuropsychiatric disorders (Muehle, 2013).

![Figure 3: Distribution of Enzyme in Rat Model (Christane, 2013).](image-url)
While ASM is expressed more generally, NSM is heavily expressed in the striatum, hippocampus and prefrontal cortex in rat models. There is a five- to ten-fold higher activity of neutral sphingomyelinase, acid ceramidase, and neutral ceramidase in brain regions than acid sphingomyelinase (Christiane, 2013).

Neutral sphingomyelinase has a separate coding region from acidic neutral sphingomyelinase. This gene is known as nSMase2. nSMase2 has been elucidated to play a number of bioactive roles. 

![Figure 4: NSMase 2 functions](image_url)

We are interested in determining the differences in the functions of neutral sphingomyelinase and acid sphingomyelinase. The goal of this thesis was to optimize a neutral sphingomyelinase enzyme activity assay and to determine the functional differences between NSM and ASM activities in various body samples including serum, plasma, and PBMCs, as well as the difference in activity between sex, age, and diseases.
Given that recent studies have exhibited increased ASM levels in serum and PBMCs in those with neuropsychiatric disorders such as major depression, Alzheimer’s and multiple sclerosis, it is hypothesized that ASM will have increased activity in the cerebrospinal fluid of patients with multiple sclerosis. It of interested to study cerebrospinal fluid distinctly from blood samples in order to determine if sphingolipid enzymes play a specific role in this region and also because of cerebrospinal fluid’s direct contact to brain region.

Research has shown that ethanol can alter the sphingomyelinase-ceramide pathway and has shown to increase ASM levels in mouse models and induce cell death (Reichal, 2011). It is hypothesized that NSM will have a similar response to ethanol as ASM and that there will be increased NSM activity in the serum of patients with alcohol dependency.
Chapter 2
Material and Methods

The material and methods will be broken up into 2 sections. The first is the description of the optimized enzyme assay, which has been developed to measure neutral sphingomyelinase activity. This protocol was adapted from the acid sphingomyelinase assay and the data from the experiments performed to optimize this method is provided in the results section. A concise description of these methods will be provided here. The second half of the methods presents the application of the developed assay to measure sphingomyelinase activity in clinical patients with neurodegenerative diseases.

Optimized Neutral Sphingomyelinase Assay:

An optimized assay base on a fluorescent substrate was adjusted and applied to measure neutral sphingomyelinase activity in peripheral blood mononuclear cells (PBMCs) and serum. For optimization of the serum and PBMCs assay, a series of Carmody buffers (mixtures of 0.2 boric acid, and 0.05 M citric acid) with pH adjusted from pH 3-10, the addition of various detergents, and the addition of different concentrations of salts, were tested. After each adjustment, enzyme activity was measured using a fluorescent test to determine which conditions were most optimal.

NSM Activity in PBMCs:

The NSM activity in PBMCs was determined using a fluorescent substrate BODIPY-FL-C12-SM (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-a-indacene-3-dodecanoyl)sphingosyl phosphocholine, D-7711, Invitrogen/Life Technologies) with 4 replicates of each. A standard reaction was optimized, which contained 2.75 ul of a 1 to 2000 dilution of the BODIPY-FL-C12-SM stock solution in a total volume of 5.5 ml of 10X Carmody buffer (Boric Acid 0.2M, Citric Acid 0.05 M, Sodium phosphate 0.1 M; pH varying depending on study, see appendix) with 0.02% NP40 detergent and 1.1 ml of 1 M stock MgCl₂. This reaction was initiated with 110 ul of PBMCs.

NSM Activity in Serum
The NSM activity in Serum was determined using a fluorescent substrate BODIPY-FL-C12-SM (N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-a-indacene-3-dodecanoyl)sphingosyl phosphocholine, D-7711, Invitrogen/Life Technologies) with 4 replicates of each. A standard reaction was optimized which contained 2.75 ul of a 1 to 2000 dilution of the BODIPY-FL-C12-SM stock solution in a total volume of 5.5 ml of 10X Carmody buffer (pH varying depending on study) with 0.05% NP40 detergent and no magnesium. This reaction was initiated with 66 ul of PBMCs.

