Transcriptional Characterization of Sepsis in a Novel LPS Pig Model

by

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ABSTRACT

Sepsis is a deadly and debilitating condition resulting from a hyperinflammatory response to infection. Most organ systems are severely impacted, including the neurological complications for survivors of sepsis. Sepsis associated encephalopathy (SAE) is characterized by dysregulated molecular pathways of the immune response impinging upon normal central nervous system (CNS) function and ultimately resulting in lasting cognitive and behavioral impairments. Sepsis predominantly occurs in a few neonates but mostly elderly individuals where they are at high risk of sepsis-induced delirium and other neurological implications that may have overlap with neurodegenerative diseases. This study seeks to identify gene candidates that exhibit altered transcriptional expression in tissues between pigs injected with saline control vs lipopolysaccharide (LPS) to model the early inflammatory aspects of the septic response. Specifically, brain frontal cortex was examined to see which genes and pathways are altered at these early stages and could be targeted for further investigation to alter the cognitive/behavioral decline seen in sepsis survivors. This experiment uses a bulk RNA-seq approach on Yorkshire pigs to identify the variance in gene expression profile. Data analysis showed several gene candidates that were downregulated in the brain in response to LPS that point to early endothelial cell disruption, including OCLN (occludin), SLC19A3 (thiamine transporter), and SLC52A3 (riboflavin transporter). Genes that were upregulated in LPS brain samples implicate endothelial cell dysfunction as well as immune/inflammatory alterations, potentially due to alterations in microglia, the primary immune cell of the brain. Several studies are now underway to understand the cellular origin of these transcriptional changes, as well as analyzing the molecular signatures

altered in response to sepsis in whole blood and kidney using bulk RNAseq. In conclusion, specific gene candidates were identified as early changes in the septic brain that could be targets to prevent long-term cognitive and behavioral changes in future studies, establishing a baseline panel to interrogate in animal models with the goal of advancing treatments for human patients who experience sepsis.

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CHAPTER 1

INTRODUCTION

SECTION 1: BACKGROUND

Diseases of the central nervous system cause a disproportionate amount of costs to our public and private health care system. 'Big players' such as Alzheimer's disease, affecting over five million people [US], are commonly associated by the public as the face of dementia and cognitive neurodegeneration. However, statistically there is a much more common source of cognitive impairment producing delirious behavior and brain dysfunction: sepsis.

With new cases approaching two million [US] and fifty million [global] annually, resulting in a twenty to fifty percent mortality rate, sepsis is one of the most common pathological conditions causing a variety of downstream organ dysfunctions through various molecular cascades. Sepsis, or systemic infection, is a life-threatening response to bacterial infection of tissues or that has entered the bloodstream, resulting in wide-scale organ dysfunction from a dysregulated host immune response. In this extreme immunological response, the body secretes an out-of-balance level of chemokines leading to body-wide inflammation. Chemokine secretion, increasing vascular permeability, is a normal element of the immune system's response to infection, but dysregulation during sepsis can lead to the damage of multiple organ systems and therefore many subsequent long-term complications. One subsequent complication of sepsis with neurological significance is sepsis associated encephalopathy (SAE) (Rudd, et. al., 2020).

When a virial, bacterial, or fungal pathogen gains access to the CNS creating an inflammatory response, also known as meningitis (a secondary infection of CNS, or

SAE), the dysregulated molecular pathways of the immune response impinge upon normal central nervous system (CNS) function and ultimately result in lasting cognitive and behavioral impairments (Pine, et. al., 2008). Symptoms of SAE include stroke, hemorrhage, and an induced delirious state that is associated with high risk for subsequent cognitive impairment (Piva, et. al., 2015).

Bacterial infection progresses to sepsis in a series of stages: from infection, to systemic inflammatory response syndrome (SIRS), to sepsis, to severe sepsis, and then finally progresses to septic shock, which has a fifty percent mortality rate. Severe sepsis is characterized by one or more organs or organ systems beginning to fail. Septic shock is a dramatic blood pressure drop due to the factors of vascular permeability from elevated chemokines, overall presenting a twenty to fifty percent chance of death, depending on demographics (Bone, et. al., 1991) and (Nguyen, et. al., 2006).

Sepsis is the most expensive healthcare condition in the US costing approximately twenty-four billion each year, and still increasing in cost. Worldwide over 50 million cases are reported each year, resulting in 11 million deaths, with 1.7 million annual cases and 270,000 deaths in the United States (Rudd, et. al., 2020).

Anyone, regardless of age, race, or sex, can develop sepsis, but it is a critical problem for elderly patients (>65yrs), infants (<1yr), immunocompromised patients, and those on dialysis or following organ transplants. Most individuals affected by sepsis are elderly and if they survive are at extremely high risk of developing cognitive impairments. Diagnostic timing is key for treating sepsis. There is a 7.6% decrease in mortality rate per hour after sepsis identification and administration of antibiotics, vasopressors, and isotonic fluids. Sepsis patients are also 2-3 times more likely to be

readmitted to the hospital, and those readmissions are more expensive than other severe conditions including heart failure, pneumonia, and COPD (chronic obstructive pulmonary disease) (Waterhouse, et. al., 2018).

While sepsis affects multiple organ systems, SAE and the immunological pathways leading to inflammatory-induced cognitive decline is a common and still a poorly understood neurological pathway with mostly unknown mechanisms. Affecting large populations and reporting high mortality rates, sepsis-induced delirium and other neurological implications of sepsis pose important avenues of research in the evolving field focused on the intersection of neurodegeneration and immune system regulation (Dombrovskiy, et. al., 2018).

SECTION 2: PREVIOUS STUDIES

Many studies concerning sepsis have looked into the disease's pathogenesis. Research into the immune system's role in sepsis have found ties to various factors such as tumor necrosis factor α (TNFα), leukotrienes, myocardial depressants, platelet activating factor, thromboxane A2, adhesion molecules, complement cascade activators, interleukins, β- endorphin, kinins, thrombin, and heat shock proteins. As sepsis is a disease with many potential causational roots and domino effects, literature is dense with studies concerning various proteins involved in the life-threatening condition. Another prominent topic in sepsis literature is treatment strategies. Current sepsis diagnosis critical criteria follow the SOFA model (sepsis-related organ failure assessment). Given the aggressiveness and rapid progression to septic shock, dynamic early goal-directed therapies have been reported and adapted with emphasis on antimicrobial application, corticosteroid administration, and source control (Trauner, et. al., 2016).

Documentation and case reports dictating the history of sepsis epidemiology is prominent throughout literature, as the story of sepsis is one of substantial health care burdens characterized by trial-and-error treatment throughout decades of medical advancement (Martin, et. al., 2003). As identification of sepsis is of utmost importance, biomarkers for diagnoses and prognostic indicators have also been investigated (Briassoulis, et. al., 2019) and (Angus, et. al., 2010). Between treatment approaches, pathologically significant protein identification, diagnostic measures, and a robust epidemiological history, literature pertaining to sepsis is as broad as the means by which the disease can be caused.

In the literature pertaining to neuroinflammation, research concerning the overlap between neurodegeneration and immune system regulation is extensive. Studies have shown that the consequences of prolonged neuroinflammation promote damaging effects on cellular mechanisms and brain function, accelerate disease progression, and promote the early development of chronic diseases such as dementia. While the innate immune system naturally induces inflammation as a defensive mechanism orchestrated by leukocytes, endothelial cells, and signaling molecules/cytokines, when inflammatory pathways become dysregulated many devastating effects can result (Zou, et. al., 2019).

Neuroinflammation describes immune responses of the central nervous system, specifically regarding the principal neural cells which include immune cells like microglia, or other cells such as astrocytes that can serve in immune roles in certain contexts. The blood-brain barrier has been shown to be permeable to pro-inflammatory mediators and further proven that with excessive and prolonged neuroinflammation, damaging effects arise concerning brain function, extending in severity from acute conditions such as delirium and postoperative cognitive dysfunction to more extreme consequences involving chronic diseases such as Alzheimer's and multiple sclerosis (Meneses, et. al., 2018). One field of research intersection with sepsis is Alzheimer’s research, where inflammatory processes have become a central focus in the pathogenesis of that and other neurodegenerative diseases (Lyman, et. al., 2013).

