EVALUATING SOLUBLE AXL AS A BIOMARKER FOR GLIOBLASTOMA

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EVALUATING SOLUBLE AXL AS A BIOMARKER FOR GLIOBLASTOMA

By

Daniel Raymond

THESIS

Submitted to
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Evaluating Soluble AXL as a Biomarker for Glioblastoma

This thesis by Daniel Raymond is recommended for approval by the student’s Thesis Committee and Department Head in the Department of Biology and by the Dean of Graduate Education and Research.

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ABSTRACT

EVALUATING SOLUBLE AXL AS A BIOMARKER FOR GLIOBLASTOMA

By

Daniel Raymond

AXL, a receptor tyrosine kinase, is known to promote malignant phenotypes in various types of cancer. AXL overexpression is commonly observed in glioblastoma and correlates with a worse prognosis. Detectable in most biological fluids, the extracellular domain of AXL, sAXL, is the product of enzymatic cleavage by ADAM10/17. Here we used ELISA, BCA and BCG assays to characterize blood serum from 23 newly diagnosed GBM patients which was collected roughly 24 hours before and after surgery as well as every three months post-surgery, corresponding to follow-up treatment visits. Immunoblotting was used to determine relative AXL expression in 13 patient tumor tissue samples. Additionally, T-1 weighted MRI scans were used to interpolate pre-operative tumor volume in all participating patients. Here we report that sAXL was elevated in the 84 GBM samples compared to the 40 control samples ($p = 0.013$). Normalizing sAXL values against corresponding serum albumin concentrations further defined the distinction between the two groups ($p < 0.0001$). While in the 19 paired, pre- and post-operative samples, sAXL did not respond significantly to surgical intervention, normalizing the values against albumin showed a significant elevation in response to surgery ($p = 0.013$). In patients whose pre-operative samples presented with sAXL elevated compared to the healthy control average (30.16 ng/mL), there was a strong positive correlation between sAXL and AXL found in the corresponding tumor tissue. Though sAXL shed from brain tumors is detectable in the serum of GBM patients, in this small series of patients, it does not correlate with tumor volume.
This project was made possible due to the collaborative support of the Upper Michigan Brain Tumor Center, the Aurora Research Institute, Excellence in Education Grant and graduate research assistantship. I would like to extend my sincerest gratitude to my thesis committee members Dr. Robert Winn, Dr. Richard Rovin, and Dr. Amber Lacrosse for their guidance and support. Additionally, I would like to thank Dr. Parvez Akhtar, Joseph Duffy, Sam Zwernik, Beau Adams, Casey Juntila, John Paul Velasco, and all of the volunteer blood donors from Northern Michigan University.
This work is dedicated in memory of Bill McBrayer and Ryan Kuzmak
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LIST OF ABBREVIATIONS

GBM - Glioblastoma Multiforme

CT - Computed Tomography

MRI - Magnetic Resonance Imaging

RTK - Receptor Tyrosine Kinase

SH2 - Src Homology 2

TAM - Tyro3/AXL/Mer

Gas6 - Growth-Arrest Specific Factor 6

PtdSer - Phosphatidylserine

VSMC - Vascular Smooth Muscle Cells

MAPK - Mitogen Activated Protein Kinase

ERK - Extracellular Signal-Regulated Kinase

MEK - MAPK/ERK Kinase

MMP9 - Matrix Metalloproteinase 9

VEGFR - Vascular Epidermal Growth Factor Receptor

NF-κB - Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells

PI3K - Phosphoinositide 3-Kinase

Akt - RAC-Alpha Serine/Threonine-Protein Kinase

TLR - Toll-Like Receptor

IFNAR - Interferon-α/β Receptor

BCL2 - B-Cell Lymphoma 2 Encoded Protein
EMT - Epithelial-to-Mesenchymal Transition

NSCLC - Non-Small Cell Lung Cancer

mRNA - Messenger Ribonucleic Acid

RNA - Ribonucleic Acid

DNA - Deoxyribonucleic Acid

IHC - Immunohistochemistry

MZF1 - Myeloid Zinc Finger 1

STAT1 - Signal Transducer and Activator of Transcription 1

HIF1α - Hypoxia-Inducible Factor 1- Alpha

AP1 - Activator Protein 1

CpG - 5'-C-phosphate-G-3'

miRNA - Micro Ribonucleic Acid

ADAM10/17 - A Disintegrin and Metalloproteinase 10/17

kDa - Kilodalton

EGFR - Epidermal Growth Factor Receptor

ECM - Extracellular Matrix

HER2/4 - Human Epidermal Growth Factor Receptor 2/4

TIMP1 - Tissue Inhibitor of Metalloproteinases

CLS - Clinical Laboratory Sciences

IRB - Institutional Review Board

rpm - Revolutions per Minute

LCB - LowCross™ Buffer

PBS - Phosphate Buffered Saline
**ELISA** - Enzyme-Linked Immunosorbant Assay

**BCA** - Bicinchoninic Acid

**BCG** - Bromocresol Green

**BSA** - Bovine Serum Albumin

**HRP** - Horseradish Peroxidase

**BSRC** – Biorepository and Specimens Resource Center

**ARI** - Aurora Research Institution

**RIPA Buffer** - Radioimmunoprecipitation Assay Buffer

**NFDM** - Non-Fat Dry Milk

**PVDF** - Polyvinylidene Difluoride

**TBS** - Tris-Buffered Saline

**TBST** - Tris-Buffered Saline+ Tween®20

**ANOVA** - Analysis of Variance

**SEM** - Standard Error of the Mean
Based on the 2016 World Health Organization’s Classification System of Tumors of the Central Nervous System, Glioblastoma Multiforme (GBM) is categorized as a grade IV diffuse glioma due to its origin in glial progenitor cells and diffuse growth pattern \(^1\). Glioblastoma is the most aggressive form of malignant primary brain cancer and affects about 3 individuals out of 100,000 each year in the United States alone \(^2\). Patients symptoms present quickly and can include headaches, abnormal physical weakness and neurological abnormalities such as memory loss, confusion, blurred vision or speech and loss of consciousness \(^3\). Identification and subsequent diagnosis of the disease is first determined by imaging techniques like CT or MRI scans followed by a craniotomy to obtain a sample for molecular diagnostic testing \(^3\). Standard treatment involves maximal tumor resection followed by radiation and adjuvant chemotherapy \(^4\). Untreated, median survival time is only about 3 months \(^2\). Even with standard treatment, median survival for GBM patients is about 15 months \(^5\). GBM patient survival is dependent on many factors including, but not limited to, age at diagnosis, sex, tumor location and treatment type \(^6\). However variable that may be, overall survival of GBM patients 5 years after diagnosis is less than 5% \(^7\). Because of the difficulties associated with treating this disease, it is of the utmost importance to develop techniques that would aid in the detection of GBM and monitor treatment progress.
Cancer cells, in part, are distinct from normal cells in the composition of surface proteins that reside on the cell membrane. Tyrosine kinases represent a minority of the protein-coding portion of our genome but have played a dominate role in cancer research since the discovery of the first proto-oncogene, src. Mutations that lead to constitutively active forms of these signaling molecules can lead to cellular transformation. In fact, deregulated signaling mechanisms are a defining characteristic of all neoplastic growths. This is represented in a central dogma of cancer research that malignant growths arise from the accumulation of mutations in both an oncogene and a tumor suppressor gene. These mutated genes or the product of their expression are frequent points of interest in the study and treatment of cancer.

One way these tyrosine kinases can be separated into categories is based on the location in which they function, the plasma membrane or the cytoplasm. Receptor tyrosine kinases (RTK) are found embedded in the plasma membrane and largely function as the primary point of contact for environmental stimuli. These proteins translate the signal from the extracellular environment through a process of enzymatic reactions to the intracellular environment. Tyrosine kinases found in the cytoplasm function as a cascade of mediators that typically transfer the signal from the membrane to a vast range of targets within the cell, resulting in a response to the original stimuli. These responses can range from translocation of transcription factors into the nucleus, in turn modulating gene expression, to the activation of cell proliferation or apoptotic mechanisms.

The process of RTK activation is initiated by the binding of a ligand to a pair of its receptor proteins, often simultaneously, resulting in activation of the dimer complex.
On the cytoplasmic side of this complex, the act of dimerization facilitates autophosphorylation and then transphosphorylation of tyrosine residues on its subunits that attract docking proteins containing SH2 domains. Cytoplasmic kinases interact with this docking complex and pass the signal further downstream to ultimately affect some kind of cellular response. While point mutations causing constitutively active kinases are one common cause of aberrant molecular signaling, simple overexpression of normal RTK’s is another.

