Investigation of Spinal Cord Injury-Induced Gastrointestinal Dysfunction and Related Microbiota, Fungal, and Intestinal Alterations in a Rat Model and Humans with Spinal Cord Injury

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INVESTIGATION OF SPINAL CORD INJURY-INDUCED GASTROINTESTINAL DYSFUNCTION AND RELATED MICROBIOTA, FUNGAL, AND INTESTINAL ALTERATIONS IN A RAT MODEL AND HUMANS WITH SPINAL CORD INJURY

By

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INVESTIGATION OF SPINAL CORD INJURY-INDUCED GASTROINTESTINAL DYSFUNCTION AND RELATED MICROBIOTA, FUNGAL, AND INTESTINAL ALTERATIONS IN A RAT MODEL AND HUMANS WITH SPINAL CORD INJURY

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Spinal cord injury (SCI) is a serious medical condition that can result in a spectrum of neurological impairments and physical disabilities. There is an average of 11-12,000 new injuries per year in the US alone, and no cure for either the paralysis, or other secondary effects. Although there have been significant strides made in the research of the pathophysiology of Spinal Cord Injury (SCI), there is still limited knowledge on the consequences of SCI in remote organs. SCI produces significant effects on the entire body including the gastrointestinal tract. Individuals with SCI often experience severe, debilitating bowel dysfunction in addition to their physical disabilities, which is of major concern due to the adverse effect on their quality of life. The aim of the studies reported in this thesis was to further understand the Gastrointestinal (GI) dysfunction in individuals with SCI and to start to identify the role and relationship of the gut microbiota, inflammation, fungi, and their associated signaling and small molecules.

This project was developed based on the hypothesis that SCI causes both local and system inflammatory states which persist long-term, resulting in sustained disturbance of gut microbiota and fungi. This dysbiosis would include altered signaling and secretion of
small molecules evidenced by a broad range of GI dysfunction and potentially contributing to other systemic dysfunctions such as autonomic and immune dysfunctions. The gut microbiome is now accepted as a determinant of human health and as such an area of intense research, especially in chronic diseases of the GI tract. However, so far there have been very limited research reports on bowel dysfunction in patients with SCI. In fact, only 1 study has been done in humans reporting alterations in gut microbial patterns in stool samples of adult chronic SCI patients. Further, another study has examined the effect of gut dysbiosis in a mouse model of SCI but only as it pertains to functional recovery of the cord injury, not systemic or secondary organ effects.

In this thesis, by employing a SCI rat model of cervical and thoracic injury we examined the effects of SCI on gastrointestinal transit, permeability, and integrity, pro-inflammatory cytokines, and bacterial quorum sensing communication to characterize the state of the GI tract in an animal model. Then, preliminary results of a human study allowed for comparison with microbiota changes, analysis of inflammation, short chain fatty acids, and bacterial quorum sensing combined with fungal species. These data provide a greater understanding of the effects of SCI on the gastrointestinal tract, highlighting the need for further investigation to elucidate the mechanism underlying these effects. Further, this study demonstrates proof of concept of ability to reliably characterize GI dysfunction on multiple levels in individuals with SCI on a large scale by the creation of a biorepository of physiological samples from these patients, with the goal to understand the mechanism of the GI problems and explore non invasive microbiome-based interventions in this population.
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Chapter 1. INTRODUCTION

1.1 Spinal Cord Injury

Definition of Traumatic Spinal Cord Injury

Traumatic spinal cord injury (SCI) is a serious medical condition which can lead to lifelong disability and currently has no cure. Traumatic SCI is usually caused by a cut or blow to the spine (usually a car crash or gunshot) compared to non-traumatic SCI, which is any spinal damage not caused by a trauma (infection, degeneration etc.). According to the National Spinal Cord Injury Statistical Center, there are currently an estimated 243,000-347,000 individuals living with traumatic SCI in the United States alone, with that number increasing by at least 10,000 each year. The US also has the highest incident rate for traumatic spinal cord injury in the world at 54 cases per million. The average age at injury has increased to 42 and non-Hispanic white males are the most commonly affected demographic. This population constitutes a significant financial burden to the patients, their families and caregivers, and the medical community as lifetime costs of the injury can exceed 4 million dollars per person. A spinal cord injury can be complete or incomplete and can occur at any level of the spine. A complete injury is one in which the entire spinal cord is severed, and no axons cross the lesion site whereas in incomplete injury some axons are spared. The spinal cord is a long bundle of nervous tissue and cells that start at the medulla oblongata (brain stem) to the lumbar region of the vertebral column. It is comprised of 31 segments; 8 cervical segments, 12 thoracic segments, 5 lumbar segments, 5 sacral segments, and 1 coccygeal segment which is mainly vestigial. More than half of all SCIs are cervical in location and incomplete, thus not all neurological communication is cut off below the lesion. The difference in level of injury usually
corresponds to a difference in symptoms. A cervical SCI can cause tetraplegia in addition to difficulty breathing and loss of other autonomic functions like body temperature, heart rate, and blood pressure\(^1\). A spinal cord injury at the thoracic level and below may result in loss of control of bowel and bladder, sexual dysfunction, and locomotor dysfunction from the waist down.