Research pertaining to the neuroinflammation in sepsis, primarily leading to brain dysfunction in sepsis associated encephalopathy (SAE) and sepsis-associated delirium (SAD), has begun to look into CNS alteration during sepsis, predominantly pathophysiology with regards to epithelial atrophy as seen in the blood-brain-barrier

breakdown, cytokine activation, and neurotransmitter deregulation (Zou, et. al., 2019). Typically, the peripheral production of proinflammatory cytokines and reactive oxygen species are involved in mechanisms for structural modifications in these brain barriers, which increase permeability and activation of glial cells such as microglia and the production of cytotoxic mediators which then act on the brain barriers, further causing damage (Michels, et. al., 2014). SAE, caused by systemic inflammation in the absence of direct brain infection, as described by widespread immune response impinging downstream on the brain, has shown changes in cerebral blood flow, metabolic alterations, and release of inflammatory molecules contribute to progressive neuronal dysfunction and cell death. Clinical studies have documented that SAD is by far the most common form of delirium acquired in the ICU, showing presentation in about 50% of septic patients (Chung, et. al., 2020). Clinical characteristics described with SAD include changes in cognition, an altered level of consciousness, reduced attention, and perceptual disturbances. Symptoms can be reversible, but seeming irreversible and prolonged neurological deficits have been observed in older patient populations that are sepsis survivors. SAD is clinically diagnosed using specific proven assessment tools such as CAM-ICU (Confusion Assessment Method for the Intensive Care Medicine) or ICDSC (The Intensive Care Delirium Screening Checklist), as well as neuroimaging studies utilizing EEG (electroencephalography) as accompaniments to clinical evaluation to define the severity of the septic state in the progression timeline. Due to this progression, a patient could walk into the hospital for a routine transplant and leave with lasting phenotypic symptoms resembling dementia (Waterhouse, et. al., 2018).

Experimental techniques utilizing bulk RNA-seq (as well as single-cell and single-nucleus RNA-seq) in pig tissue have also been implemented in a few studies. Expression analyses comparing different computational mapping methods and recognition of allele-specific expression have followed these studies to create a more understood picture of pig transcriptomics for biomedical research application and to further understand gene expression changes in disease models (Jacquier, et. al., 2011) and (Wirka, et. al., 2018). The pig genome still poses a challenge due to poor annotation, but progress is being made in this model. Studies have also aimed to identify candidate genes and pathways associated with targeted approaches for investigation via RNA-seq analysis, where genes were also clustered according to connectivity to modulate co-expression patterns resulting in tissue-specific candidate genes identified for further studies (Wu, et. al., 2017). RNA-seq in pig models has also been applied to study the diverse physiology of the brain and its complex organization at cellular and molecular levels with comprehensive brain mapping projects. Transcriptomics projects with single-cell RNA sequencing have been utilized, as well as in situ hybridization and antibody-based protein profiling to map molecular profiles (Mulder, et. al., 2009). These studies, centered around RNA-seq technology, produce data analysis results consistent with a preserved fundamental brain architecture pattern seen during mammalian evolution and local gene expression profiles. Emergent RNA-seq technology at both single-cell and bulk tissue resolution provide promising potential avenues to explore gene expression as well as treatment-specific transcription profiles in the next frontier of biomedical research (Maslove & Wong, 2014) and (Klem, et. al., 2014).

SECTION 3: MICROARRAY vs BULK RNA-SEQ vs SINGLE-CELL/NUCLEUS APPROACHES

Microarray technology's prevalence in genomic expression studies, profiling predefined transcripts and genes through hybridization, have a relatively long history across literature and increased dramatically after its initial introduction in the 1990s. Microarray-based gene expression studies of sepsis have shown multifaceted pathogen recognition and insights to signaling pathways involved in inflammation that characterize sepsis (Maslove & Wong, 2014). Microarray approaches have largely been supplanted by the more consistent and quantitative RNA-seq, an approach that is also far less prone to false negative and false positive signals.

RNA-seq requires aligning reads to a reference genome and then looking for differential expression to identify molecular pathway dysfunctions resulting in changes of gene expression. These techniques are useful for studying cellular responses and expression profiles across a multitude of model types, including disease pathology. Single-cell, single-nucleus, and bulk RNA-seq each have their own advantages and limitations, with bulk RNA-seq having more established computational pipelines and far less costly than the newer single-cell/nucleus RNA-seq techniques that have newly evolving computational approaches and remain 5-10x more costly per sample than bulk RNA-seq (Hadfield, 2020).

Bulk RNA-seq uses a population of cells for its sequencing library providing gene expression of an entire sample, measuring the average expression level for each gene across a large population of all cells within a sample. Hence bulk RNA-seq illustrates gene expression within a whole organism or tissue type, ideal for studying the

pathological effects on an organ system. Bulk RNA-seq is useful for comparative transcriptomics and quantifying expression signatures (disease studies). Bulk RNA-seq has limitations for studying heterogeneous tissues like the brain and is difficult to assign specific transcriptional changes to any one cell type (Cardoso, et. al.,2017).

Single-cell RNA-seq [scRNAseq] uses sequencing libraries representing single cells, differentiating among cell types within the sample itself. ScRNA-seq measures the expression level dispersion for each gene across cell populations, illuminating cell-specific changes in transcriptome allowing for cell-type identification and heterogeneity responses and it has revolutionized our understanding of cell-specific changes (Haque, et. al., 2017) and (Saunders, et. al., 2018).

Single-nucleus RNA-seq has the advantage over single-cell in that it can be used on archived tissue (where the freeze-thaw process results in lysis of cellular membranes) and an advantage over bulk by being able to distinguish distributions of cellular populations. In single-nucleus, tissue is thawed, undergoes nuclear prep, and is placed in a mild environment for stripping cell membranes and non-nuclear material before nuclei purification in a sucrose gradient, prior to undergoing the then same steps as single-cell when being placed into the 10X instrument and partition with oil drops for oil bead amplification.

This study was designed with plans to expand to single-cell and single-nucleus studies in the future, but these analyses are outside the specific focus of this report and will be performed in a subsequent study. This initial investigation solely utilizes bulk RNA-seq and then a subsequent study with single-nucleus RNA-seq will be performed on

preserved, extracted samples from this study. The results from the current study will be used to determine representative samples to perform subsequent snRNAseq.

CHAPTER 2

METHODOLOGY

SECTION 1: THIS EXPERIMENT'S APPROACH

This project investigates the impact of sepsis on the brain at bulk RNA-seq resolution, and will eventually be compared to the response that occurs in other peripheral organs such as kidney and lung. To investigate potential inflammatory pathways that could contribute to spiraling cognitive decline during the sepsis response, this study used Yorkshire pigs injected with LPS i.v.

Pigs were selected for this study in lieu of other animals for a multitude of reasons. Current FDA guidelines recommend all new drug applications provide data from two species (one as non-rodent condition). Additionally, there is a meaningful unmet demand for large animal models of sepsis that more accurately represent human vulnerability to infection. Biologically, the pig's immune system has over 80% similarity to humans (<10% for mouse), pig's blood coagulation system is similar to humans, and pig size and blood volume allows for concurrent monitoring and chronological clinical evaluation of sepsis pathway progression (Pabst, 2020) and (Brownell, 2018). Additionally, pigs have a gyrencephalic brain with folds similar to humans and a grey to white matter ratio closer to humans than other animal models, especially rodents (Howells, et. al., 2010) and (Meurens, et. al., 2011).

LPS, lipopolysaccharide (endotoxin composing the outer membrane layer of gram-negative bacteria), is a component of bacterial cell walls commonly used to model the inflammatory aspects of sepsis. Sepsis was induced in the pigs by injecting LPS intravenously. The corresponding controls were given a saline injection in their

counterpart of mirrored five step anesthetized administration process [Telazol, Xylazine, Glycopyrrolate, Plasmalyte, then the LPS or saline injection]. Under septic circumstances, circulating endotoxin acts as a pathogen associated molecular pattern (PAMP) which stimulates the innate immune system and some non-immune cells that results in the initiation of inflammatory processes, mediating both local and systemic inflammatory response (Yücel, et. al., June 2017). LPS was selected for this preliminary investigation based on it being a well-controlled and reproducible system. The next phase of this investigation will incorporate E. coli injections, where an actual pathogen will be used for sepsis induction in a follow-up experiment to this one (Yücel, et. al., 2017).

The neurological implications of sepsis will be looked at through a bulk RNA-seq lens, to analyze brain tissue from the frontal cortex, along with several other tissue types that will be analyzed later. Since organ failure is a hallmark of sepsis, and as the kidneys are often the first organs affected, a bulk RNA-seq approach will also be applied for renal tissue analysis in a subsequent investigation, as well as blood, lung, liver, and heart tissue (Ramayo-Caldas, et. al., 2018) and (Ramos-Ibeas, et. al., 2019).

SECTION 2: SCIENTIFIC METHOD BREAKDOWN

Theories: It is clear that sepsis causes brain dysfunction and neuroinflammation. The neuroinflammatory transcriptional pathways leading to SAE and changes in gene expression during sepsis specifically in the brain is still largely unknown in large model organisms or in humans.

Question: Which genes and pathways are altered in the brain during sepsis?

Hypothesis: This experiment broadly hypothesizes that we expect to see robust and significant differences in gene expression between LPS (aka endotoxin) and saline control groups. Specifically, we hypothesized that we would see vascular and immune pathways impacted in the pig brain during sepsis. As this is the lab's first of many studies on sepsis, in animal models (pigs, sheep, and mice) as well as post-mortem human tissues from patients who died from sepsis, this project will be crucial for developing more directed and robust hypotheses in the future. Importantly, it will set our baseline understanding of how well, or not, this pig model is able to recapitulate the molecular and cellular changes that occur in the CNS of human sepsis cases.