After incubation for 24 hours at 37 degrees Celsius, reactions were stopped by freezing them in -20 degrees Celsius and then stored until processing. These samples were spotted on silica gel thin layer chromatography plates. Ceramide and uncleaved sphingomyelin were separated over a distance of 3 cm using a mixture of ethyl acetate with 1% HAc as a solvent.

![Figure 5: Sphingomyelinase converts Sphingomyelin to Ceramide](image)

The Typhoon Trio scanner (GE Healthcare; 488nm excitation, 520 nm emission, 285 V, 100 um resolution) with the ImageQuant software (GE Healthcare) was used to quantify the data. Enzymatic activity is presented as the hydrolysis rate of SM (fmol) per time (h) and per sample volume (ul)

Collection of Mononuclear Cells (PBMCs) and Serum Samples from Human Patients:
Human PBMCs and Serum were obtained by taking blood from patients and controls. Blood was collected in polypropylene tubes, centrifuged at 2000xg for 10 minutes to pellet cells and stored at -80 degrees Celsius until use.

**Clinical Testing:**

**Alcohol Study Patients:**

The total study included 48 individuals, 24 individuals in the control group (12 males and 12 females) and 24 individuals in the alcohol group (12 males and 12 females). The individuals in the alcohol group suffered from alcohol dependence according to the ICD-10 criteria. Participants were active drinkers (abstinence <36 hours) with a reported history of alcohol consumption ranging from 2 to 3 years. All patients were seen as patients in the detoxification unit of University Hospital of Erlangen. Blood was taken from patients. Blood samples were collected in polypropylene tubes, centrifuged at 2000xg for 10 minutes to pellet cells and stored at -80 degrees Celsius until use. Serum NSM assay protocol was followed to measure enzyme levels in patients.

**Approval of Studies:**

This research was conducted in compliance with the Helsinki Declaration and approved by the Ethics Committee of the Friedrich-Alexander-University of Erlangen-Nuremberg. Written informed consent was obtained from all person prior to participation. All participants underwent physical examination at the beginning of the study.

**ASM in Multiple Sclerosis Patients:**

Acid sphingomyelinase activity was measured in cerebrospinal fluid of multiple sclerosis patients. This procedure followed the protocol of Christiane Muehle, which was published in her recent protocol paper on the characterization of acid sphingomyelinase activity in human cerebrospinal fluid (Muehle, 2013). The protocol is as follows: The fluorescent substrate BODIPY-FL-C12-SM with 3-4
replication was used to measure activity. A standard reaction included 58 pmol substrate (1:2000 dilution of the 1.16 stock solution in DMSO) in a total volume of 100 ul 200mM sodium acetate buffer (pH 5.0) with 500mM NaCl, 0.02% Nonidet P-40 detergent and 500 uM ZnCl2. This reaction was initiated by the addition of 0.6 ul of cerebrospinal fluid. After an incubation of 24 hours at 37 degrees Celsius, reactions were put into freezer at -20 degrees Celsius until further processing. During processing, samples were spotted on silica gel chromatography plates and ceramide and uncleaved sphingomyelin were separated over a distance of 4 cm using a mixture of chloroform: methanol (80:20 v/v). The Typhoon Trio scanner (GE Healthcare; 488nm excitation, 520 nm emission, 285 V, 100 um resolution) with the ImageQuant software (GE Healthcare) was used to quantify the data. There were 25 Multiple Sclerosis patients and 15 controls from the University Hospital Erlangen.

**Collection of Cerebrospinal Fluid Sample (CSF) from Human Patients**

Human CSF was obtained through lumbar puncture of patients being tested for various inflammatory diseases of the central nervous system. Those who tested negative were considered controls. CSF was collected in polypropylene tubes, centrifuged at 2000xg for 10 minutes to pellet cells and stored at -80 degrees Celsius.

![Image](human_serum.png)

*Figure 6: Enzyme Assay Human Serum Sphingomyelin Conversion to Ceramide Converted to Fatty Acid*
Chapter 3

Results

Characterization of Neutral Sphingomyelinase in Plasma and PBMCs

Neutral sphingomyelinase activity had never been characterized and optimized. Varying a number of conditions including pH, detergent, magnesium concentration and incubation time, provided a more thorough understanding on the optimal conditions to measure neutral sphingomyelinase.