Identifying the phases of a spinal cord injury is crucial toward understanding what happens in each phase and improving recovery. SCI affects the whole body on a cellular level via many different processes at least 25 of which have been identified through extensive scientific research. Knowledge of these stages and processes primarily helps researchers to identify targets for intervention strategies. Generally speaking, SCI is best described as having 2 main phases, a primary injury phase, which consists of the mechanical injury itself and lesion formation, and a secondary injury phase comprised of all events triggered in the primary injury phase. The injury can further be broken down into 5 stages, from primary immediate to chronic, each with unique pathological events, challenges, and therapeutic strategies.

The first phase of SCI is the primary immediate phase, which constitutes the first 2 hours after injury and represents the primary injury phase. The most common form of SCI is a compressive or contusive injury caused by a physical shearing, laceration, stretching, or penetration of the spinal cord. All of the above mechanisms result in traumatic severing of axons and the site of the injury leading to immediate death of neurons and neuroglia as well as spinal shock. Within the first 2 hours pathological changes can be observed such as swelling and hemorrhaging at the site of injury causing immediate necrosis due to mechanical disruption of normal membrane and vascular structures \(^2\). Interestingly, the
overall gross histopathological changes are not usually visible at this point except in the case of extremely severe compressive injury or complete severing of the cord. Nevertheless, cellular response to the injury begins within minutes including drastically increased secretion of pro-inflammatory cytokines TNF-α and IL-1β, activation of microglial cells, and cytotoxic levels of extracellular glutamate.

There are four phases of secondary injury. The first is the early acute phase that takes place less than 48 hours post-injury. This is the phase wherein the secondary effects become dominant and severity of the injury becomes more apparent and is therefore the phase targeted for many neuroprotective interventions. The next phase involves the secondary processes that are initiated beyond the hemorrhaging, swelling, and inflammation. Calcium ion, Ca$^{2+}$, dysregulation is common at this point and induces further cell death through multiple cascade pathways, including the activation of calpain and free radical production. Lipid peroxidation is an even more destructive free radical reaction after SCI as it is self-perpetuating and causes membrane damage through increase in reactive oxygen species (ROS) levels peaking at 12 hours post-injury. These high ROS levels can take longer than a month to return to normal and are associated with both immediate ischemia and the following reperfusion. The importance of ROS in the pathophysiology of SCI is further evidenced by the neuroprotective effects of antioxidant drugs such as tempol. The field of neuroinflammation research in SCI has grown drastically and begun to uncover some of the intricacies of the process and to understand which aspects of the inflammatory response exacerbate the injury and which ones provide a regenerative environment. In the early acute phase, the resident neuroglia are still active and inflammatory cells are in peak infiltration mode, releasing cytokines. An excellent example
of the Janus nature of the inflammation process in SCI is found in the multiple roles of TNFα. Inhibition of TNFα has been shown to improve functional neurological recovery after CNS injury. However, TNFα signaling has a demonstrated neuroprotective effect. TNFα and IL-1β are also known to have a significant effect on vascular permeability and are believed to be the major contributors to the detrimental increase in blood brain barrier (BBB) permeability seen in the acute phase of SCI [4]. Both necrosis and apoptosis are mechanisms of cell death seen in traumatic SCI. Neuron death usually occurs by necrosis where as more ischemia-sensitive cells like oligodendrocytes undergo apoptosis. A hallmark of SCI is demyelination of axons around the site of injury which can hinder functional recovery [5]. Remyelination is possible but can be best accomplished by mature oligodendrocytes which are sensitive and undergo apoptosis early in the injury, so other mechanisms of remyelination are a topic of research interest. The third is the subacute phase occurring between 2 hours to 2 weeks after injury, where cell-based therapies are being explored. During this phase the astrocytic “scar” forms at the lesion site due to astrocytes at the periphery becoming unusually energetic and proliferate increasing filament protein production to form multiple large interwoven processes with microglia. Glial scars can be both beneficial and detrimental to healing. The presence of the scar is a physical and chemical barrier that prevents axon regeneration and may be a therapeutic target, but consequences of inhibiting astrocytic scaring in SCI has not yet been studied. On the bright side, phagocytic activity is at a maximum during the subacute period which helps to remove cell debris from the injury and promote axonal growth. In the intermediate phase, from 2 weeks to 6 months, the astrocytic scar continues to develop and sprouts axons, which is encouraging evidence of regeneration in severe SCI despite no functional
significance. The last and chronic phase of SCI is 6 months or more after the injury. During the first 1-2 years of the chronic phase, the scar has fully matured and maximum functional recovery is achieved. In up to 30% of patients the lesion can change causing delayed neurological dysfunction and further paralysis. Therapeutic strategies at this stage are geared toward promoting plasticity and improving the function of the demyelinated and disrupted axon.