Prediction: This experiment hypothesis follows current literature in predicting expectations to see changes in gene expression between groups.

Previous studies: Others have profiled septic-like injury in humans, but only of blood or brain tissue of rodents in LPS or pathogen-induced sepsis models.

Short-Term Goals of this Study: Identify gene candidates to target during sepsis modulation in pigs that are also applicable to mice and humans for future studies.

Long-Term Goals of this Study: The Fryer Lab's ultimate goal from this study is to eventually identify pathways and molecular cascades resulting from alterations in gene

expression under septic conditions, where these early changes in the septic brain could be targeted to prevent long-term cognitive and behavioral changes. Another direction of research could involve pinpointing specific molecules as biomarkers/targeted novel therapeutics. Similar in concept to previous studies in the lab, lipocalin-2 (LCN2) was identified as a critical factor in protecting the CNS in a sepsis mouse model. The Fryer Lab potentially seeks to discover new drugs that target LCN2 or other molecules for concurrent therapy to patients at the time of infection, thereby preventing CNS inflammation and subsequent cognitive impairment (Kang, et. al., 2017). Additionally, separate studies in the Fryer lab will perform dual RNA-seq from blood to determine patient transcriptional response and active pathogen transcripts for pathogen identification and presence of antibiotic resistance and identify key host and pathogen transcripts across a wide range of sepsis types that could be used to develop a custom diagnostic panels. Since there are no great biomarkers for sepsis, this experiment investigates significant differences in gene expression between sepsis and control groups, serving a crucial role for developing more directed hypotheses in the future as the beginning of a chain of successive studies aimed to eventually pinpoint specific molecules as biomarkers/targeted novel therapeutics and to develop assays for rapid/inexpensive clinical diagnosis panel, similar for example to that of the Prosigna PAM50 Breast Cancer Prognostic Gene Signature CodeSet licensed to NanoString.

Projection: This sepsis model is intended to show differential gene expression from control groups to achieve the short-term goal of identifying gene candidates to target during sepsis in pigs. It will be compared directly to an E. coli-induced sepsis study.

SECTION 3: SUBJECTS

Twelve 5-month-old pigs of Yorkshire breed (n=6 for each group: control and sepsis treatment, average body weight 70kg) [see Table 1] were utilized. Samples collected include blood/plasma, kidney (cortex and medulla), lung, liver, heart, and brain (prefrontal cortex, both grey and white matter). The rate of LPS injections was 0.5 - 0.75 mL/kg. A prior acute dosing study was done at Mayo Clinic to test the lipopolysaccharide (LPS) induced inflammatory response, resulting in death within 5-10 hours. In this investigation, tissue was immediately extracted, with part of the collection immediately frozen on dry ice and part of the tissue was preserved for histology by placing in formalin, then in ETOH 70% (4ºC). The study was performed in accordance with the National Institute of Health Guide for the care and use of laboratory animals and under the approved protocol [#A00004511] for animal welfare as enacted by Mayo Clinic. One sample per tissue for each sacrificed pig (one brain sample and one kidney sample, both fresh frozen -80C) was collected and analyzed.

ID	GROUP	BODY WEIGHT (KG)	DOSE OF INJECTION	TIME OF INJECTION	TIME OF DEATH
2	LPS	78.0	1 ml/hr	08:05	11:15
3	Control	76.7	1 ml/hr	08:00	12:23
4	Control	76.7	1 ml/hr	06:40	14:05
6	Control	79.6	.50 ml/hr	11:00	16:10
7	Control	77.8	.50 ml/hr	10:00	15:10
8	LPS	74.1	.75 ml/hr	08:20	12:40
9	LPS	78.5	.75 ml/hr	07:56	10:01
10	Control	78.4	.75 ml/hr	08:30	13:35
11	Control	73.7	.75 ml/hr	08:32	13:32
12	LPS	71.2	.75 ml/hr	08:00	14:30
13	LPS	72.0	.75 ml/hr	08:10	10:30
14	LPS	77.7	.50 ml/hr	07:53	17:12

[Table 1] Table of subjects

SECTION 4: MATERIALS AND PARADIGM

The general methodology of this experiment entails dissecting tissue samples, extracting total RNA, running bulk RNA-seq, and then analyzing the data through computational genomics pipelines in R programming language.

This investigation utilized a variety of materials and machines. Equipment and supplies were provided by Mayo Clinic's joined research laboratories. Machines and apparatus used in the biological preparation of tissue sample include: 15 mL falcon tube, cell pellets, rotator/rocker, pipettes, centrifuges, and other lab equipment.

Chemical materials used in the biological preparation of tissue sample include: Na2SO4, K2SO4, HEPES, glucose, MgCl2, protease (p5380, Sigma), papain (Worthington, LK003153), 0 L-cysteine, EDTA, actinomycin D (Sigma, A1410), K2SO4, trypsin inhibitor (Sigma, T6522), BSA (Sigma, A2153), ovomucoid protease inhibitor (Worthington, LK003153), and 0.01% BSA (Life Technologies, AM2616).

Extracted sample chunks were frozen and sent to Yale University's core facility for tissue lysing and RNA extraction/preparation/sequencing, where real time analysis (RTA) software converted signal intensities to individual base calls using Illumina's CASAVA 1.8.2 software suite, with error rate is less than 2% and strong distribution of reads per sample/lane. RNA-seq data was obtained with post-sequencing computational processes, through data analysis on the NovaSeq platform with R packages used for standard filtering, cutoff, and graphical illustration.

SECTION 5: BIOLOGICAL PROCEDURE

Whole chunk samples were dissected and collected after death in regions of interest. Brain tissue samples were sent to Yale for total RNA purification and sequencing on Illumina's microarray platform with bulk RNA-seq. RNA purification methods follow Yale's Center for Genome Analysis (YCGA) protocol for Bulk RNA sequencing (Chomczynski & Mackey, 1995).

Required chemical reagents include: 70% ethanol, TRIzol reagent (Invitrogen), PBS, cell scraper, DEPC-treated water (Ambion), and isopropyl alcohol. Equipment and supplies used include: refrigerated centrifuge, vortex mixer, microcentrifuge, aerosol-barrier tips, micropipettors, powder-free gloves, and centrifuge tubes.

Per YCGA protocol, tissue samples were homogenized in 1 ml of TRIZOL reagent per 50 to 100 mg of tissue using a glass-Teflon. The samples were minced and rinsed with ice cold PBS. Samples were directly lysed by adding TRIZOL reagent and grated with cell scraper. Cell lysate was passed several times through a pipette and vortexed thoroughly. Samples were spun for 5 min at 300 X g and after removing media cells were suspended in ice cold PBS. Samples were pelleted by spinning at 300 X g for 5 min. Samples were lysed with TRIZOL reagent by repetitive pipetting/passing through syringe and needle. The homogenized sample was incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes and then centrifuged to remove cell debris. The supernatant was transferred to a new tube and 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added for phase separation. Samples were vortexed vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. Samples were then centrifuged at less than 12,000 x g for 15 minutes at 2 to 80

C. Following centrifugation, the mixture separated into lower red, phenol-chloroform phase and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase and the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol (0.5 ml isopropyl alcohol per 1 ml of TRIZOL reagent). The samples were then incubated at 15 to 30 degrees Celsius for 10 minutes and centrifuged at less than 12,000 x g for 10 minutes at 2 to 4 degrees Celsius. The RNA precipitated, forming a gel-like pellet on the side and bottom of the tube. The supernatant was completely removed and the RNA pellet was washed once with 75% ethanol. RNA was redissolved when samples were mixed by vortex and centrifuged at less than 7,500 x g for 5 minutes at 2 to 8 degrees Celsius. Leftover ethanol was removed and RNA pellets were vacuumed for 5-10 minutes.

For analysis, 1 µl of RNA was diluted with 39 µl of DEPC-treated water (1:40 dilution). Using 10 µl microcuvette, optical density (OD) was taken at 260 nm and 280 nm to determine sample concentration and purity. Total RNA quality control was calculated by estimating A260/A280 and A260/A230 ratios through nanodrop (optimal purity level is between 1.8 and 2). RNA integrity was determined by running an Agilent Bioanalyzer gel, measuring ribosomal peak ratios. Samples with RIN values of 7 or higher were eligible for library preparation (>8 ideal for RNA-seq). The total RNA isolated was then further cleaned using a cleanup protocol and subsequently underwent sequencing by the Illumina bead array platform.

SECTION 6: RNA-SEQ

After completing the tissue sample collection and RNA purification described above, the samples underwent bulk RNA-seq where the transcriptome of gene expression patterns encoded by RNA are quantified.