One such case is that of receptor tyrosine kinase AXL (Ufo, Ark), a member of the TAM kinase family along with Tyro3 (Sky) and Mer, and serve as key players in numerous normal and malignant cellular functions. In 1988, the gene that codes for AXL was first characterized as a novel proto-oncogene in human myeloid leukemia cells. Canonical activation of the AXL receptor dimer complex involves the binding of its natural ligand, Growth-arrest specific factor 6 (Gas6), as well as interacting with the phospholipid, phosphatidylserine (PtdSer). However, studies have suggested that in the cases in which AXL is overexpressed, heterotypic activation and even ligand-independent receptor activation can occur. When expressed normally and often in concert with the other members of the TAM family, activation of AXL promotes various normal cellular behaviors seen across different cell types. These include apoptotic cell clearance, dampening of cytokine induced inflammation, migration and survival of certain types of neurons and Vascular Smooth Muscle Cells (VSMC) cells, differentiation and maturation of NK cells, normal platelet aggregation and spermatogenesis.
Whereas standard expression and activation of AXL leads to normal cellular functions, overexpression has been shown to cause aberrant signaling that results in malignant phenotypes (Figure 1)\(^{35,36}\). Described below, these phenotypes include increased survival, proliferation, migration, invasion, angiogenesis, immune suppression and decreased sensitivity to chemotherapeutic intervention. AXL signaling has been shown to stimulate cellular proliferation and protect from apoptosis through the MEK/ERK\(^{35}\) and PI3K/Akt/NF-κB\(^{37,38}\) pathways. Invasion and migration are controlled through pathways like Rac/p38 MAPK and ERK/MMP9 respectively. Angiogenesis has been connected to AXL through in vivo studies demonstrating cross-talk between AXL and VEGFR-2 activated Src family kinases (SFK), leading to downstream activation of the PI3K/Akt pathway\(^{39}\). Immune suppression, primarily in the form of dampening the responses of the immune system caused by cytokine release and TLR activation, is facilitated by AXL negative regulation of the TLR and cytokine-associated receptors (eg. IFNAR)\(^{24,11,37}\). Decreased drug sensitivity is a result of AXL-related mechanisms, one of which involves upregulation of signaling proteins and transcription factors such as BCL-2 and Twist\(^{40}\). Furthermore, the upregulation of various AXL-related pathways result in epithelial to mesenchymal transition (EMT) in various cell types\(^{41–43}\).

Studies have been undertaken to understand the implications of aberrant AXL signaling for patients with different cancers. In short and as summarized by Rankin and Giaccia\(^{44}\), AXL plays a significant role in the phenotypic expression of different cancer cells and is therefore a valuable therapeutic target. High AXL expression has been correlated with worse survival rates in at least 14 types of cancer, including GBM\(^{45–59}\). Additionally, AXL expression has been shown to confer drug resistance to cells in at
least five types of cancer\textsuperscript{40,59–64}. Furthermore, AXL has been demonstrated to be an important conduit to the molecular rewiring necessary for EMT and/or the maintenance of this state in GBM\textsuperscript{65}, breast cancer\textsuperscript{41–43}, ovarian cancer\textsuperscript{66}, NSCLC\textsuperscript{67} and pancreatic cancer\textsuperscript{68}. Taken together, these studies have demonstrated the significant role that aberrant AXL signaling may play in numerous cancers and has bolstered efforts to target AXL when treating these diseases.

Due to the similar effects of AXL overexpression and activation across a variety of cancer subtypes, increased focus has been placed on using AXL-targeted drug therapy. Various AXL inhibitors are at different stages of development with promising preclinical results\textsuperscript{69,70} suggesting that in specific cancers, these drugs can significantly supplement the effectiveness of current treatments. Use of AXL-targeting small-molecule inhibitors such as, Bemcentinib (BGB324; previously R428), and others are currently at different phases of clinical trials in patients with GBM\textsuperscript{71} (NCT03965494), pancreatic cancer\textsuperscript{72} (NCT03649321), NSCLC\textsuperscript{73,74} (NCT02922777, NCT02424617) and AML/MDS\textsuperscript{75} (NCT02488408). However, despite the advanced stage of some of these clinical trials, the long term efficacy of AXL targeting has not been reported. Although these clinical efforts oriented towards treatment strategies are valuable, identification of the disease at its earliest stages would drastically change the outcomes for the majority of patients.

Specifically in GBM, AXL and Gas6 have been reported to be moderate to highly expressed at both the mRNA and protein level in in the majority of cases. Similarly, in patient tissue samples with high AXL levels, determined by IHC, AXL and Gas6 expression were correlated with shorter time to disease progression and was associated
with a poor survival rate. Furthermore, AXL protein expression was only found to be present in glioma tumor tissue and its vasculature, but absent in non-neoplastic tissues\textsuperscript{45}. Another study reported that AXL was elevated in GBM and was correlated with a worse prognosis and showed that AXL overexpression directly correlated with drug resistance and contributed to tumorigenesis\textsuperscript{59}. There are numerous studies reporting significant expression of AXL in various regions of the brain, however, a comprehensive study performed in 2015 resulting in the Human Protein Atlas, indicates that of all the organs where AXL protein expression is found, it is least abundant in the brain\textsuperscript{76}. These studies highlight the role AXL plays in GBM, from its origin through the treatment and outcome of the patient.

Cellular regulation of AXL can be found at all levels of expression, from transcriptional controls to post-translational deactivation through enzymatic cleavage. Environmental stimuli trigger the cell to release transcription factors, several of which have been demonstrated to act upon the \textit{axl} gene. Several transcription factors like MZF1\textsuperscript{77}, STAT1\textsuperscript{11}, HIF1\textalpha\textsuperscript{78} and AP1\textsuperscript{79} have been shown to induce \textit{axl} transcription. However, methylation of CpG islands adjacent to the transcription factor binding sites have been demonstrated to play an antagonistic role in the generation of \textit{axl} mRNA\textsuperscript{80}. Once transcribed, \textit{axl} mRNA does not always result in translation. In fact, despite relatively equivalent expression of \textit{axl} mRNA in different cell types, protein level AXL expression is significantly different between cell types\textsuperscript{76,81}. Post-transcriptional regulation can take the form of ribosomal blockades by miRNAs such as miR-199a/b\textsuperscript{82} and miR-34a\textsuperscript{82,83}, preventing the translation of the AXL mRNA. Even after translation, the AXL protein can also be subject to ubiquitination and subsequent degradation\textsuperscript{84}. Another post-
translational means of protein regulation is modification by ADAM proteins that have been shown to regulate AXL activity by enzymatically cleaving the extracellular domain\textsuperscript{21,85}. The regulatory process for protein creation and function is a complex and finely tuned system that nevertheless offers various points to target for detection or treatment of AXL-influenced diseases.

This soluble form of AXL, denoted sAXL, is nearly ubiquitous in the body since it is a product of membrane AXL, whose expression is nearly ubiquitous on some level. This is also partially due to the small size of sAXL (43 kDa) making it capable of permeating certain blood vessels\textsuperscript{86}. Presumably due to a lack in uniformity of testing, there is a range of reported sAXL concentrations in blood serum from healthy control populations. Regardless, sAXL can be found in the majority of the relevant biological fluids such as blood serum\textsuperscript{87} or plasma\textsuperscript{88,89}, cerebrospinal fluid\textsuperscript{90}, urine\textsuperscript{91} and peritoneal effusions\textsuperscript{92}. Evidence supporting the existence of a soluble AXL extracellular domain (sAXL) generated due to proteolytic cleavage, as opposed to alternative transcript splicing, was first published in 1995. Based on their evidence and previous studies examining soluble forms of EGFR and other RTKs, the authors theorized that sAXL acted as an regulator of full-length RTK function by binding the receptor’s natural ligand and down-regulating its signaling function\textsuperscript{93}. Further studies confirmed that cleavage of the extracellular domain of AXL can serve as a dual-function regulator of AXL signaling activity. By releasing the ligand binding domain, sAXL prevents the initiation of dimerization\textsuperscript{94} and it can bind Gas6 in the ECM, further preventing initiation of the signaling cascade\textsuperscript{78}. 
Evidence has been put forward to suggest the important role this regulatory mechanism may play in malignant diseases. As reported by Miller et al., certain kinase inhibitors can cause reduced ADAM-mediated RTK cleavage, including AXL, and was inversely correlated with progression-free survival in a cohort of melanoma patients. In this study, the use of mitogen-activated-protein-kinase (MAPK) inhibitors showed significantly decreased proteolytic cleavage of AXL, MET and HER4. In melanoma xenograft models, the inhibition of TIMP1, the natural inhibitor of ADAM proteins, restored sheddase activity. When combined with MAPK inhibition, there was a synergistic effect, resulting in a reduction of tumor growth and metastasis.