Figure 7: Detergent Assay PBMCs NSM Conditions
Detergent conditions were varied with different concentrations, high activity was observed using Triton X 100 at 0.010% detergent with 55% enzyme concentration. However, this measurement was viewed as an outlier in its activity with over 35% more activity. The detergent NaDoc has a similar curve pattern as the other detergents but has the highest peak at 0.02%. NaDoc was considered optimal and used for future experiments. A concentration between 0.010% and 0.100% is most optimal for nearly all detergents.

![Figure 8: pH Assay for PBMCs for NSM Conditions](image)

Highest activity is observed between pH 7 - pH 8. Low neutral sphingomyelinase activity is observed in the acidic range for pH. Enzyme activity drops at pH of 9 and increases again in the alkaline range. As expected, a neutral pH was optimum.
NSM is dependent on magnesium as a cofactor. The PBMCs have the highest activity at a concentration of 75 mM from every group in the neutral pH range. A concentration of 150 mM and above magnesium for neutral pH ranges decreased the enzyme activity. There was a slight increase enzyme activity at 300 mM MgCl₂ over 150 mM MgCl₂. 75mM MgCl₂ in neutral conditions was considered optimal to measure NSM activity in PBMCs.
Reliable enzyme activity is considered to be between 30% and 60% activity. In this case, 1.2 ul of PBMCs incubated between 20 to 40 hours would be optimal.

The optimal conditions for measuring neutral sphingomyelinase in PBMCs are exhibited to be pH 7.2 using carmody buffer, 0.2% NaDoc detergent, no zinc, and 75 mM Magnesium.
Characterization of Neutral Sphingomylinase in Serum:

Figure 11: Serum pH and Magnesium Assay NSM conditions

Highest neutral sphingomyelinase activity was observed in a pH range between 6.5 and 7.5. There was very little activity in the acidic range between pH 3-5 and also very little activity between pH
8-11. This condition contained no zinc but some magnesium.

Figure 12: Effect of Magnesium and EDTA on serum NSM (pH 7.4)

Highest activity is observed between 0.1 and 1.0 mM of MgCl₂ and MgSO₄. EDTA chelates with Magnesium. With the addition of EDTA, the activity of the enzymes drops significantly, which is not surprising given that Mg++ is a cofactor for the enzyme.
Figure 13: Influence of pH, Zn$^{2+}$ and Mg$^{2+}$ on Sphingomyelinase

Highest activity is observed in neutral range pH of 7.0 and 7.4 when magnesium is not present. For serum, the high concentration of 200mM magnesium inhibits activity. NaDoc detergent was used in this experiment. NaDoc detergent inhibited activity of acid sphingomyelinase in the acidic pH range.
Concentrations of magnesium beyond 1.0 mM began to decrease Neutral sphingomyelinase activity in serum. Concentration of 100 mM of both MgCl₂ and MgSO₄ showed activity approaching 0. Using MgSO₄ consistently showed higher NSM activity than when using MgCl₂. 0.1 mM MgSO₄ is considered optimal.

The optimal conditions for measuring neutral sphingomyelinase activity in serum are exhibited to be pH 7.2 using carmody buffer, 0.2% NaDoc detergent, no zinc, and 0.1 mM magnesium.
NSM in Clinical Samples

Serum NSM in alcohol dependent patients (NOAH)

Figure 15: Serum NSM Activity in Alcohol Dependent Patients

Neutral sphingomyelinase activity is significantly higher for both males and female alcohol dependent patients in comparison to controls individuals’ NSM activity. For males, the p-value is 0.010 for females the p-value is 0.004.
Serum Acid Sphingomyelinase in alcohol dependent patients

Figure 16: Acid Sphingomyelinase activity in alcohol dependent patients

ASM activity is significantly higher in male alcohol dependent patients with a p-value of 0.016.

In female patients, ASM activity is not significantly different between alcohol dependent patients and controls.
There was a sample size of 18 males and 22 females. Females with multiple sclerosis had a significant decrease in acid sphingomyelinase activity in comparison to the control group of females. Males with multiple sclerosis also had decreased ASM activity however this was not statistically significant.