The RNA-seq workflow is composed of library preparation, insertion into sequencer for clonal amplification and cluster generation, bridge PCR, sequencing by synthesis, and then analysis. As mRNA (RNA of interest, corresponding to genomic profile) only composes two to three percent of cellular RNA, poly-A enrichment (barcoded bead binding on mRNA transcripts for eukaryotic cells) was applied for targeted mRNA isolation (McKnight & Amieux, 2009).

RNA is initially converted to cDNA and then fragmented for sequencing. Adaptor ligation is applied so the DNA fragments can bind to the sequencer itself during cluster generation and bridge PCR, where dsDNA is synthesized and bends in the library clusters designed to be sequenced (Chukrallah, et. al., 2020).

The RNA-seq libraries are a reference sequence composed of converted double-strand cDNA reads for nucleotide sequencing with designated read lengths that allow quantification, discovery, and profiling of RNAs. These libraries are used for bulk RNA-seq. The RNA-seq library was prepared by purifying mRNA from approximately 200ng of total RNA with oligo-dT beads and sheared by incubation at 94C in the presence of Mg (Kapa mRNA Hyper Prep Cat# KR1352). Succeeding first-strand synthesis with random primers, second strand synthesis and A-tailing were implemented with dUTP, which generated strand-specific sequencing libraries. Adapter ligation with 3’ dTMP overhangs were ligated to library insert fragments. Library amplification magnifies

fragments with the appropriate adapter sequences at both ends (strands marked with dUTP were not amplified). Indexed libraries that met appropriate cut-offs were quantified by qRT-PCR (KAPA Biosystems) and inserted size distribution was determined with the LabChip GX/Agilent Bioanalyzer. Samples with a yield of $\geq$0.5 ng/ul were used for sequencing. While bulk RNA-seq was used here, a comparable method with microarrays is possible, where fluorescent dyes attached to nucleotides are used during the synthesis reaction and then applied to spotted oligonucleotide arrays. Fluorescent emission is read by the scanner and used to infer gene expression levels.

In preparation for sequencing, sample concentrations were normalized to 1.2 nM and loaded onto an Illumina NovaSeq flow cell at a concentration yielding 25 million passing filter clusters per sample. Samples were sequenced using 100bp end sequencing on an Illumina NovaSeq6000 according to Illumina protocols. The 10bp dual index was read during additional sequencing reads. Data generated during sequencing runs were simultaneously transferred to the YCGA high-performance computing cluster. A positive control (prepared bacteriophage Phi X library) provided by Illumina was spiked into every lane at a concentration of 0.3%, monitoring sequencing quality in real time.

Once the raw biological output data from RNA-seq is obtained and transferred to FASTQ format (text-based format for nucleotide depiction) where it is normalized in various ways (e.g., read depth differences or normalize number of reads per sample and reads per gene since genes are different lengths and equal expression comparison is wanted), the computational genomics aspect of this study is done in R’s analysis software, described in the section below (Vladimir, 2019).

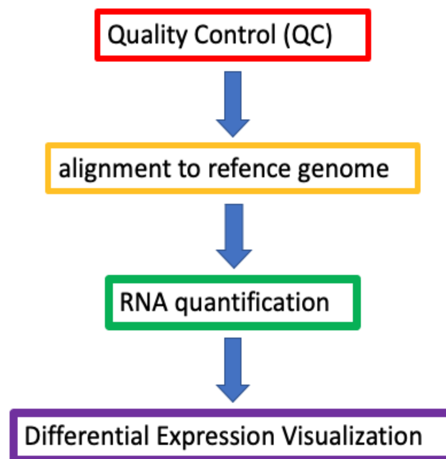
SECTION 7: COMPUTATIONAL GENOMICS ANALYSIS

In R, a computational genomics package designed for nucleic acid sequenced data analysis was utilized. Data processing underwent computational pipelines to downsize data, cluster expressions, and provide statistics for the biological RNA raw data.

Ensembl gene annotation techniques were used in data analysis pipelines. Trimming involved removing adaptors, duplicate sequences, and outliers. After trimming adapter content off of the raw fastq files, data was re-analyzed using fastqc to assess the quality of the samples. Reads were paired for each sample and then aligned to the Sus scrofa pig reference genome (HISAT2). HISAT2 is a rapid and sensitive alignment program for mapping sequence reads (both RNA and DNA) to a human genome reference population in addition to a single reference genome; the program was used as the reference genome in the original processes of this study. After alignment, the number of gene features were counted using the sus scrofa annotation file, then complement of gene counts into single count matrices for gene identification. STAR, a splice aware aligner, was used to align the reads to the genome, as well as HISAT2. Counts were condensed by summing technical replicate samples then lowly expressed genes were filtered out using a cutoff 3 CPM (counts per million) in minimum 3 samples being compared. The data was then normalized for library size differences and computed differential expression between controls and LPS.

From this data, many different quantified visuals and graphic analytics can be formed. Heat maps are used to visualize changes in expression between control and experimentally treated samples to depict increases or decreases in gene expression. Principal component analysis is used to see if any of the samples have different changes

in gene expression that may correlate and be related to the treatment conditions they underwent. Functional annotation of DEG (differentially expressed genes) is used to look at genes that have an increased expression, decreased expression, or remain the same and look to see if there are any similar pathways or processes, that might be involved in that pattern (such as metabolism, inflammation or immune response) reflecting gene expression change in treated/diseased groups. In this report we look at three particular figures: QC Report, MDS Plot, and Volcano Plot. Figure 1 below illustrates a simplified overview of the computational pipeline used in this study.



[Figure 1] Simplified Computational Pipeline

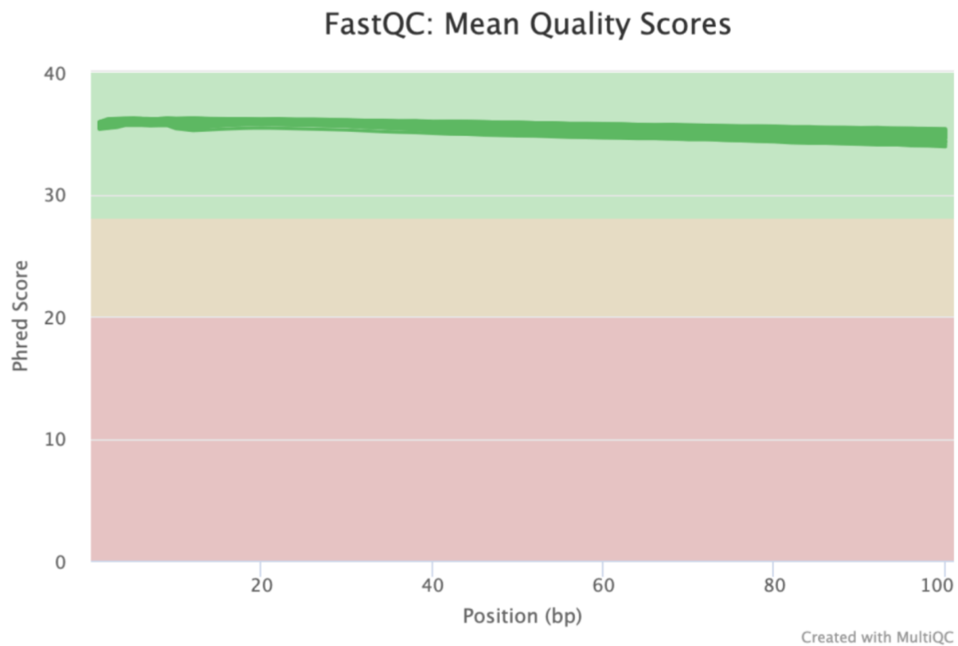
A QC report was used to show the quality for the data. The MDS plot was used to look for batch effects; despite four samples being re-sequenced, the MDS plot does not cluster by batch, suggesting that we can incorporate the resequenced samples into the analysis. MDS plots were also used to see if the samples cluster by group (control vs. LPS), where the plot does cluster by control vs. LPS; both outcomes represent good data. MDS plots were examined with CPM and logCPM to determine any clear outliers in the data. The volcano plots were used to illustrate and identify gene candidates for control vs LPS groups.