Due to the demonstrable link between AXL overexpression and various diseases, numerous studies have examined localized AXL tissue expression and the release of sAXL as potential means of detecting diseases or monitoring treatment. Elevated sAXL levels have been seen in chronic liver diseases such as liver fibrosis and cirrhosis and hepatocellular carcinoma. Another significant study demonstrated a strong association between sAXL and tumor progression, as well as patient survival, in renal cell carcinoma. Similar studies have connected sAXL, or its natural ligand Gas6, to a myriad of other diseases or physiological conditions such as melanoma, ovarian carcinoma, systemic lupus erythematosus, large abdominal aortic aneurysms, severe preeclampsia, tumor burden in Neurofibromatosis Type 1, myocardial infarction and heart failure. A possible correlation exists between sAXL and GBM however, based on available records, direct evidence of this has not been reported. This project will be the first to investigate the possible distinctive presence of circulating sAXL in GBM.
Figure 1: AXL Signaling Pathway Overview
(Top Left) Illustrations representing the immunosuppressive effects of AXL activation in several types of immune cells. (Bottom Right) Simplified illustration showing the general phenotypic expressions that result from AXL activation and downstream signaling through several significant pathways. Taken from “Giving AXL the Axe: Targeting AXL in Human Malignancy” Gay CM, Balaji K, Byers LA. Br J Cancer Lond. 2017;116(4):415-423.
Figure 2: Regulating RTK Signaling Through Sheddase Activity
(A) Illustration representing ADAM-dependant sAXL shedding as a means of disrupting the AXL/Gas6 complex, therefore dampening the effects of AXL signaling. (B) Inhibition of ADAM proteins bypasses this regulation of AXL, leads to the accumulation of ADMA-related RTK proteins and serves to amplify signaling cascades.
AIMS AND GOALS

Glioblastoma lethality is in part due to the aberrant molecular signaling pathways facilitated by RTKs. The RTK AXL promotes malignant phenotypes in GBM and other cancers through myriad intracellular signaling pathways. Due to a unique type of regulatory mechanism, facilitated by ADAM10 and ADAM17, the extracellular portion of AXL is found circulating in the blood. This is true in the case of cancer patients and healthy populations alike. The aim of this study is to compare the relative abundance of sAXL in the blood of GBM patients and healthy volunteers then to use this as a means of establishing sAXL as a novel biomarker for GBM.

I hypothesized that circulating sAXL in blood serum collected from GBM patients would be elevated compared to a volunteer control population. I further hypothesized that sAXL levels in samples collected after surgery would be lower than paired samples collected prior to surgical intervention. It was also predicted that a change in circulating sAXL levels would be reflected in samples collected while patients were undergoing chemo- and radiation therapy. Lastly, I hypothesized that circulating sAXL levels would reflect AXL expression in corresponding tumor tissue as well as tumor volume calculated from contemporary MRI scans.
METHODS AND MATERIALS

Obtaining Blood Serum Samples

Blood serum samples from 29 Northern Michigan University undergraduate students enrolled in CLS 100 (phlebotomy) were a generous gift provided for this research during the Winter 2019 semester. Blood samples were collected during the course while practicing basic phlebotomy techniques. Samples were collected in Gold-top serum separator tubes that were labeled with a de-identified number that corresponded to each student along with their age and sex. Tubes were centrifuged at 1000 rpm for 5-10 minutes then the supernatant was collected and aliquoted into 0.65 mL Eppendorf tubes. Tubes were labeled and stored at -80° C. To expand this control population, approval from Northern Michigan University’s Institutional Review Board (IRB) was obtained in order to recruit 13 faculty members to donate blood for this study. A student, experienced in phlebotomy, obtained the samples and used similar labeling as described to maintain volunteer confidentiality. These blood serum samples were treated as described previously.

Approval from the Aurora Health Care IRB was obtained for the collection of blood serum, tissue specimens, and supplemental research information. Blood serum samples were collected from GBM patients undergoing surgery at Aurora St. Luke’s Hospital in Milwaukee Wisconsin. Of the 27 patients enrolled in the study, 23 of them had confirmed GBM tumors by the Hospital’s pathology lab. Blood serum samples from these patients were collected before surgery, after surgery, and up to 7 time points
corresponding with follow-up visits approximately every three months. These samples will hereafter be referred to as pre-operative, post-operative, and BL1-7, respectively. A sum of 84 GBM samples from 23 GBM patients was acquired for this study. Half of these were from the pre- and post-operative time points, the other half from the bi-monthly “treatment timeline”. Of the 42 pre- and post-operative samples, 38 were collected from the same patient before and after surgery. All samples were labeled with a de-identified number and the corresponding time point.

*Dilution Scheme for Serum Samples*

The blood samples were prepared as 1:200 dilutions, resulting in two 500µL aliquots by a two-step serial dilution in both Low-Cross Buffer™ (Candor Biosystems) or Phosphate Buffered Saline (Dulbeco). These samples were stored at -80°C for subsequent testing. Additionally, three 50µL aliquots of blood serum were stored to mitigate freeze-thaw cycles of the original blood samples. These smaller aliquots would be used for BCG assays to determine the total serum Albumin concentrations while the 1:200 dilutions in either LCB or PBS would be used for either ELISA or BCA assays respectively.
Figure 3: Serum Dilution Scheme.
The general plan used to mitigate freeze-thaw cycles and inter-assay variation caused by dilution. From the same serum sample, multiple aliquots were prepared in LCB (1:200), PBS (1:200) or undiluted for the three assays to be implemented (ELISA, BCA and BCG).
Preparation of Human AXL ELISA Kit

Human AXL DuoSet ELISA kits (R&D Biosystems) were purchased along with the corresponding Ancillary Reagent Kits (DY008). The AXL capture and detection antibodies were allowed to sit at room temperature for 15 minutes prior to diluting in PBS, aliquoting in single-use portions and storage at -80° C. Following the manufacturer’s instructions, 1mL of PBS was added to the vial containing capture antibody in order reconstitute it, resulting in a 360µg/mL stock solution which was sufficient antibody for at least 15 assays. Similarly, the detection antibody vial was reconstituted with 1mL of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2µM filtered), resulting in a 9µg/mL solution that was sufficient to perform at least 15 assays. The recombinant AXL protein standards came in three separate vials containing 80ng of lyophilized protein each. After warming to room temperature, one vial was reconstituted in 500µL of room temperature LCB to a final stock concentration of 160ng/mL. Each solution was aliquoted into single-use quantities and stored at -80° C. The streptavidin-HRP solution came in a light-resistant vial and was stored undiluted at 4° C. As a part of my optimization of the ELISA protocol, the working concentration standards were later prepared in one large serial dilution to the final concentrations of 4000, 2000, 1000, 500, 250, 125, 62.5 and 0 pg/mL in LCB at a final volume of 6mL each. These solutions were then split into 14 aliquots and frozen at -80° C. This would ensure that all samples are being compared to the exact same standards despite repetitions being performed on separate dates.
Enzyme-linked Immunosorbent Assay

The night before each assay, an aliquot of AXL capture antibody was thawed on ice and was diluted 1:180 in PBS, to a final volume of 10mL at a concentration of 2.0 µg/mL. Using an 8-well multichannel pipette, 100µL of this solution added to each well of a 96-well plate provided with the ancillary reagent kits. The plate was sealed with a transparent adhesive strip, centrifuged briefly at 3000rpm to ensure even coating of all wells. The plate was then allowed to sit at room temperature overnight on an even surface.

The following morning, the plate was inverted to expel the solution into a waste container and blotted against a clean paper towel. The plate was then washed three times with 300µL of wash buffer (0.05% tween® 20 in PBS, pH 7.2-7.4), inverting, and blotting the plate against a clean paper towel each time to ensure complete removal of fluid. The plate was then blocked by adding 300µL of reagent diluent to each well and allowed to sit at room temperature for 2 hours. During this time, diluted samples in LCB and diluted AXL standards were thawed on ice, vortexed, and centrifuged briefly to ensure even distribution. Next, 160µL of each sample was transferred to a designated 96-well “transfer plate”, mirroring the intended locations on the ELISA plate. Using a “transfer plate” rather than pipetting individual samples into 94 wells served to minimize any variation of sample volume added between wells and exposure time to capture antibody. After the blocking step, the plate was washed three times exactly as before. Using the 8-well multichannel pipette, 100µL was transferred from each column of the “transfer plate” to the ELISA plate, changing tips for each set of samples. The plate was centrifuged briefly to ensure even distribution of the samples and allowed to incubate at
room temperature for 2 hours. The wash step was then repeated as before. This was followed by the addition of 100µL of AXL detection antibody (50.0 ng/mL) to each well using a multichannel pipette. The plate was centrifuged briefly and allowed to incubate for 2 hours. Following another wash step, 50 µL of Streptavidin-HRP was warmed to room temperature and diluted 200-fold in reagent diluent. Then 100µL of this solution was added to each well and allowed to incubate for 20 minutes. The plate was washed and then coated with 100 µL of a 1:1 mixture of color reagent A (hydrogen peroxide) and color reagent B (tetramethylbenzidine). After a 20-minute incubation, the reaction was halted by the addition of 50µL of stop solution (2N sulfuric acid). The plate was immediately transferred to a spectrophotometer to measure the optical density of each well at two wavelengths, 450 and 540nm. The 540nm measurement was used as a wavelength correction by subtracting these values from the 450nm values. After adjusting for background values by normalizing against the blanks, a four-parameter logarithmic curve-fit was generated in GraphPad Prism based on the standard curve and use to interpolate the concentrations of the samples. Standards on each plate were tested in duplicate and each sample was tested in triplicate and repeated at least once, either on a separate day or on a duplicate plate.