Figure 17: S-ASM in CSF of patients with Multiple Sclerosis
Chapter 4
Discussion: Neutral Sphingomyelinase Activity in Serum and PBMCs

Optimizing PBMCs Assay

Through this experimental process it was determined that there are specific conditions that are optimal when measuring the activity of neutral sphingomyelinase in PBMCs. The data above allowed the optimal conditions to be elucidated. Before these experiments, measuring neutral sphingomyelinase in any of these samples was not possible. Being able to measure an enzyme in blood allows for a minimally invasive enzyme assay, which can lead to a further understanding of the role of neutral sphingomyelinase in both patients with full health and those with a disease or disorder.

\textit{pH}

The ideal pH range when measuring the activity of NSM in PBMCs was 7.35 exhibited in Figure 9. This was the expected measurement given the initial discovery of neutral sphingomyelinase occurred in the neutral range activity. The acidic range was not active indicating that acid sphingomyelinase activity was not being recorded.

\textit{Detergent}

The next condition that needed to be optimized for PBMCs was detergents. The highest enzyme conversion rate was observed using Triton X, however this was viewed as unusual due to the fact that there was such an extreme jump in activity at 0.01% detergent. Therefore, the second highest measured activity with NaDoc was considered to be the true optimal detergent to measure NSM in PBMCs. The activity for NaDoc followed a normal curve with the other detergents and had an enzyme conversion rate of about 20% with a detergent concentration of 0.015%. Additionally, it was determined in previous experiments that NaDOC inhibits acid sphingomyelinase activity. This is useful to ensure that it is strictly neutral sphingomyelinase activity recorded. The graph below exhibits the inhibition of acid
sphingomyelinase in NaDoc detergent conditions.

![Graph showing S-ASM activity (% max) vs. detergent (% v/v) for different detergents.](image)

**Figure 18 NaDoc Inhibits Acid Sphingomyelinase**

**Magnesium**

It is known that neutral sphingomyelinase is dependent on magnesium (Hannun, 2008) however it was never previously determined at which concentration of magnesium neutral sphingomyelinase activity was optimal for PBMCs. For this reason, the magnesium concentration were tested and the results are shown in **Figure 10**. Varying magnesium concentrations at varying pHs were observed to determine the most optimal concentration of magnesium for neutral sphingomyelinase. It was clear that in the neutral pH range from 7.0 to 7.5 that a magnesium concentration of 75 mM has the highest activity.

**Volume and Time**

The final condition to determine optimal conditions for measuring the activity of neutral sphingomyelinase were to see what volume of PBMCs sample should be used for the test and how long the reaction should incubate for. Reliable enzyme activity is considered to be between 30% and 60% conversion rate. In this case, looking at Figure 11, one can see that the optimal volume of PBMCs is 1.2 ul and then allowing that to be incubated between 20 to 40 hours would be optimal. We incubated for 24
hours from this experiment forward. A 24-hour incubation cycle also allows scientists to execute experiments regularly and efficiently.

Optimizing Serum Assay:

\textit{pH}

The ideal pH range when measuring the activity of NSM in serum was between 6.5 and 7.5 exhibited in Figure 12. The acidic range was not active indicating that acid sphingomyelinase activity was not being recorded. The alkaline range was also very low. As expected given the current understanding of neutral sphingomyelinase, its highest activity was within the neutral pH range. Additionally in this pH experiment, no zinc was added to the mixture. The lack of zinc eliminated the possibility of acid sphingomyelinase activity because s-ASM activity is dependent on zinc.

\textit{Magnesium}

It was important to determine that neutral sphingomyelinase activity was being recorded and not acid sphingomyelinase activity.

To help elucidate this, an experiment was conducted under neutral conditions with magnesium, and then with magnesium and the addition of EDTA. EDTA chelates with magnesium. As shown in Figure 13, the addition of EDTA led to a dramatic drop in enzyme activity; at the optimal activity of 1.0 mM Magnesium, the activity dropped from 23\% to 5\%. It is known that neutral sphingomyelinase is magnesium dependent; therefore this experiment clearly supports that neutral sphingomyelinase activity is being recorded. Further this experiment showed that the optimum amount of magnesium for neutral sphingomyelinase activity in serum is around 1.0 mM with higher concentrations of magnesium being inhibitory. To better determine the optimal magnesium concentrations, further magnesium experiments were conducted. Figure 15 shows that concentrations beyond 1.0 mM begin to decrease neutral sphingomyelinase activity in serum, with concentrations of 100mM of both MgCl$_2$ and MgSO$_4$ showing activity approaching 0. 0.1mM MgSO$_4$ was considered optimal and used in further experiments.
Detergent

To elucidate neutral sphingomyelinase activity from acid sphingomyelinase activity, the detergent NaDoc was used. It was known that acid sphingomyelinase activity is inhibited by the detergent NaDoc (Muehle, 2013). This was exhibited by the fact that there was very little activity in the acidic range for this experiment (less than 0.5% conversion) while the neutral range activity with the correct ion combination was between 2.5 to 3.5% conversion.