CHAPTER 3

RESULTS

SECTION 1: DATA AND MULTI-QC REPORT

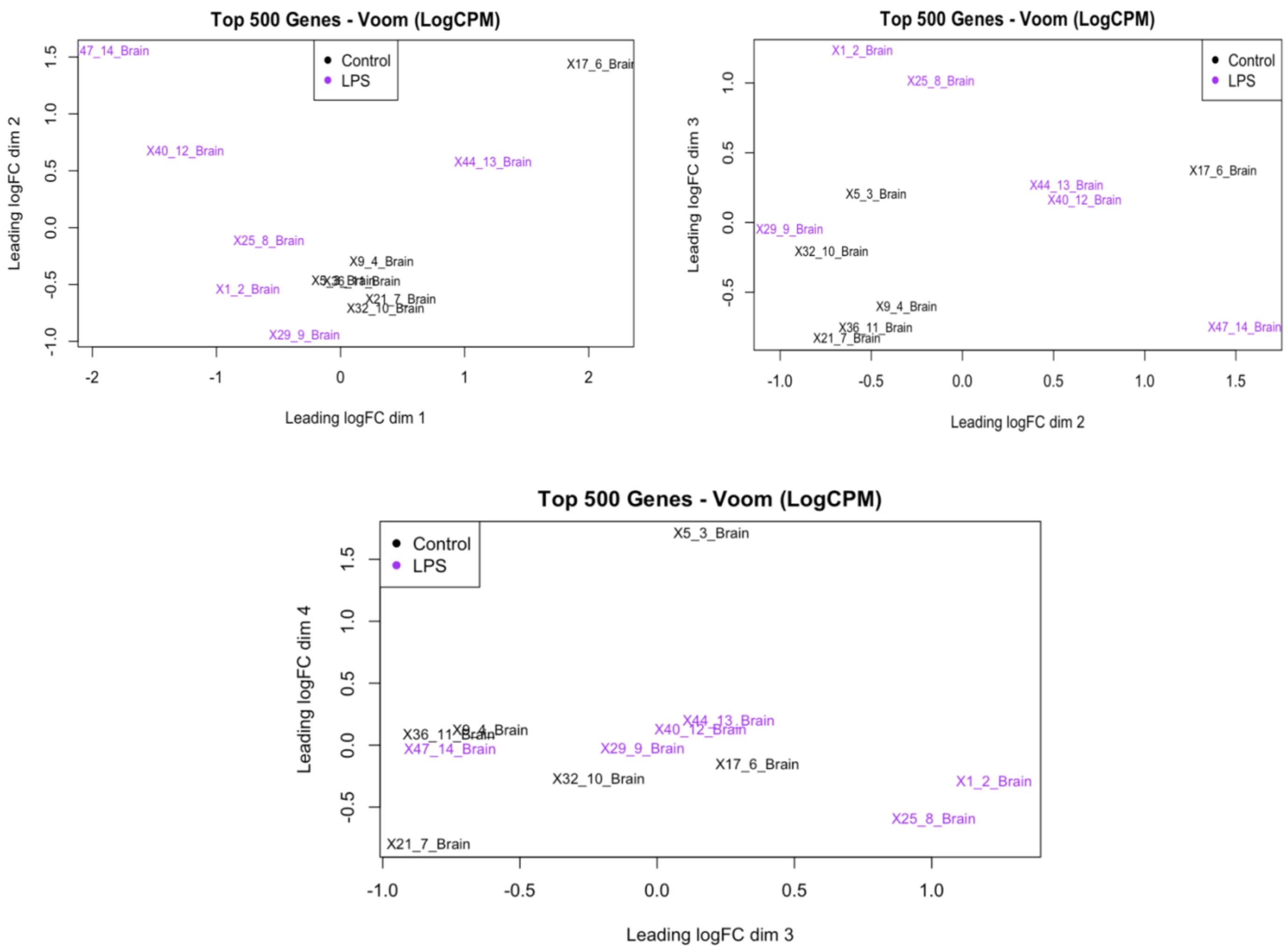
The QC report measures Phred Score (quality) vs position (first to hundred and first base pair). The data showed a steady quality score above 35 (40 indicating the potential miscall of one base pair per ten thousand on a log scale), for 29 samples. The high-quality report of this data is shown before trimming [see Figure 2].



[Figure 2] QC Report

SECTION 2: MDS PLOTS

MDS plots allow for multidimensional scaling (gene count matrices) with the visualization of similar individual cases in a data set. After being aligned to reference genome (HISAT2), the following MDS Plot figures show data after filtering and normalization (technical replicate batches and outliers removed, top 500 and 100 genes are illustrated, with N=11) [see Figure 3]



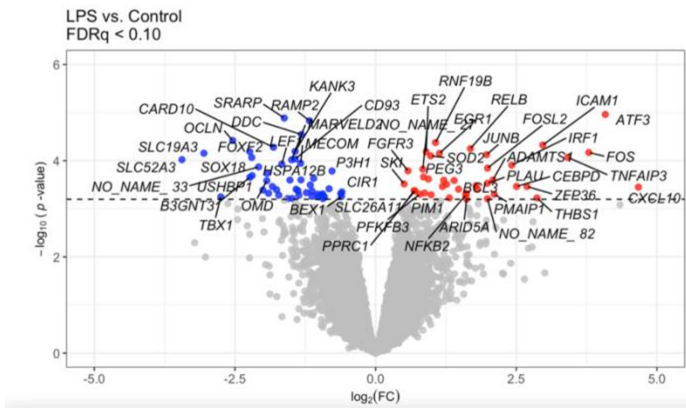
[Figure 3] MDS Plots

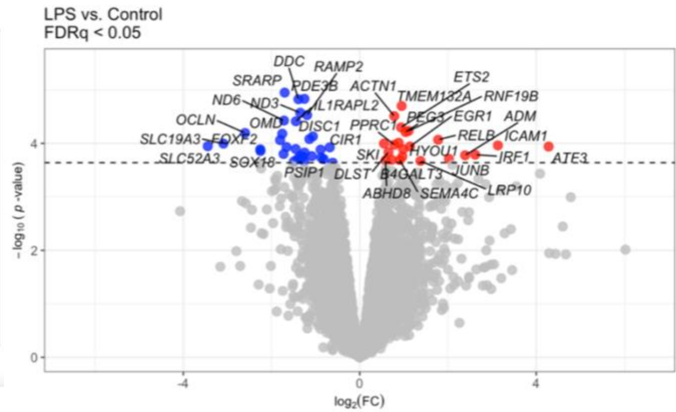
SECTION 3: VOLCANO PLOTS

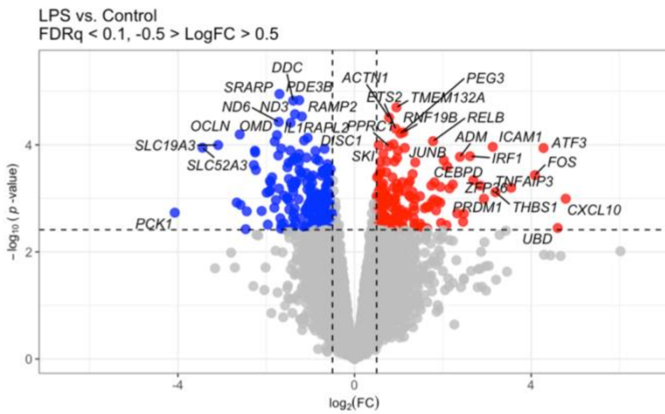
Volcano plots are characterized by their scatterplot format, showing statistical significance (p-value) on the y-axis against the magnitude of change (fold change) on the x-axis, allowing for the visualization and the identification of genes with large fold changes that are also statistically significant, allowing us to prioritize genes for validation and further experiments.

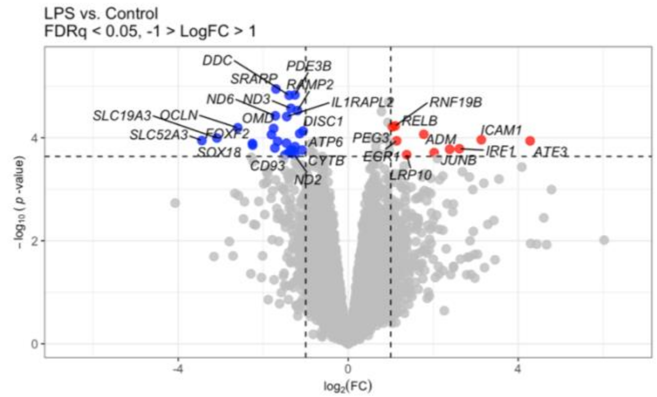
The volcano plots below show differentially expressed genes (DEGs). Each point on the plot represents a single gene. On the x-axis, the log2(FC) is plotted. A log2(FC) of 1 means that the gene shows twice as much expression in the LPS group compared to the control. On the y-axis the -log10(P-Value) is graphed. The DEGs cutoff is set to various constraints such as FDRq < 0.1 & $-0.2 > \log2(FC) > 0.2$. The dashed vertical and horizontal lines define this differential cutoff.

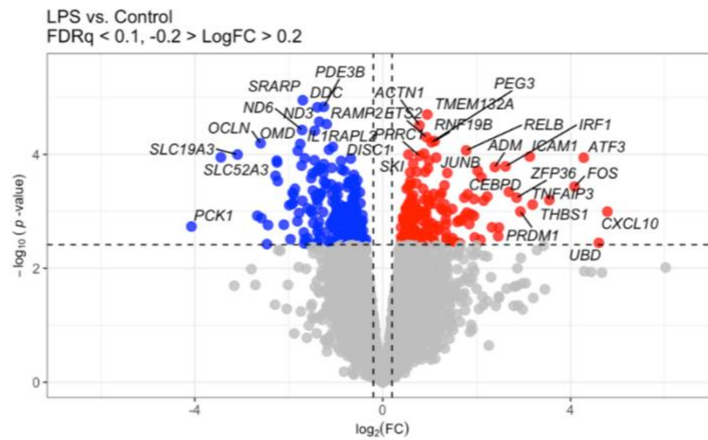
Red-colored genes on the right side of the figure (quadrant I) signify that the gene is upregulated in the LPS pig brains compared to the controls. Blue data points on the left of the volcano plot (quadrant II) represent genes that are down regulated in the brain of LPS pig brains compared to normal. Various cutoffs are included here as specific criteria will be established in the future and upcoming studies narrow the lens on biological significance [see Figure 4].