**Optimizing the AXL ELISA for Ideal Interassay Variation**

In the earliest stages of this project, samples were tested only once in triplicate but due to early results suggesting promise for this assay it was decided that repeating measures would offset limited sample availability as well as demonstrate another critical assay performance characteristic, inter-assay variation. However, repeating the assay on separate dates revealed an objectively high level of inter-assay variation, calling into
question the results of our previously collected data. To counteract this phenomenon, several strategies were implemented, the first of which included preparing the samples in the appropriate buffers and dilutions prior to testing and storing them at -80°C as duplicate aliquots (see figure 3).

A simple experiment was first implemented to ensure that storing the diluted samples at -80°C would not affect protein interpolated concentrations. For 13 randomly selected serum samples, 1:200 dilutions in LCB and PBS and three smaller, undiluted, aliquots were prepared. These samples were stored together at -80°C until the day of testing. On the day of testing, the previously undiluted samples were prepared identically to its counterpart samples and tested for sAXL and total protein using the standard ELISA and BCA protocols, respectively. Samples diluted on the day of testing and those stored frozen collectively showed no significant difference in protein concentration in either the ELISA (figure 4) or BCA assay (figure 5).

All samples were then prepared as seen in Figure 3, however, after the first ELISA was performed it was evident that the inter-assay variation was still present (figure 6). From the protein stability experiment, it became apparent that the most significant missing variable was the standards to which the diluted samples were compared. Afterward, a batch preparation of AXL recombinant protein standard was serially diluted but, on a scale, large enough to perform each of the remaining ELISAs twice. The diluted standards were aliquoted and stored at -80°C together and thawed on the day of testing. Together, preparing the diluted samples and standards resulted in a drastically reduced inter-assay variation and was confirmed by a paired t-test (figure 7).
**Bicinchoninic Acid (BCA) Assay to Determine Total Serum Protein**

Samples diluted 1:200 in PBS were thawed on ice, mixed via vortex and centrifuged briefly. BCA standards (Thermofisher) previously were prepared at concentrations of 2000, 1500, 1000, 750, 500, 250, 125, and 0 µg/mL in molecular grade water. After samples and BCA standards were warmed to room temperature, 50µL of the standards were added to a transfer plate in duplicate. Next, 100µL of the diluted samples were added to the plate in triplicate, mirroring the sample layout used in the ELISA experiments. Using an 8-well multi-channel pipette, 25 of each sample was transferred to a clean 96 well plate, one column at a time. Next, 200µL of a mixture of BCA reagents A and B (1:50) was added to each well. After a 30-minute incubation at room temperature, the plate was measured for optical density at 562nm. Standards used were tested in duplicate and each of the samples was tested in triplicate. Concentrations were interpolated from a standard curve, adjusted by the dilution factor, and were converted from µg/mL to g/dL by dividing by a factor of 10,000.

**Bromocresol Green (BCG) Assay to Determine Total blood albumin**

Frozen aliquots of blood serum were thawed on ice, diluted 1:2 in ultra-pure water, and transferred to a 96-well plate. Frozen BSA standards at concentrations of 5, 4, 3, 2, 1.5, 1, 0.5, and 0 g/dL were thawed on ice and transferred to the 96-well plate. Next, 200µL of BCG reagent was added to each well and incubated for five minutes at room temperature. The plate was then read for optical density at 620nm. Using GraphPad Prism, a four-parameter logarithmic curve-fit was generated and used to interpolate the sample concentrations. Standards used were tested in duplicate and each sample was tested in triplicate. The BCG assay functions as a specific quantitative measurement of
albumin due to the formation of a chromophore upon the interaction of BCG and albumin.

**Tissue Sonication**

Frozen tumor tissue samples that were acquired were obtained by the ARI Biorepository and Specimen Resource Center (BSRC) who stored them at -80°C until ready for transfer to the ARI lab. Frozen tissues were briefly thawed on ice prior to being transferred to a sonication tube, also on ice, with clean forceps. The original tubes were rinsed with ice-cold RIPA buffer containing protease inhibitors and transferred to the sonication tube. Tubes were weighed before and after to determine tissue mass. Ice-cold RIPA buffer was added to a final volume of 500 µL in each tube. Samples were then sonicated twice at 10% amplitude for 10 seconds and three times at 15% amplitude or until the homogenate became transparent. Samples were immediately placed back on ice in between sonication bursts and great care was taken to keep the sonication probe in the middle of the mixture to avoid frothing. These homogenates were subsequently centrifuged at 14,000 rpm at 4°C for 10 minutes to pellet any insoluble material. The remaining supernatant was removed with a pipette, and 50 µL aliquots were stored at -80°C.

**Western Blotting**

Protein concentration for each of the 15 tumor lysates was determined by BCA assay (Thermofisher) using minimal lysate (10µL/ well). Using the interpolated concentration was then used to calculate the volume needed to load 40ug of total protein per well. Ice-cold RIPA buffer was used to normalize the volume and a consistent
amount of 4x LSB (BioRad) containing 10% β-mercaptoethanol was added to each tube. Samples were denatured at 95°C for 5 minutes and immediately chilled on ice. Denatured samples were loaded in a 4-12% Criterion™ Bis-Tris gel (BioRad) and ran at 80v for approximately 1.5 hours. The proteins were transferred to a PVDF membrane using the iBlot® 2 (Thermofisher) semi-dry transfer system. The membrane was blocked in 5% molecular grade NFDM blocking buffer (BioRad) for 1 hour. It was then washed three times for 5 minutes in 1x TBS (BioRad). The membrane was covered in 5% blocking buffer containing AXL antibody diluted 1:200 and incubated overnight at 4°C on a shaker. The next morning the membrane was washed three times in TBST (0.1% tween) for 10 minutes. Secondary, mouse anti-goat, antibody diluted 1:2000 in blocking buffer was added to the membrane and was allowed to incubate for one hour (RT), with shaking. The membrane was washed as before and developed by covering it drop-wise with a 1:1 mixture of clarity peroxide reagent and clarity luminol reagent (BioRad) and incubating for five minutes. The blot was then immediately transferred to a LiCore imaging system and read for 2 minutes on the chemiluminescent channel and 5 minutes on the 700nm channel. The membrane was then stripped of antibodies by coating the membrane in OneMinute® Stripping Buffer (GM Biosciences), blocked for one hour in 5% NFDM and washed three times in TBST (0.1% Tween20). The membrane was coated in an rabbit anti-actin primary antibody (1:2000 in 5% NFDM) incubated overnight at 4°C on a shaker. The following morning the membrane was washed three times for 10 minutes in TBST (0.1% Tween20). The membrane was then coated in anti-rabbit secondary antibody (1:2000 in 5% NFDM) for 1 hour at room temperature. This was followed by three washes for 10 minutes in TBST (0.1% Tween20). The membrane was
then developed by covering it drop-wise with a 1:1 mixture of clarity peroxide reagent and clarity luminol reagent (BioRad) and incubating for five minutes at room temperature. The blot was then immediately transferred to a LiCore imaging system and read for 2 minutes on the chemiluminescent channel and 5 minutes on the 700nm channel.

Pre-Operative MRI analysis

MRI scans taken for each of the 27 enrolled patients were completely de-identified and made available in BrightMatter Plan (Synaptive Medical, Toronto, CAN), the software used by Aurora St. Luke’s to plan surgeries. Training in the software was conducted by an experienced neuroradiologist, how to distinguish tumor tissue from other enhancing regions of the brain. BrightMatter Plan was used to analyze all of the pre-operative T-1 weighted scans of the patients and build a 3D model of the tumor. The software automatically calculates the tumor volume. Each analysis was reviewed and modified when necessary to ensure their accuracy.

Data Analysis

Determination of statistically significant variation between two groups was performed using a two-tailed, unpaired student’s t-test unless noted otherwise. For datasets with three or more groups were analyzed using repeated measures, mixed-model, one-way ANOVA with Dunnett’s post hoc. Graphical representation of datasets were performed in GraphPad with error bars representing either 95% confidence intervals or mean ± SEM unless noted otherwise.
RESULTS

Characterizing sAXL in GBM and Healthy Control Populations

It was hypothesized that sAXL would be significantly elevated in patients with GBM as a result of elevated membrane AXL release from tumor tissue as a result of enzymatic cleavage. Investigating this in the broader sense meant performing ELISAs on GBM and control samples using the optimized methodology previously mentioned. Since the sample size was somewhat limited compared to other studies investigating protein biomarkers, a somewhat larger data set was generated by testing each sample in triplicate and on two separate occasions. Including other variables, such as serum albumin or total serum protein, would also allow for a more powerful statistical analysis of these two populations. After testing 124 samples as previously described, sAXL was found to be significantly elevated in the GBM population (35.77 ± 1.25, n=84) compared to the healthy population (30.16 ± 1.88, n=40; \( p = 0.013 \)) (Figure 8). Upon further comparison of each population, normalizing the sAXL values against serum albumin (Figure 11) and total protein (Figure 12), the distinction became even more apparent in both cases \( (P < 0.0001) \). This increased distinction was due to the finding that GBM patients had significantly lower serum albumin \( (P < 0.0001) \) (Figure 9) and total serum protein \( (P < 0.0001) \) (Figure 10) compared to the control population. Additionally, sorting the samples by gender in each category (healthy or GBM), no significant difference was seen in any group as determined by a one-way ANOVA (Figure 13).
Figure 4: Protein Stability Test: ELISA

sAXL concentrations (ng/mL) for paired samples previously diluted and stored at -80°C (33.8±3.24, n=13) versus samples prepared the day of testing (34.06±3.52, n=13). Statistical significance of variation was calculated using a two-tailed, paired student’s t-test. Concentrations and error bars presented as mean ± SEM.

ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 5: Protein Stability Test: BCA assay
Total protein concentrations (g/dL) for paired samples previously diluted and stored at -80°C (6.17±0.26, n=12) versus samples prepared the day of testing (6.41±0.20, n=12). Statistical significance of variation was calculated using a two-tailed, paired student’s t-test. Concentrations and error bars presented as mean ± SEM.

ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 6: ELISA Inter-assay Variation: Individual Protein Standards
Sample ID vs. sAXL concentrations for samples prepared and stored identically then tested on two separate dates. AXL recombinant protein standards used were prepared on the day of testing. Statistical significance of variation was calculated using a two-tailed, paired student’s t-test. Error bars presented as mean ± SEM.
ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 7: ELISA Inter-assay Variation: Batch-Prepared Protein Standards
Sample ID vs. sAXL concentrations for samples prepared and stored identically then tested on two separate dates. AXL recombinant protein standards used were prepared as a batch and aliquoted for multiple tests. Statistical significance of variation was calculated using a two-tailed, paired student’s t-test. Error bars presented as mean ± SEM.
ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Table 1: sAXL Summary Data

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>N</th>
<th>sAXL (ng/mL)</th>
<th>Albumin (g/dL)</th>
<th>sAXL/Albumin Ratio</th>
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<tr>
<td>Healthy</td>
<td>(n=40)</td>
<td>30.16±1.88</td>
<td>5.89±0.16</td>
<td>5.38±0.44</td>
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<td>F (n=27)</td>
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<td>28.67±1.92</td>
<td>5.86±0.17</td>
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<td>M (n=13)</td>
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<td>33.26±4.2</td>
<td>5.97±0.35</td>
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<td>GBM</td>
<td>(n=84)</td>
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<td>4.24±0.07</td>
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<td>F (n=26)</td>
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<td>30.16±1.88</td>
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<td>34.57±2.46</td>
<td>4.17±0.08</td>
<td>9.01±0.44</td>
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<tr>
<td>Pre-Op</td>
<td>(n=20)</td>
<td>30.74±1.96</td>
<td>4.35±0.12</td>
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<td>F (n=8)</td>
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<td>29.22±3.35</td>
<td>4.25±0.17</td>
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<td>31.75±2.48</td>
<td>3.92±0.14</td>
<td>7.69±0.75</td>
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<tr>
<td>Post-Op</td>
<td>(n=22)</td>
<td>32.32±2.26</td>
<td>3.76±0.1</td>
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<td>9.66±1.1</td>
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<td>BL1</td>
<td>(n=9)</td>
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<td>4.83±0.14</td>
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<td>4.77±0.21</td>
<td>7.52±1.35</td>
</tr>
<tr>
<td>BL2</td>
<td>(n=6)</td>
<td>49.11±6.98</td>
<td>4.32±0.29</td>
<td>11.52±1.57</td>
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<td>5.35±0</td>
<td>12.39±0</td>
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<td>45.68±7.44</td>
<td>4.11±0.24</td>
<td>11.35±1.91</td>
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<td>BL3</td>
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<td>9.12±1.09</td>
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<td>10.03±1.11</td>
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<td>BL5</td>
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<td>4.38±0.23</td>
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<tr>
<td>F (n=1)</td>
<td></td>
<td>34.92±0</td>
<td>4.74±0</td>
<td>7.37±0</td>
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<tr>
<td>M (n=5)</td>
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<td>35.99±4.16</td>
<td>4.3±0.26</td>
<td>8.55±1.2</td>
</tr>
<tr>
<td>BL6</td>
<td>(n=3)</td>
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<td>4.48±0.22</td>
<td>10.18±0.86</td>
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<tr>
<td>F (n=1)</td>
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<td>4.89±0</td>
<td>10.11±0</td>
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<td>4.32±0.39</td>
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<tr>
<td>F (n=0)</td>
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<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
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<tr>
<td>M (n=5)</td>
<td></td>
<td>32.95±4.56</td>
<td>4.32±0.39</td>
<td>7.95±1.6</td>
</tr>
</tbody>
</table>

Descriptive statistics table outlining concentrations of sAXL (ng/mL), Albumin (g/dL) and the corresponding ratio between them. Groups first separated based on cohort (GBM vs. Healthy), further separated based on collection time points and then by gender.
Figure 8: sAXL in GBM and Healthy Blood Serum

Serum sAXL concentrations are elevated in GBM patient samples (35.77 ± 1.25, n=84) compared to healthy volunteer samples (30.16 ± 1.88, n=40; *p = 0.013). Grouped sAXL concentrations are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance of variation was calculated using a two-tailed, unpaired student’s t-test.

ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 9: Serum Albumin in GBM and Healthy Blood Serum
Serum albumin concentration are elevated in the healthy volunteer samples (5.89 ± 0.16, n=40) compared to the GBM patient samples (4.235 ± 0.07, n=84) ($P < 0.0001$). Collective Albumin concentrations are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance was calculated using a two-tailed, unpaired student’s t-test.
ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Figure 10: Total Protein in GBM and Healthy Blood Serum

Total protein concentration are elevated in the healthy volunteer samples (7.43 ± 0.15, n=40) compared to the GBM patient samples (5.98 ± 0.093, n=74) ($P < 0.0001$). Collective total protein concentrations are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance was calculated using a two-tailed, unpaired student’s t-test.

ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Figure 11: sAXL/Albumin Ratio in GBM and Healthy Blood Serum

Values from each sample’s measured sAXL concentration normalized against the average of the corresponding serum albumin concentration for each sample. Ratios for GBM patient samples (8.68 ± 0.34, n=84) are elevated compared to the healthy volunteer samples (5.38 ± 0.44, n=40) (P < 0.0001). Collective ratios are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance was calculated using a two-tailed, unpaired student’s t-test.

ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 12: sAXL/Total Protein Ratio in GBM and Healthy Blood Serum

Values from each sample’s measured sAXL concentration normalized against the average of the corresponding total protein concentration for each sample. Ratios for GBM patient samples (6.20 ± 0.23, n=74) are elevated compared to the healthy volunteer samples (4.14 ± 0.23, n=40) (P < 0.0001). Collective ratios listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance was calculated using a two-tailed, unpaired student’s t-test.

ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001
Figure 13: Serum sAXL Concentration in Males and Females Separated by GBM Status
Serum sAXL concentrations for healthy (solid fill) and GBM (diagonal pattern) samples separated by male (blue) and female (red). Concentrations were not significantly different between genders in either the healthy or GBM cohorts. Error bar represent 95% confidence interval. Statistical significance of variation was calculated using a one-way ANOVA.
Using sAXL as a Predictive Biomarker for GBM Presence

In order to test the hypothesis that sAXL was a predictive biomarker for the presence of GBM, pre-operative serum samples (as opposed to all the GBM serum samples) were compared to the healthy controls. First, comparing sAXL in the pre-operative and post-operative samples to the healthy control would serve to distinguish sAXL as a marker for the presence of GBM at the point of surgery. However, considering only sAXL, neither the pre-operative or post-operative samples compared to the healthy control showed a significant difference (Figure 14). Making this same comparison with serum albumin alone showed that healthy samples had elevated concentrations of serum albumin as compared to both groups ($P < 0.0001$) (Figure 15). For this reason, normalizing sAXL against serum albumin was able to distinguish sAXL in both pre-operative ($P = 0.013$) and post-operative populations ($P < 0.0001$) from the control population (Figure 16). This suggests that sAXL concentration is not sufficient to predict GBM presence but comparisons to other serum proteins may be sufficient in making the distinction between these populations.
Figure 14: sAXL Concentrations: Control, Pre-operative and Post-operative samples
Serum sAXL concentrations are not elevated in pre-operative (30.84 ± 2.07, n=19) or post-operative samples (32.04 ± 2.44, n=19) compared to the healthy control samples (30.16 ± 1.88, n=40). Similarly, sAXL concentrations did not significantly change in response to surgery. Concentrations for each group are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance between healthy and GBM samples were calculated using an unpaired t-test. Statistical significance between paired GBM samples was calculated with a paired t-test.

ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Figure 15: Serum Albumin Concentration: Control, Pre-operative and Post-operative samples

Serum albumin concentrations are significantly elevated in the healthy control population (5.89 ± 0.16, n=40) compared to both pre-operative (4.34 ± 0.12, n=19) (\(P < 0.0001\)) and post-operative samples (3.70 ± 0.10, n=19) (\(P < 0.0001\)). Additionally, serum albumin concentrations are elevated in the pre-operative samples compared to post-operative samples (\(p = 0.0002\)). Concentrations for each group are listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance between healthy and GBM samples were calculated using an unpaired t-test. Statistical significance between paired GBM samples was calculated with a paired t-test.

ns: not significant, * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\), **** \(P < 0.0001\)
Figure 16: sAXL Normalized by Albumin: Control, Pre-operative and Post-operative samples

Serum sAXL concentrations normalized by albumin concentrations from the healthy control (5.384 ± 0.44, n=40), pre-operative (7.32 ± 0.59, n=19) and post-operative samples (8.84 ± 0.77, n=19). Values calculated as a ratio between individual sAXL concentrations and the mean albumin concentration for each sample, collectively listed as mean ± SEM. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance between healthy and GBM samples were calculated using an unpaired t-test. Statistical significance between paired GBM samples was calculated with a paired t-test.

ns: not significant, * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \), **** \( P < 0.0001 \)
**Serum sAXL in Response to Surgical Intervention**

Since the first step in the treatment of GBM is the maximal amount of tumor resection, it would follow that removal of sAXL releasing tumor tissue should result in an overall reduction in serum sAXL following surgery. It was shown that pre-operative samples tested only for sAXL showed no significant difference compared to the healthy controls (Figure 14), but when normalizing by serum albumin a distinction was made (Figure 16). Despite this, it became apparent that there was no significant difference in sAXL as a result of surgical intervention, in roughly half the patients sAXL levels rose and in the other half, they fell with no noticeable trend (Figure 17). However, performing this same comparison with sAXL concentrations normalized to serum albumin showed a distinct upward trend as determined by a two-tailed, paired t-test between the 19 matched samples ($P = 0.019$) (Figure 18). Moreover, an interesting correlation appeared from this dataset when interpolated tumor volumes were included in the analysis. To represent the change in sAXL between each time point, pre-operative sAXL values were subtracted from their corresponding post-operative values ($n=19$). These values were then plotted against pre-operative tumor volume, fit with linear regression and evaluated for a correlation using the Pearson method (Figure 19). The result was a significant correlation ($P = 0.013$) between tumor volume and net change in circulating sAXL. The trend shows that after surgery, patients with larger tumors tended to result in a net increase in sAXL after surgical intervention and smaller tumor volumes tended to result in a net decrease in sAXL.
Figure 17: Change in Serum sAXL: Pre-Post Surgery
No significant change is seen in response to surgery as determined by a two-tailed, paired t-test. Individually matched serum sAXL concentrations from patient samples before (n=19) and after (n=19) surgery. Each dot represents the average of 6 replicates and each line connects the paired samples.
Figure 18: Change in Normalized Serum sAXL: Pre-Post Surgery

Serum sAXL/albumin ratios are significantly elevated after surgery ($P = 0.019$) as determined by a two-tailed, paired t-test. Each dot represents the average of 6 replicates and each line connects the paired samples. Individually matched serum sAXL concentrations from patient samples before (n=19) and after (n=19) surgery.
Figure 19: Pre-operative Tumor Volume vs. Change in sAXL
Elevation in serum sAXL levels observed after surgery directly correlates with tumor volume. Solid blue line represents best-fit line determined by standard linear regression. Dotted line represents baseline, no change, in sAXL. Correlation (r) and significance (P) values calculated using Pearson method.
Serum sAXL in Response to Treatment

Looking past the point of surgery (ie. Post-operative time point) to the treatment time points was attempted to test the hypothesis that sAXL was capable of tracking disease progression and treatment response. It can be seen that the number of samples acquired by each patient during this phase dropped precipitously. Despite this, it is also apparent that sAXL tended to be at its highest during the period following surgery once standard treatment regimens had begun (Table 1, figure 20). However, upon characterizing the treatment timeline samples and comparing against the post-operative samples did not showed any significant effect as determined by a mixed-model one-way ANOVA (Figure 20). Similar to previous comparisons, sAXL concentrations were normalized against serum albumin but did not show any significant effect in treatment timeline samples from the healthy controls and the surgical samples as determined by a mixed-model one-way ANOVA (Figure 21). Together these data suggest, but cannot confirm, that the significant elevation of sAXL in the GBM population as a whole compared to the control population (Figure 8), is primarily in response to either treatment or disease progression.
Figure 20: sAXL at Each Treatment Timepoint and Post-operative Samples
Serum sAXL concentrations from GBM patient samples, separated by treatment time point showed no significant effect compared to the post-operative samples. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance of variation for paired samples was calculated using a mixed-model one-way ANOVA.
ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Figure 21: sAXL Normalized by Albumin at Each Timepoint and Postoperative Samples
Serum sAXL concentrations normalized by serum albumin from GBM patient samples, separated by treatment time point showed no significant effect compared to the post-operative samples. Values calculated as a ratio between individual sAXL concentrations and the mean albumin concentration for each sample. Violin plots above represent the group median (solid line) and quartiles (dashed lines). Statistical significance of variation for paired samples was calculated using a mixed-model one-way ANOVA.
ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$
Associations between sAXL, AXL and Tumor Volume

In all, 16 tissue samples were made available for western blotting. Of these, 14 were GBM, one was an astrocytoma, and one was a tumefactive demyelinating process (MS). One of the GBM tissue samples was so small, a high enough concentration of protein could not be extracted for western blotting. With the remaining 15 samples, the protein concentration of the homogenates was estimated using BCA assays. Next, 40 µg of protein was loaded in a 4-12% Criterion™ Bis-Tris gel (BioRad), and separation of protein was achieved via gel electrophoresis and transferred to a PVDF membrane. This membrane was then blocked in 5% NFDM blocking buffer and blotted for AXL (1:2000) and β-Actin (1:2000). The membranes were developed using BioRad chemiluminescent substrate solutions, imaged and analyzed using Image Studio software (Li-COR Biosciences) (Figure 24). The optical density (OD) of the bands was interpreted and by dividing the AXL O.D by the actin OD, a value representing relative AXL expression was obtained.

Pre-operative tumor volumes for all 23 GBM patients enrolled in the study were determined using de-identified T1-weighted MRI scans using BrightMatter Plan software (Figure 25). This was done by scrolling up and down through the axial plane of the scans, highlighting enhancing regions with the brush tool that logically corresponded to tumor tissue (i.e. excluding ventricles and apparent blood vessels). All analyses were reviewed and when necessary edited, by a board-certified neuroradiologist. All scan files were labeled with the de-identified number and time point that corresponded with its matched serum sample. Tumor volumes interpolated from these scan files were transcribed by
hand, cross-checked, and manually transferred into a composite sAXL excel spreadsheet used to perform all statistical analyses.

Determining correlations between membrane AXL (mAXL) was performed using GraphPad software by inputting matched patient data, such as pre-operative sAXL and membrane AXL expression values, then performing a correlation analysis using the Pearson method. As a whole, comparing mAXL expression to pre-operative sAXL concentrations yielded no clear correlation ($r= 0.15$) (Table 2). However, when patient samples are broken into groups based on sAXL concentration, a new pattern emerged. Similar to methods performed in Hutterer et al.\textsuperscript{45}, those patients with pre-operative sAXL concentrations $\geq 30$ ng/mL (the average healthy sAXL concentration) were placed in the “sAXL High” group and the remainder in the “sAXL Low” group. Repeating these comparisons with each group then revealed a number of interesting correlations, the most significant of which being pre-operative sAXL in the “high” group is directly correlated with membrane AXL expression ($r= 0.85$) (Table 3, Figure 26).

A somewhat weaker correlation was made between pre-operative sAXL/albumin ratio and mAXL ($r= 0.65$) (Table 2). Moreover, in the “Low” group, there were several, moderate to strong, negative correlations made between mAXL and the following characteristics: pre-operative sAXL, pre-operative sAXL/albumin ratio, post-operative sAXL, post-operative sAXL/albumin ratio and pre-operative tumor volume (Table 2). These negative correlations indicate that larger mAXL expression results in smaller values in the corresponding variable. This is most interesting in the case of pre-operative tumor volume as it suggests that in the largest tumors lies the greatest retention of AXL on the plasma membrane, perhaps as a result of reduced proteolytic cleavage.
Evaluating associations with pre-operative tumor volume was performed as before but using volume as the dependent variable. In the collective GBM sample group (n=13), the strongest correlation was a moderate one with post-operative sAXL/albumin ratio group (Table 3). Once separated into “High” and “Low” groups, tumor volume showed no strong correlations. Of the moderate correlations, tumor volume was negatively correlated with pre-operative sAXL and normalized sAXL and positively correlated with post-operative sAXL and normalized sAXL (Table 3) but only in the “high” group. In the “low” group, volume was moderate-positively correlated with pre-operative, post-operative sAXL values and as previously mentioned, mAXL expression (Table 3). Based on the limited dataset, this suggests that the strongest associations lie between tumor volume and mAXL, as well as between tumor volume and post-operative sAXL/albumin ratio.
Figure 22: Membrane sAXL Expression in GBM Tumor Lysates