Significance of Optimal Conditions

In conclusion, the optimal conditions for measuring neutral sphingomyelinase in PBMCs are exhibited to be pH 7.2 using Carmody buffer, 0.2% NaDoc detergent, no zinc, and 75 mM Magnesium and the optimal conditions for measuring neutral sphingomyelinase activity in serum are exhibited to be pH 7.2 using carmody buffer, 0.2% NaDoc detergent, no zinc, and 0.1 mM Magnesium.

These optimized conditions are the conditions that were used to measure our clinical samples. The development of this novel NSM assay will hopefully aid in better understanding the role of NSM in the body and to better distinguish its role from other sphingolipid enzymes.

Future NSM Studies

While distinct NSM activity was measured in serum there are further steps to be taken to validate this finding. It would be important to check the serum and plasma of heterozygous NSM knockout mice. The gene nSMase2 could be knocked out. This would ensure that truly NSM levels are being recorded. Under the same optimal conditions outlined above, it is expected that no activity will be recorded in the neutral range for knock out mice. Also in future studies it would be interesting to measure NSM in CSF and then compare NSM to ASM levels in CSF.
Chapter 5

Discussion: Clinical Samples

Clinical trials:

These clinical trials were to serve as the first step in understanding the role of sphingolipid enzymes in disease specifically within multiple sclerosis and alcohol dependency. Altered levels of ASM and NSM in patients indicate their role in pathology.

ASM in CSF of patients with Multiple Sclerosis.

An increased activity of ASM was hypothesized for patients with Multiple Sclerosis as exhibited in Figure 18. However in the female MS patient group, there was a significant decrease in their ASM activity. Additionally, while not significant, the male group of MS patients also showed decreased ASM activity. This is different than the acid sphingomyelinase levels measured in serum from other studies, which showed increased activity. This difference and significant decreased in ASM activity in the CSF of MS patients raises a number of questions and possibilities. One possibility is that the enzyme levels in CSF are different than the levels in serum. While this was unexpected, it may actually lead to insights in how decreased enzyme activity plays a role in pathology. It is possible that in distinct regions of the body that different enzyme levels exist but from our knowledge this has never before been previously reported. In this light, instead of a strict perspective that increased levels of ASM contribute to multiple sclerosis, it may be more appropriate to consider that altered levels of enzymes are what contribute to the disease.

That being said, it has been observed that the therapeutic FTY720 is a blocker of spingosine 1-P and that is reduces the clinical symptoms within multiple sclerosis patients (Ludwig, 2006). FTY720 function as a sphingosine-1-phosphate receptor modulator. By inhibiting the receptor, it prevents the cells from signaling toward secondary lymphoid tissues and limits the release of lymphocytes (Ludwig, 2006).
Because a decrease in symptoms is associated with inhibition of S1P, it would be expected that decreased levels of sphingomyelinase would also decrease symptoms, following the sphingolipid pathway (Arundhati et al. 2010). For this reason, it is important to further investigate why decreased levels of ASM were observed in our study.

With this, an important consideration is that these decreased levels in in CSF could be the therapeutics these MS patients are taking. In future studies it would be imperative to record which drugs patients are on. For example, any MS patients on FTY720, which is known to inhibit acid sphingomyelinase by a mechanism similar to tricyclic antidepressants, would exhibit decreased levels of ASM (Ludwig, 2006). Therefore it is unclear whether the measurements reflect that natural case of enzyme activity for the disease or whether this has been affected by drug intake.

Independent of whether the clinical trials were affected by therapeutics, there is clear evidence that a rebalancing the sphingolipid rheostat could have a therapeutic basis. This research revealed a recording of decreased ASM however questions whether the alteration of enzymes could be both increased and decreased and how to consider that with a therapeutic.