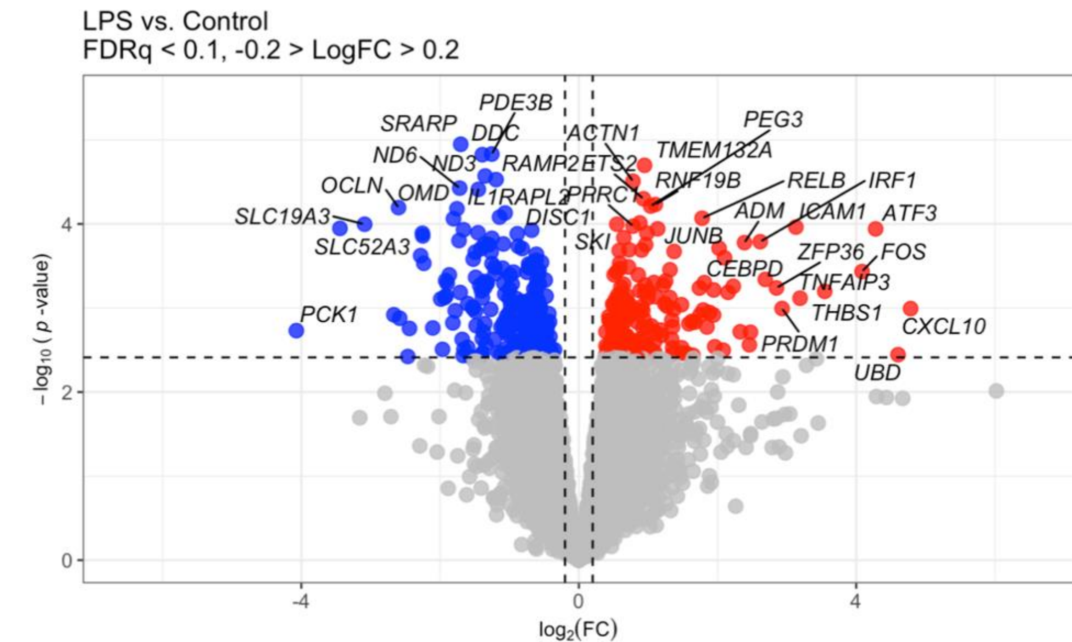


Note: The graph is the exact same when the logFC is 0

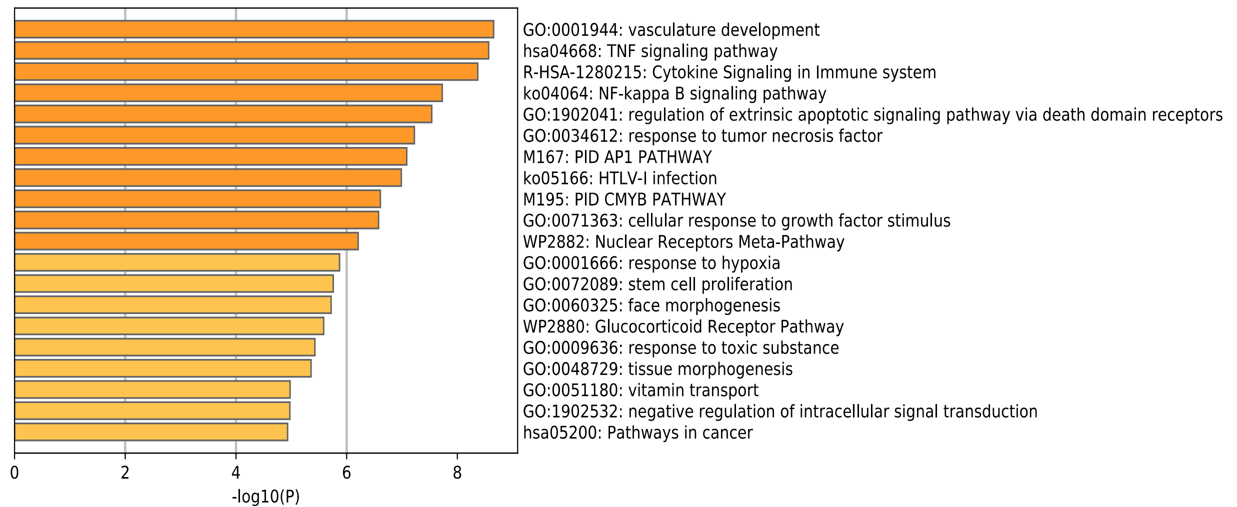
[Figure 4] Volcano Plots

SECTION 4: CELL POPULATIONS

The main volcano plot used for discussion is seen in Figure 5. Additionally, Figure 6 illustrates a Metascape visual. This data comes from a database of gene ontology referenced to known genes and pathways in the human genome. Significant, differentially expressed genes from our results were used to query this database, and the figure notes the overlap of what functions and enrichment pathways the majority of our significant, differentially expressed genes have. As illustrated in figure 6, most of these genes belong to pathways involved in vascular development and cytokine and inflammatory pathways, as predicted by our hypothesis which expected to see changes in vascular and immune pathways impacting the pig brain during sepsis.