Western blots for 15 tissue homogenates, 13 of which are GBM. Membrane AXL expression found at ~140kDa (Top). Housekeeping gene, β-Actin expression for corresponding tissue samples found at ~40kDa (Bottom). Optical densities of these bands were calculated using Image Studio software (Li-COR Biosciences) and used to calculate relative AXL expression.
Figure 23: T1 Weighted MRI Scan of GBM Tumor
Example of tumor volume analysis in BrightMatter Plan software. Enhancing regions showing high contrast material uptake were highlighted using the brush tool in the transverse plane (bottom left panel) at various increments of distance. Once layers of tumor were highlighted top to bottom, tumor volume was interpolated, and a corresponding value would appear in the top right section.
Table 2: Associations with membrane AXL

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Pearson correlations between membrane AXL as determined by western blotting and other patient sample characteristics separated into three groups, all samples with available tissue samples, patients with high sAXL (≥30ng/mL) and patients with low sAXL (<30ng/mL). † Number of comparisons made. ‡Pearson r value: ±0.3-0.7 is considered a moderate correlation; > ±0.7 is considered a high correlation *P values: ≤0.05 is considered statistically significant.
<table>
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<td>7   -0.32  0.48</td>
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Pearson correlations between pre-operative tumor volume and other patient sample characteristics separated into three groups, all samples with available tissue samples, patients with high sAXL (≥30ng/mL) and patients with low sAXL (<30ng/mL).

† Number of comparisons made. ‡Pearson r values: ±0.3-0.7 is considered a moderate correlation; > ±0.7 is considered a high correlation *P values: ≤0.05 is considered statistically significant.
Figure 24: Pre-operative sAXL is Associated with Membrane AXL

The 11 patients with both tissue and pre-operative serum samples available were separated into two groups, sAXL high (≥ 30 ng/mL, blue) and low (< 30 ng/mL, red) based on pre-operative sAXL concentrations. The pre-op sAXL concentration for both of these groups were plotted against relative AXL expression determined by western blotting. These values were fitted using linear regression and correlations were calculated using the Pearson method.
DISCUSSION

The receptor tyrosine kinase AXL has been well characterized with regard to its implications in various types of cancer, including GBM. However, until now, the soluble form of this protein (sAXL) resulting from the proteolytic cleavage by ADAM proteins had yet to be explored as a potential biomarker for GBM. Due to the evidence that AXL is frequently overexpressed in GBM and that the protein is subject to this unique form of post-translational regulation resulting in its release into the circulatory system, it seemed possible that this could serve as a means of detecting GBM in a minimally-invasive manner.

While this study was able to generate some supporting evidence, we did not find that sAXL can be used to predict the presence of GBM near disease onset. Granted, there is a lack of uniformity in the reported values associated with circulating sAXL, in other studies in other cancer types examining sAXL as a predictive biomarker. However, those studies found a larger difference between the control and study populations. For example, sAXL is highly elevated in a number of liver diseases. As reported by Dengler et al.\textsuperscript{9} in a large multicenter study, sAXL was elevated in hepatocellular carcinoma (78.69 ng/mL, n= 347) compared to healthy controls (40.15 ng/mL, n=75)\textsuperscript{97}. Our results did not have this pronounced difference between healthy and disease but showed some promise for using sAXL as a biomarker for GBM, particularly when combined with multiple variables.
The systematic dilution scheme (see figure 3), where samples were stored diluted in order to mitigate freeze-thaw cycles on the limited number of serum samples, showed no significant effect on interpreted sAXL concentrations (see figure 4). This approach was inspired by the Dengler et al.\textsuperscript{108} optimization study where they evaluated the stability of sAXL when exposed to multiple freeze-thaw cycles. In the same study, they demonstrated the necessity of performing a 1:200 dilution of serum in the proprietary buffer LowCross™ buffer in order to effectively reduce the masking effect of protein-rich solutions like blood serum. However, due to high inter-assay variation, it was necessary to prepare the AXL protein standards as a batch such that they very closely mirror each other in assays performed on different days. This effectively increased the assay’s precision as demonstrated by the dramatically reduced inter-assay variation (see figures 6 and 7). Further optimization of the assay’s performance characteristics would entail acquiring internal controls in the form of purified human AXL protein to assess the reliability of the standards being used. Also, to address any concerns regarding accuracy, having a set of samples tested at a separate facility using the same protocol would be a means of bolstering the confidence of the results.

Here it was demonstrated that compared to a volunteer population, sAXL is elevated in blood serum collected from GBM patients (see figure 8). While sAXL concentration was able to distinguish the GBM and control populations in general, the bulk of that difference came from the treatment time points, long after the disease had become apparent. Incorporating a second characteristic, serum albumin, helped further distinguish GBM from healthy controls in nearly all cases. When normalized against serum albumin, the pre-operative values were significantly elevated compared to the
control group but lower than that of the paired post-operative samples (see figure 16). However, the use of albumin, which can change due to something as simple as being properly hydrated, is not reliable without confirmation of these findings on a much larger scale.

In order to bolster the predictive capacity of this sort of assay I would suggest incorporating other proteins that are more relevant to tumor pathophysiology and are similarly subject to sheddase activities. For example, CD147\textsuperscript{109}, TNF\textsuperscript{110}, or HER\textsuperscript{111} proteins all play significant roles in cancer cells and are also subject to post–translational regulation in an ADAM-dependent manner. In this combinatorial approach as seen in Miller et al.\textsuperscript{94}, building a biomarker panel of tumor-relevant proteins could serve to increase the predictive efficacy not achieved with sAXL alone or the sAXL/albumin ratio. Implementing the protocol in an automated fashion or using another methodology such as LC-MS could prove to be a more high-throughput approach, especially if it could analyze multiple targets in a similar timeframe.

Initially, the lack of a distinct trend from pre- to post-operative samples suggests that surgical intervention did not significantly impact sAXL levels in paired samples (see figure 17). However, there was a modest elevation in sAXL/albumin ratio in these same paired samples, suggesting that there was an impact but not significant enough to notice by assessing sAXL alone (see figure 18). In general, the blood-brain barrier (BBB) is known to be highly restrictive with respect to which macromolecules are able to pass. This is partially due to the tight junctions that create a tight seal between the specialized ependymal cells that make up the blood-brain barrier\textsuperscript{112}. This is why only lipid soluble\textsuperscript{112} and very small molecules (i.e. \textless 400 Da)\textsuperscript{113} are able to readily pass the BBB without
active transport. While the BBB often has increased permeability in glioma for a number of reasons\textsuperscript{114}, there does not seem to have been any direct evidence that suggests sAXL is restricted from or capable of crossing the BBB. In this study however, sAXL/albumin ratios were elevated, instead of decreased, after surgery and the data that showed tumor volume correlated with the net change in sAXL after surgery. One explanation for this would be that the blood-brain barrier is hampering the release of sAXL in general but not completely and only the largest tumors have produced such an excess of sAXL that it has become “backed up” and is then released as a result of surgery. One way to test this hypothesis would be to collect blood that is aspirated from the surgical site where the tumor is located then compare it to blood serum collected contemporaneously from a more peripheral blood vessel like the median cubital vein.

Theoretically, if sAXL were being released from the tumors in high enough quantities to make up a noticeable portion of the total serum sAXL concentration, the post-operative concentrations should have decreased relative to pre-operative values. However, this is built upon the premise that circulating sAXL is cleared or filtered out fast enough to notice a decrease in a timeframe of 24 hours. To date, there do not appear to be any publications supporting or refuting this idea directly. A small number of urine samples were collected from GBM patients and were tested for sAXL alongside the serum samples. Interestingly, sAXL was highly elevated in one of the four samples (data not shown), but did not seem related to either the pre-operative or post-operative samples and seemed likely related to kidney issues as has been reported before\textsuperscript{91}. One direction I would venture in order to explore this idea would be to track the release of sAXL in urine, which is found at similar concentrations as those reported for blood serum\textsuperscript{91},
throughout the course of a day in order to assess a normal rate of sAXL release from the body.

Considering that sAXL concentrations were highest during the treatment timeline, the elevation could simply be due to upregulated AXL induced by chemotherapeutic drugs as demonstrated in the past\textsuperscript{40}. On the other hand, AXL has also been shown to be upregulated in drug-resistant forms of numerous cancer subtypes\textsuperscript{40,67,94,115} and this could be another example of that. This study, however, was not designed to evaluate the prognostic potential of sAXL and therefore, the curious elevation seen during the treatment timeline is a phenomenon left mostly unexplored. A retrospective study of outcomes from the patients enrolled could be implemented to assess these effects. Regardless, the results of this study suggest that on its own, sAXL would not serve as an ideal predictor of GBM prior to the emergence of symptoms.