Analyzing the neuropathology of Neumann Pick’s disorder provides another potential reason for decreases levels of ASM. In Neumann Pick’s disorder there is a depletion of Acid Sphingomyelinase, which leads to a build up of sphingomyelin fat in the brain. This build up of lipid leads to brain damage in patients. It is possible that a similar mechanism may occur within patients with multiple sclerosis and could explain the decreases levels of NSM in their cerebrospinal fluid.

Overall because the sphingolipid pathway induces apoptosis, differentiation, proliferation and growth arrest depending on the enzyme activity involved, it is important to continue to determine how the specific activity of each enzyme in patients influence the disease.

Future ASM in CSF in Multiple Sclerosis Patient Studies

It is important to repeat these studies to validate the decreased levels of acid sphingomyelinase in cerebrospinal fluid in patients with multiple sclerosis. Also, this study only had 40 individuals with 24
patients with multiple sclerosis. A larger sample size would ensure more accurate results in the future. Further, it would be important to determine if and what therapeutics each patient is on to determine how that may influence measured enzymes levels (Jana, 2010).

**Discussion of NOAH patients for NSM samples in comparison to ASM samples**

There was a significant increase in NSM activity for both male and female alcohol patients supporting the initial hypothesis. This increased NSM activity has been associated with inflammation in multiple research studies. Specifically an increase in neutral sphingomyelinase has been associated with oxidative stress and neuronal apoptosis (Jana, 2007). This stress could lead to long term neural and cognitive effects for those with alcohol dependency (Jana, 2010). While alcohol dependency is sometimes considered a psychiatric and mental disorder, this research reveals a possibility of the neurophysiology behind alcohol dependency (Moussas, 2009). Through this research, we are unable to determine whether increased NSM levels are a result of alcohol use or a biological characteristic of those who are more predisposed to be alcohol dependent. It would be important to conduct a long-term study measuring NSM levels of alcohol dependent patients directly after alcohol intake and over a time period of sobriety (Reichel, 2011).

Additionally, these results with NSM in alcohol patients are different from those in the ASM in alcohol dependent patients study (Reichel, 2011). This supports the hypothesis that NSM and ASM have distinct roles.

**Importance of clinical sample trials for ASM and NSM**

Ultimately sphingolipid enzymes are not yet well understood but with this evidence, it is appropriate to hypothesize their role in disease pathomechanisms. It is evident that both ASM activity levels are altered in Multiple Sclerosis, and that NSM and ASM activity levels are increased for patients with alcohol dependency. This catalyzes a number of future research questions and clinical application. For example, for patients with multiple sclerosis who already have altered sphingolipid enzymes affecting
their inflammation mediators, it may be critically important to ensure they are not regularly drinking alcohol to ensure their sphingolipid enzymes are not further altered from the sphingolipid enzyme rheostat. Further research should be done to determine these mechanisms. With more research, these enzyme levels could also serve as biomarkers for disease diagnosis and eventually could present as therapeutic drug targets for various neurodegenerative diseases.

Further, through the multiple clinical trials it is evident that ASM and NSM have distinct roles and are affected differently for pathology. There was a significant difference between NSM and ASM levels in both patients with MS and in the study of patients with alcohol dependency. The correlation of ASM-NSM is only at with a r-value of 0.469 and with a p-value of 0.0077. Moving forward more attention should be made to further elucidate the difference in their roles in pathology and it should not be assumed that they have the same roles in simply different pH regions.


ACADEMIC VITA

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EDUCATION

The Pennsylvania State University, The Schreyer Honors College
University Park, PA

Eberly College of Science Graduation: May 2016
Bachelor of Science Biology-Neuroscience with Honors

RESEARCH EXPERIENCE

Children’s Hospital of Philadelphia Philadelphia, PA
Medical Observer: Radiology
May 2015-August 2015

- Worked under Dr. Lisa States observing and rotating through various medical departments
- Developed operational efficiency analysis for interventional radiology surgeries to effectively shorten transition times between surgeries
- Attended weekly conferences covering complex medical cases

DAAD RISE Fellowship, Kopfkliniken des Uni-Klinikums, Neurobiology Laboratory
Erlangen, Germany
Undergraduate Researcher with Dr. Christiane Muehle
May 2014-August 2014