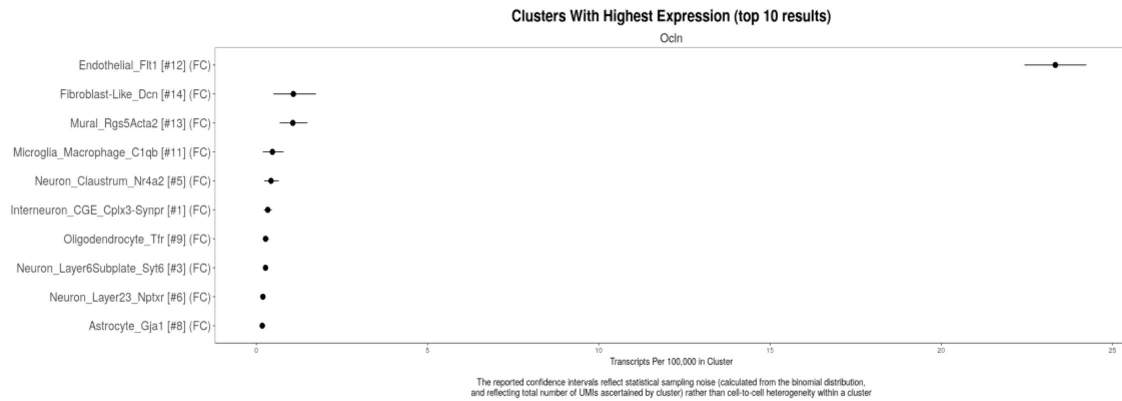
[Figure 5] Principal Volcano Plot

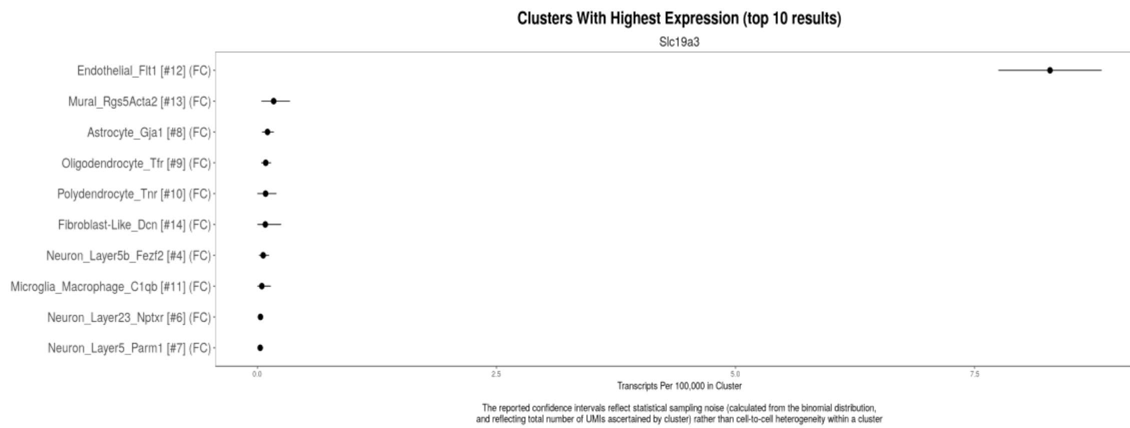


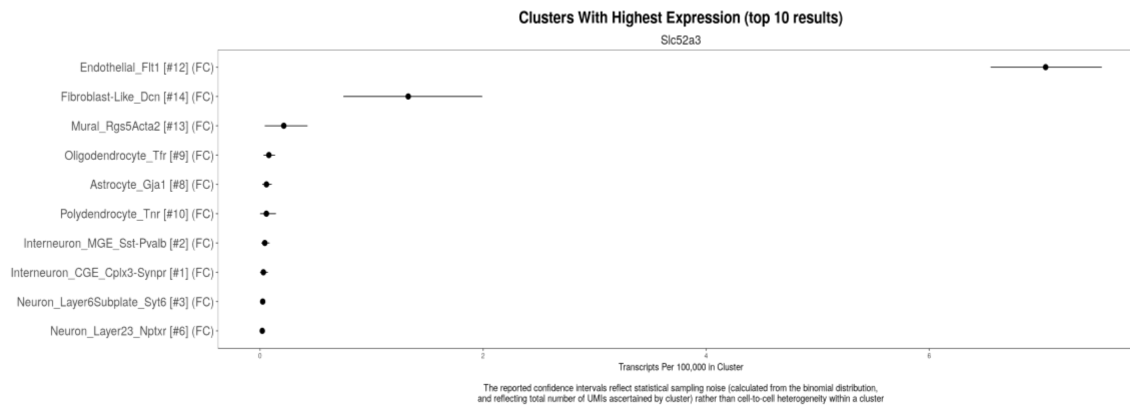
[Figure 6] Metascape Gene Enrichment Pathway Visual

Three significant genes to note that are down regulated in LPS induced sepsis pig brains include OCLN (occluding), SLC19A3, and SLC52A3. Occludin encodes a membrane protein required for cytokine induced regulation of tight junction permeability barriers; SLC19A3 is a thiamine transporter, and SLC52A3 is a riboflavin transporter. Figure 7 introduces another graphic from a single-cell RNAseq database for mouse brain cells. Here we note the same trend, where the significant down regulated genes are almost exclusively confined to endothelial cells (Sjöstedt, et. al., 2020).

Endothelial cells compose the lining of our body's blood vessels. They serve a variety of functions, but most significantly they are involved in bloodstream and surrounding tissue exchange, such as tight junctions in the blood brain barrier, and they are also impacted by chemokines; which is significant given that septic shock is defined by a dramatic decrease in blood pressure from a chemokine cascade increasing vascular permeability (Félétou, 2011).



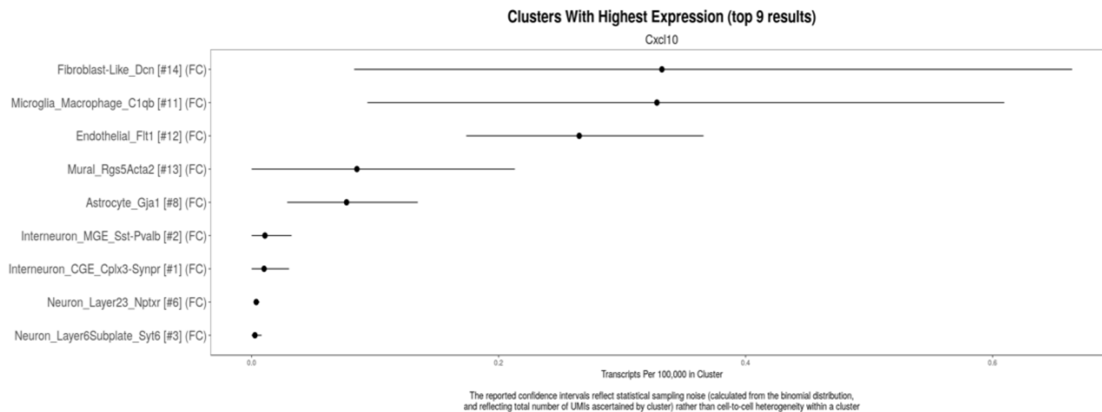


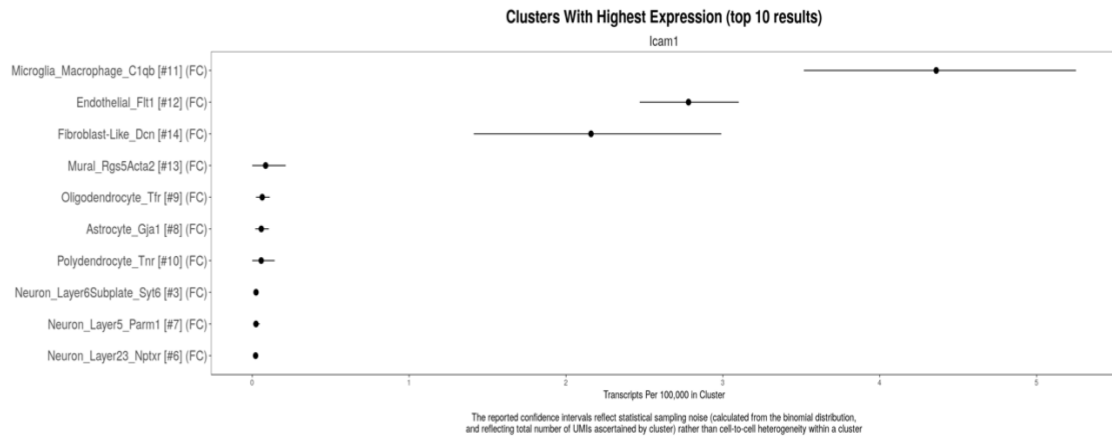


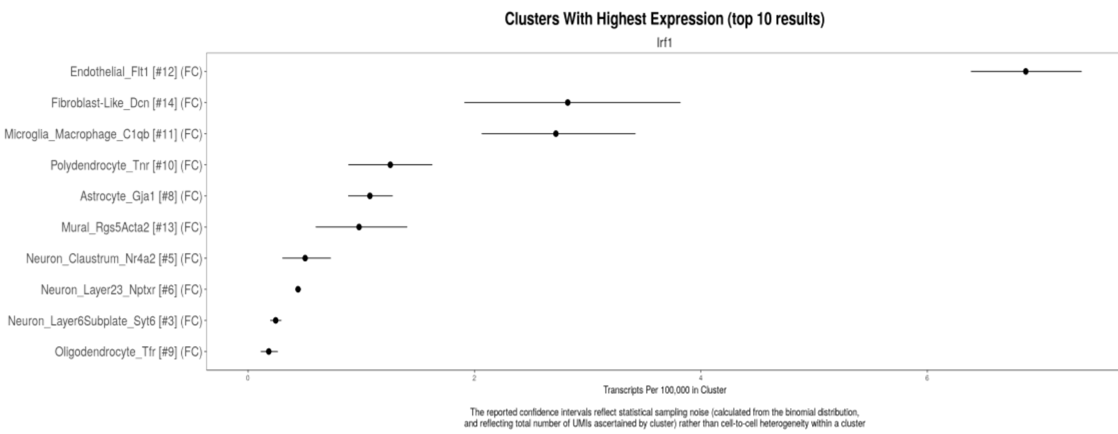
[Figure 7] Down Regulated Gene's Cell Population Expression Identification (in order: Occludin, SLC19A3, and SLC52A3, respectively)

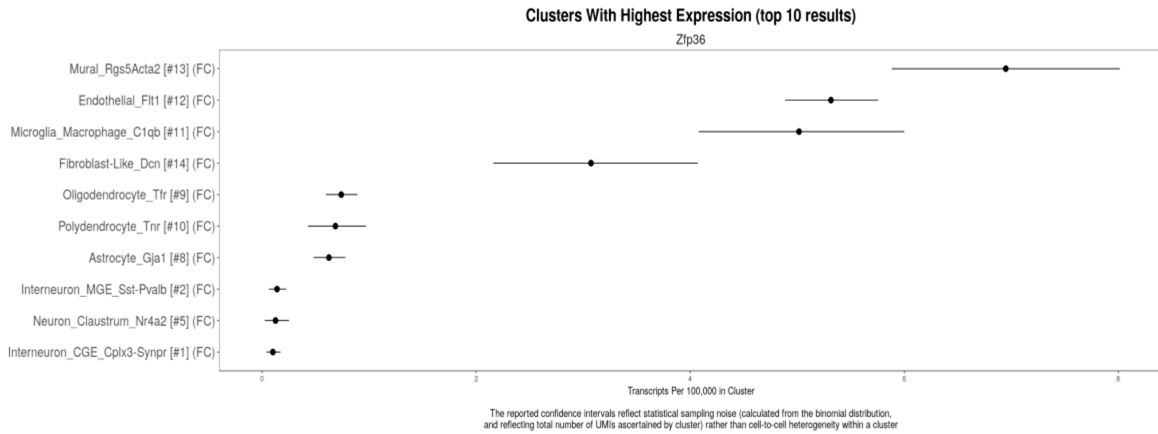
Four significant genes to note that are up regulated in LPS-induced sepsis pig brains include ICAM1, IRF1, CXCL10, and ZFP36. ICAM1 is a cell surface glycoprotein

typically expressed in endothelial immune cells (Bui, et. al., 2020). IRF1 is a transcriptional regulator involved in innate and acquired immune responses (Kuriakose, et. al., 2018). CXCL10 encodes a chemokine and corresponding receptor (Chevigne, et. al., 2021). ZFP36 is involved in inflammatory regulation (Zhang, et. al., 2019). We note that the expression of these genes are also usually found in endothelial cells, as well as mural cells (which are the smooth muscle cells of larger blood vessels), and microglia (the residence immune cells of the brain). This correlation again confirms our initial hypothesis of the vascular and inflammatory pathways involved in the early septic response. Figure 8 illustrates where these genes are normally expressed, at least in rodent brains (no single-cell studies have been performed in pig brains).