One of the most significant limitations in this study was the sample size and collection consistency. Understandably, obtaining tissue specimens was not always feasible because there is a prioritization hierarchy to which biopsied samples are made available. First, the pathology department obtained whatever size specimen was needed for diagnostic testing. Second, tissue samples were used to generate primary cell lines and this study received whatever was left. Similarly, obtaining blood serum was not always possible. In nearly every enrolled patient, some samples were missed due to complications in the collection procedure, patients opting out of treatment, etc. Additionally, it now appears that sonication on the tumor tissue samples likely resulted in the degradation of proteins. Instead, a gentler, more precise method of protein extraction should have been implemented. Using either an electric homogenizer or a simple
dissociative agent, followed by differential centrifugation should have been used to isolate the membrane fractions and would have served our purposes much better and produced higher quality western-blots. This would have allowed us to assess AXL expression in tumor tissue in a manner comparable to the experimental design seen in Hutterer et al.45.

These shortfalls can be compensated for in a number of ways. Instead of obtaining biopsied specimens and performing western blots, a better approach would be obtaining sections of formalin-fixed paraffin-embedded (FFPE) tissue and performing immunohistochemistry (IHC). This offers several major advantages to obtaining frozen tissue, primarily in that it is collected whenever possible and is typically not in short supply. Also, fixed tissues can be tested for molecular characteristics using IHC years after its collection greatly extending the “shelf-life” of precious samples. Since a complete collection of blood serum from all designated indicated time points were difficult to obtain for a number of reasons, a viable option would be to supplement these samples patient blood serum that is commercially available. Furthermore, the collection of non-GBM blood samples including patients with lower grade brain tumors, non-GBM tumors that are associated with sAXL like HCC98 would make us better equipped to elucidate sAXL’s potential as a GBM biomarker.

Three of the four hypotheses were rejected: Circulating sAXL in patient blood serum was not significantly decreased following surgical intervention, it was not decreased in response to chemotherapy and radiation therapy, and pre-operative sAXL did not significantly correlate with tumor volume. Despite this, it is important to consider that this was performed on a small scale and with populations that weren’t matched.
ideally based on age, sex or other demographic characteristics. Due largely to time constraints, multifaceted analysis of the MRI scans was not feasible and could have answered several of the questions left unanswered here. In the future, the MRI scans will be re-examined for further characteristics such as extent of necrosis, cell density of the tumors, and infiltrating cells that would not have been readily detected using the T1-weighted scans. Also, evaluating the relative expression and activity of the sheddase ADAM 10/17 in the tumor tissue is a major component of sAXL release that could explain the lack of correlation between tumor volume, membrane AXL expression, and soluble AXL.
CONCLUSIONS

In this study, we have demonstrated that there is potential for sAXL as a non-invasive biomarker for GBM but could not confirm it outright. Blood serum concentrations of sAXL were found to be elevated in the group of GBM samples collected prior to diagnosis and throughout treatment. Utilizing serum albumin concentrations as a second characteristic to normalize sAXL against served to distinguish pre-operative GBM blood serum from the healthy controls, albeit only modestly. Given the highest sAXL levels seen were during treatment instead of prior to diagnosis, sAXL alone would not likely function well as a predictive marker. Perhaps most promisingly, in a subset of patients, grouped based on the abundance of sAXL relative to the control population’s average, pre-operative sAXL levels showed a significant correlation with AXL found in corresponding tumor tissue. This would suggest that sAXL is in fact being released from tumor tissue and is subsequently detectable in the blood. While the natural fluctuations of sAXL may mask this effect, designing a panel of similar proteins could prove effective in predicting GBM where sAXL alone could not. Based on the evidence presented here, we believe that repeating this study in a larger and modified fashion is merited.
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APPENDIX A

NMU IRB Approval letter for the collection of Blood Serum

IRB Approval: HS19-1033

Janelle Taylor <jantaylo@nmu.edu>
To: draymond@nmu.edu, "Winn, Robert J." <rwinn@nmu.edu>
Cc: Bethney Bergh <bbergh@nmu.edu>, Derek Anderson <dereaende@nmu.edu>, Lisa Schade Eckert <leckert@nmu.edu>

Tue, Apr 2, 2019 at 2:01 PM

Memorandum

TO: Daniel Raymond
   Biology Department

CC: Rob Winn
    Biology Department
    College of Arts and Sciences

DATE: April 2, 2019

FROM: Lisa Schade Eckert, Ph.D.
       Interim Dean of Graduate Education and Research

SUBJECT: IRB Proposal HS19-1033
IRB Approval Dates: 4/2/19 – 4/1/20
Proposed Project Dates: 4/2/19 – 5/14/20
“Evaluating sAXL as a Biomarker for Glioblastoma (GBM)”

Your proposal “Evaluating sAXL as a Biomarker for Glioblastoma (GBM)” has been approved by the NMU Institutional Review Board. Include your proposal number (HS19-1033) on all research materials and on any correspondence regarding this project.

A. If a subject suffers an injury during research, or if there is an incident of non-compliance with IRB policies and procedures, you must take immediate action to assist the subject and notify the IRB chair (dereaande@nmu.edu) and NMU’s IRB administrator (leckert@nmu.edu) within 48 hours. Additionally, you must complete an Unanticipated Problem or Adverse Event Form for Research Involving Human Subjects.

B. Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding. Informed consent must continue throughout the project via a dialogue between the researcher and research participant.

C. If you find that modifications of methods or procedures are necessary, you must submit a Project Modification Form for Research Involving Human Subjects before collecting data.

D. If you complete your project within 12 months from the date of your approval notification, you must submit a Project Completion Form for Research Involving Human Subjects. If you do not complete your project within 12 months from the date of your approval notification, you must submit a Project Renewal Form for Research Involving Human Subjects. You may apply for a one-year project renewal up to four times. Failure to submit a
APPENDIX A

NMU IRB Approval letter for the collection of Blood Serum

3/5/2020

Northern Michigan University Mail - IRB Approval: HS19-1003

Project Completion Form or Project Renewal Form within 12 months from the date of your approval notification will result in a suspension of Human Subjects Research privileges for all investigators listed on the application until the form is submitted and approved.

All forms can be found at the NMU Grants and Research website: http://www.nmu.edu/grantsandresearch/node/102

Janelle N. Taylor
Graduate Education and Research
Northern Michigan University
906-227-1407
1401 Presque Isle Ave, Marquette, MI 49855
http://www.nmu.edu/graduatesstudies/
APPENDIX B

Aurora IRB Approval letter for the collection of Blood Serum

27 May 2015 [REVISED]

Natalie Polinske
Biorepository Center
Aurora St. Luke's Medical Center
2900 W Oklahoma Ave
Milwaukee, WI 53215

RE: #14-79: Prospective Biospecimen Collection, Storage, and Distribution

Dear Ms. Polinske:

On behalf of the Aurora IRB1 Biomedical, I am pleased to inform you that the above-named protocol (v. 9/10/14), Informed Consent (IRB approved consent final version date 8/20/14) and Supplemental Research Information/Authorization (IRB approved final version date 9/18/14), as well as any submitted surveys, questionnaires, recruitment, and written materials to include diary cards and clinical trial cards, meet the IRB’s criteria for approval and were approved on 10 September 2014. You will receive a stamped copy of all approved recruitment and written materials to be seen by subjects for your research record.

An IRB Waiver of HIPAA Authorization is also granted pursuant to 45CFR164.512 since an Honest Broker will be responsible for de-identifying protected health information for research purposes.

The IRB has determined and documented at a convened meeting that the research involves no greater than minimal risk, and provided no additional risks are identified, continuing review can be conducted via expedited review (Category #9).

The approval period is effective on 30 September 2014 (date IRB conditions of approval were determined to be met) and expires on 09 September 2015. The internal IRB tracking number assigned to your research study is 14-79. Please be sure to reference that number in any future correspondence with the IRB.

A summary of your obligations as Principal Investigator are outlined in the attached IRB Approval Letter Addendum. Feel free to contact me with questions regarding any aspect of your human subject research. I can be reached at 414.219.7744 or via e-mail at <michelle.maternowski@aurora.org>.

Sincerely,

Michelle Maternowski, BS, CIM
Interim Director, Research Subject Protection Program
Sent on behalf of the Aurora IRB Biomedical

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Aurora IRB Compliance Statement: The Aurora Health Care Institutional Review Boards (Aurora IRBs) comply with all applicable laws, guidelines, and federal regulations that oversee the operation of Institutional Review Boards, specifically 45CFR46 and 21CFR50 and 56, including International Conference of Harmonisation GCP Good Clinical Practice guidance (ICH GCPs). The Aurora IRBs are duly constituted (fulfilling federal requirements for diversity), have written procedures for initial and continuing review of clinical trials, prepare written minutes of convened meetings, and retain records pertaining to the review and approval process. In accordance with these regulations [45CFR46.107(a) and 21CFR56.107(a)], the Aurora IRBs prohibit any member from participating in the IRB’s initial or continuing review of any study in which the member has a conflicting interest, except to provide information requested by the IRB. Our policy is to require a voting member of the IRB to leave the room for final discussion and voting on a protocol in which the member is an investigator, or has any conflict of interest. In addition, the Aurora IRBs have received FUL accreditation by ACRPP (valid through September 2016).