- Selected as 1 of 300 students for DAAD Rise Fellowship from over 2,000 applicants to research in Germany
- Researched the role of sphingolipid enzymes in Multiple Sclerosis and Depression
- Developed novel enzymatic assay for Neutral Sphingomyelinase using bio-fluorescent BODIPY
- Measured activity of Neutral Sphingomyelinase in clinical patients and compared measurements to healthy controls.
- Observed significantly decreased amounts of this enzyme in patients with depression and female patients with MS

Pennsylvania State University, Neurobiology Laboratory
University Park, PA
Undergraduate Researcher with Dr. Byron Jones  September 2012-May 2014
• Researched host susceptibility to paraquat neurotoxicity and its relevance to Parkinson’s disease
• Analyzed the genetic differences in populations exposed to paraquat and which genes affected their susceptibility

Drexel College of Medicine, Microbiology Laboratory  Philadelphia, PA

High School Researcher under Dr. Thomas Edlind  June 2011–August 2012
• Researched Elucidate function of RLM1 in the MAP-Kinase pathway in cellular homeostasis in C. albicans to identify potential therapeutic targets. Research poster is attached*

LEADERSHIP EXPERIENCE

Ukweli Test Strips Venture, Pennsylvania State University  University Park, PA
Project Developer and Research Member  January 2015-December 2015
• Worked to develop low cost diagnostic tools for developing world contexts specifically Nyeri, Kenya in May 2015
• Redesigned inkjet printers to print reactant chemicals instead of ink, and printed reliable low cost test strips to screen for urinary track infections and diabetes
• Worked with editorial team and Khanjan Mehta to write, edit, and design the book, “Solving Problems That Matter”, attached*
• Published paper in Global Humanitarian Technology Journal, attached*

Leadership JumpStart  University Park, PA
Teacher/Life Coach  August 2013-January 2015
• Presented students with dynamic tools on how to develop their leadership skills and empower students to believe they make an impact on campus and in society
• Meet one on one with students to dissect challenges faced, empathize and provide potential solutions
• Created space for students to foster internal motivation, question their leadership philosophy and take action

MedRhythms, Inc., Neurologic Music Therapy Startup  University Park, PA
Business Development and Research Intern  October 2015-Present
• Focused on the intersection between neuroscience, music and technology, providing interventions to achieve optimum outcomes in sensorimotor, speech and language and cognitive goals in patients.
• Worked closely with CEO and President to develop business development plans, collect and compile neuroscience journal literature, and create patient testimonial videos
Presidential Leadership Academy  
University Park, PA  
Member  
March 2013-Present

- Selected as one of the top 25 Freshman on Penn State’s Main Campus  
- Took classes with the President of the University, President Erickson and President Barron, and the Dean of the Honors College, Dean Christian Brady.  
- Participated in 3-year leadership program to develop and demonstrate critical thinking, analysis and decision making

Apollo, THON Special Interest Organization  
University Park, PA  
Vice President, Treasurer  
May 2014-June 2015

- Provided both moral and financial support to the Four Diamonds children and their families in their fight against pediatric cancer  
- Organized fundraisers, community events and presented to potential donors  
- Raised over $157,00 in the 2014-2015 year for the Four Diamonds Fund

One Team International  
University Park, PA  
Treasurer, Co-Founder  
April 2013-January 2015

- Created a forum for students to discuss current women’s issues and used the knowledge gained from these discussions, to coordinate advocacy and fundraising events that empower student through sport and physical activity  
- Supported the program Naz India in Delhi, whose goal is to educate and empower young women through sport  
- Traveled to Delhi, India in July 2013 to better understand cultural context and develop partnership with Naz India

HONORS/AWARDS

- Eric and Tara Keiter Science Honors Scholarship  
- Schreyer Honors College Academic Excellence Scholarship  
- Phillip and Barbara Schumacher Honors Scholarship  
- Mosakities Family Trustee Scholarship for the Schreyer Honors College  
- Germantown Academy McNeil Financial Award
Can We Manufacture Diagnostic Test Strips Using an Inkjet Printer?

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Abstract—Developing countries face an increasing double disease burden of chronic health conditions like cancer, cardiovascular disease, and diabetes alongside infectious communicable diseases like tuberculosis, HIV/AIDS, and malaria. Rural patients often cannot afford to visit established medical centers because of the cost or time associated with travel. Poor education and social stigma prevent patients from seeking medical attention or getting tested for curable illnesses. Access to affordable and accessible tools for screening and diagnosis of common illnesses can greatly reduce the strain on fledgling health care systems. This article provides a proof-of-concept for manufacturing dipstick test strips that can be used for a variety of diseases.