[Figure 8] Up Regulated Gene's Cell Population Expression Identification
(in order: CXCL10, ICAM1, IRF1, and ZFP36, respectively)

CHAPTER 4

DISCUSSION

With stringent threshold cutoffs, ten gene candidates upregulated in LPS brain samples include: RNF19B, PEG3, RELB, ICAM1, EGR1, ATF3, IRF1, JUNB, LRP10, and ADM. Of these, genes up-regulated in the LPS samples show enrichment for T-cell activation: EGR1 (early growth response 1), ICAM1 (intercellular adhesion molecule 1), IRF1 (interferon regulatory factor 1), and RELB (viral oncogene homolog).

A few prominent genes of interest commonly associated with immune system regulation are of notable importance. IRF-1 is involved in the initial response to viral and bacterial infection, as well as tumors (Taniguchi and Takaoka, 2002). It plays a role in the up-regulation of the innate (non-phagocytes: natural killer and mast cells, eosinophils, basophils, as well as the phagocytes: macrophages, neutrophils and dendritic cells), and adaptive (lymphocytic T and B cells) immune responses. ICAM-1 is typically up-regulated in an inflammatory state (e.g., due to TNF-a, IL-1, LPS), causing expression of the protein adhesion molecules (integrins) on immune cell membranes (Zoppo, et.al. 2006) AND (Zheng & Yenari, 2004)

Additionally, selectins respond to the same cytokines (TNF-a, IL-1, and LPS) and are up-regulated on the luminal surface of vascular endothelial cells. Together, integrins and selectins enable leukocytes to escape vasculature into damaged tissue, then proceed to follow chemokine signals to the site of injury/inflammation. The proteins that ICAM-1 upregulates can assist in this inflammatory response transportation at specific locations. EGR-1 is involved in memory formation (specifically long-term fear memory and anxiety), neuronal plasticity, synaptic signaling, and potentiation of the inflammatory

response (macrophage enhancement) (Sun et al. 2019), (Duclot and Kabbaj 2017), and (Trizzino et al. 2021). It is relevant to note that RELB and EGR1 are also circadian genes, with transcriptional expression regulated with an additional component interval timing. The intersection of the immune system and neurodegeneration with these genes show promise for future work to identify cascading mechanisms that could lead to the observed cognitive decline following sepsis.

These genes have robust expression in endothelial cells, which form the single cell layer that lines all blood vessels and regulate exchanges between the bloodstream and surrounding tissues. This is important in sepsis as endothelial cells are directly impacted in septic shock when body-wide vascular permeability reaches a critical threshold from the high levels or circulating chemokines and cytokines. Signals from endothelial cells also organize the growth and development of connective tissue cells which form the surrounding layers of the blood-vessel wall; such is seen in the permeable barrier for the blood brain barriers (leading to potential avenues to investigate SAE mechanisms) and other processes such as wound healing, angiogenesis, leukocyte trafficking, regulation of vascular tone, immunity inflammatory processes, diabetes, and cardiovascular diseases. This also possesses potentially exciting exploratory pathways to investigate the mechanism of other sepsis-related complications, in addition to cognition or delirium.

Additionally, with a less strict threshold, HYOU1 is up-regulated in the LPS samples. The HYOU1 protein has been reported to be up-regulated in tumors, and this gene belongs to the heat shock protein family. Previous research has shown up regulation of heat shock proteins during the septic process (Miliaraki et al. 2018). PEG3 also stood

out as an interesting gene. In humans, EG3 is thought to be involved in tumor suppressor activity and tumorigenesis in glioma and ovarian cells (Feng, et. al., 2008).

Overall, this expression pattern suggests endothelial cell dysfunction in the genes that are downregulated in the LPS group. The biggest fold changes are in endothelial cell marker genes, including: occludin, SLC52A3 (riboflavin transporter), and SLC19A3 (thiamine transporter). Microglial alterations might also have occured in the LPS group, given that they are the initial responders to changes in the brain and convey inflammatory and damage signals to other brain cells on the abluminal side of the BBB. This avenue poses a directed approach to further investigating SAE and pathways to cognitive decline during sepsis.

In total, 26 genes were observed to be up-regulated in the controls compared to the LPS samples [see Table 2 to the right], based off the cutoff of 3 CPM (counts per million), where a negative logFC indicates higher expression in LPS than the control and a positive logFC indicates higher expression in control than LPS. With a less strict threshold, the number of potential gene candidates broadens to 442 gene expression differences.

gene_name	adj.P.Val	logFC	expression
SRARP	0.046686462	1.701316225	up in control
PDE3B	0.046686462	1.25394509	up in control
DDC	0.046686462	1.389929762	up in control
ND3	0.046686462	1.349260494	up in control
RAMP2	0.046686462	1.192676192	up in control
ND6	0.046686462	1.716709667	up in control
IL1RAPL2	0.046686462	1.447555748	up in control
OCLN	0.046686462	2.600100425	up in control
DISC1	0.046686462	1.062692044	up in control
OMD	0.046686462	1.756450032	up in control
ATP6	0.046686462	1.141971745	up in control
CARD10	0.046686462	1.806088424	up in control
ND5	0.046686462	1.660644805	up in control
KANK3	0.046686462	1.452354559	up in control
SLC52A3	0.046686462	3.444597395	up in control
SLC19A3	0.046686462	3.090121888	up in control
ND4	0.047541341	1.252316183	up in control
LEF1	0.047541341	1.725979501	up in control
CYTB	0.047541341	1.08628823	up in control
COX1	0.047541341	1.296494117	up in control
MECOM	0.047541341	1.355460575	up in control
SOX18	0.047541341	2.249905033	up in control
CD93	0.047541341	1.411026209	up in control
ND2	0.047541341	1.263864208	up in control
MARVELD2	0.047541341	1.491688911	up in control
FOXF2	0.046686462	2.255071527	up in control
RNF19B	0.046686462	-1.104425789	up in LPS
PEG3	0.046686462	-1.037603139	up in LPS
RELB	0.046686462	-1.776826063	up in LPS
ICAM1	0.046686462	-3.130910485	up in LPS
EGR1	0.046686462	-1.138367694	up in LPS
IRF1	0.047541341	-2.615321576	up in LPS
ATF3	0.046686462	-4.279432396	up in LPS
JUNB	0.047541341	-2.020746503	up in LPS
LRP10	0.047541341	-1.37480402	up in LPS
ADM	0.047541341	-2.387074628	up in LPS

[Table 2] Differentially Expressed Genes

CHAPTER 5

CONCLUSION

This experiment began with the question regarding which genes and pathways are altered in the brain during sepsis. As described above, specific gene candidates were identified and will be used as targets for future studies regarding sepsis pathways, molecular mechanisms, biomarkers, and expression profiles across organ systems, with an aim to develop therapeutics to prevent CNS damage. We noted significant expression changes in LPS induced sepsis, with emphasis on vascular and immune pathways, when compared to control pig brain tissue.

Questions for future research include several factors that are still under consideration and the direction/specifics of future studies soon to come. For example: Which organs should be sampled? What are the time points (since wide-ranging transcriptional changes in leukocytes transpire within a few hours of an inflammatory molecule introduction)? What other symptoms and vitals should be monitored? Is there a lack of clinical application due to the fact that <40% of clinical diagnosis cases have negative bacterial cultures (LPS present)?

The limitations and potential pitfalls of this experiment include temporality, since patients are admitted to the ICU at various stages of sepsis and most gene expression studies have only looked at one time point. As well, this experiment involved healthy, young swine, without antibiotic treatment, which also lacked comorbidities commonly associated with human septic patients. Finally, only a single agent (LPS) was administered and there is difficulty measuring the standardized sepsis diagnostic criteria SOFA as well as changes in the pig brain from observation (i.e., pig mental status).

The significance of this experiment pertains to the potential avenues of biomedical intervention following sepsis diagnosis, where these identified gene candidates could lead to novel therapeutic targets to counteract the changes in the CNS that can lead to cognitive and behavioral impairments.

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