Moreover, the health care situation is further compromised by the double burden of infectious diseases alongside the increasing prevalence of chronic illnesses [5] [6]. For example, in sub-Saharan Africa every woman has a 50% chance of attaining a urinary tract infection in her lifetime [7] [8]. Additionally, given major changes in lifestyles diabetes has become an epidemic in sub-Saharan Africa [9]. There is a large gap between the need for healthcare and the accessibility to and affordability of healthcare to community members. In low resource settings, access to healthcare is hard to come by. Just getting to a clinic or hospital can take a person a few hours.

Selection for Echinocandin Resistance: Loss of Heterozygosity in Polymorphic Transcription Factor Rlm1 of Candida albicans

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Department of Microbiology and Immunology, Drexel University College of Medicine

ABSTRACT

Like-producing infections with opportunistic yeast Candida albicans have increased in recent years due to the increase in number of individuals who are immunocompromised in association with AIDS, organ transplants, and antibiotics for cancer and autoimmune diseases. UNICEF recently reported a 12% increase in candidiasis in children aged 1-10 years, which are neurological and severe in which children are infected. Echinocandins, including caspofungin and micafungin, are prominent new antifungal drugs. They have fungicidal activity and low toxicity, consistent with their mechanism of action being cell wall synthesis inhibition. High-level echinocandin resistance (MIC ≥16μg/ml) in C. albicans has been reported, but rare cases. We observed, however, that C. albicans strains exhibiting low-level resistance could readily be isolated in the laboratory. A simple colony isolation method on plates devoid of drugs resulted in the isolation of several low-level resistant isolates. The Data shows that the RLM1 is heterozygous due to polyG polymorphism, see that echinocandin resistance leads to loss of heterozygosity due to mutation of the two alleles. To detect, RLM1 from the clinical isolate was amplified and sequenced. Heterozygosity was observed for C. albicans strain 20710-050. Sequencing revealed loss of 304 heterozygous, consistent with the duplicated alleles containing echinocandin resistance. Future studies will test the effects of loss of heterozygosity or Rlm1 alleles, and whether or not this occurs in patients during echinocandin therapy.

RESULTS

Hypothesis: Growth on echinocandin-containing medium will select for Rlm1 loss-of-heterozygosity mutants

Representative plates showing selection for echinocandin resistant C. albicans strain 20710-050

RLM1 sequence from C. albicans 20710-050 wild-type parent: Heterozygosity following first polyG repeat

C. albicans Rlm1 is highly polymorphic due to variable length of its polyG region

Cytosine alignment of Rlm1 from strains genome-sequencing strains MK2314 and WC1-1

Sequencing reveals RLM1 heterozygosity following the polyG (repetitive) region in 6 of 8 C. albicans strains

Materials & Methods

Source: C. albicans strains employed in this study and their sources are indicated in the Table.

DNA isolation: Overnight cultures (2 ml) were plated, suspended in 100 μl of 4X TE, and vigorously shaken with glass beads for 1 min. Following heating in a boiling water bath for 5 min, samples were cooled 15 min, and 1 μl of proteinase K for 1 h.

PCR: The DNA was amplified in 20 μl reaction mixtures containing 5 μl of 10X PCR buffer (Promega), 0.2 μM of each of the primers, and 2 μl of DNA and 1.25 μl of Taq DNA polymerase. Sequencing reactions were performed using BigDye terminator cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. Sequencing reactions were performed on an automated sequencer (Applied Biosystems 3730xl) and sequence data were analyzed using Applied Biosystems GeneScan and Seqscape software.

Conclusions & Future Directions

- The loss-of-heterozygosity suggests that one of the two RLM1 alleles in the parent provided a selective advantage in echinocandin resistance.
- In further research, this selection could be repeated with other echinocandins, as well as other antifungals that do not target the cell wall (fumagillin, amphotericin B).
- Another critical experiment will be to disrupt each allele of RLM1 to see if this gives the same effect on drug susceptibility, since it is formally possible that different genes fighting RLM1 also lost their heterozygosity and could be responsible for echinocandin resistance.