Measures of severity and treatment

The pathophysiology of traumatic spinal cord injuries remains complex and heterogeneous so appropriate classification of injury severity (completeness) is important for maximum recovery. The current gold standard is the American Spinal Injury Association international standards for neurological classification of SCI (ISNCSCI) developed in 2006 based on the Frankel scale. To classify a spinal cord injury using these standards requires detailed process including determination of sensory, and motor levels for both sides of the body, analysis of neurological level of injury, and completeness of the injury. All this information is then combined to determine ASIA (American Spinal Injury Association) impairment score which medical professionals can use as an indication of severity. Using this method, impairment is scored on a 5-point ordinal scale from A to E, where A indicates no motor or sensory function in the lowest spinal segment (S4-S5), and E indicates that sensory and motor function are normal (Figure 1.1.1).
This analysis is very reliable and valid with a high correlation to other scales that assess neurological, and physical impairment. ASIA scoring also identifies whether the injury is complete (no signals can pass the site of injury) or incomplete (some signals can still be transmitted). In addition to an ASIA impairment score, it is important to also note the spinal level of the lesion and individuals with SCI are usually identified as paraplegic or quadriplegic. Quadriplegia results from injuries at T1 and above and affects all 4 limbs.
and in severe cases, basic autonomic processes such as breathing. Paraplegia results from injuries below T1 and patients experience varying levels of paralysis below the waist.\(^8\)

Spinal cord injury research is extraordinarily complex even in an animal model. The current overall goal of acute medical care of an SCI is primarily to prevent the injury from getting worse. After physical stabilization of the spine and initial analysis of the extent of the injury, the next step is to control inflammation. For pharmacological control of inflammation in SCI there have been many clinical trials, but none have proven effective enough to be recommended as the standard of care. For example, until 2013, intravenous (IV) administration of the steroid MPSS (methylprednisolone sodium succinate) was the recommended treatment in the acute stage of SCI, however, in the past decade it has become very controversial.\(^9\) In 2002, the American Association of Neurological Surgeons and the Congress of Neurological Surgeons (AANS/CNS) recommended IV MPSS for either 24 or 48 hours as the standard of treatment for the management of acute SCI. However, in 2013 this portion of the same guidelines was updated to state that administration of MP for the treatment of acute SCI is not recommended. This change in recommendation was due to the lack of evidence for any remarkable benefit in multiple prospective randomized studies, as well as the significant negative side effects that it can cause, including wound, respiratory, and other infections resulting in longer hospitalization, increased chance of surgery, and higher instance of death due to infection.\(^10\) Nevertheless, decrease of inflammation remains a high priority and at the physician’s discretion and patient autonomy, MPSS as well as minocycline, and erythropoietin, are currently used for this purpose.
In the initial stages of a spinal cord injury, a major goal is to decrease the extent of secondary damage to catalyze functional recovery. Surgery is another intervention that has shown potential for treatment of SCI with its own controversies concerning the role and timing of use. Surgical decompression and stabilization are accepted as valid options for treatment of SCI based on class II clinical evidence of their safety and efficacy. Timing and choice of surgery have been shown to be the biggest determinants of effectiveness of the treatment and no consensus has yet been reached on either \(^{11}\). In the case of a compressive injury, the spinal cord is deformed or shifted frequently causing significant pressure or pinching of the spinal nerves. Decompression surgery is the opening or removal of the body portion of the spine to relieve this pressure so that the nerves are no longer being crushed. Clinical studies have indicated that decompression surgery can improve functional recovery and decrease hospitalization time. Most studies have given evidence that surgery within 24 hours of injury is most beneficial to function and neurological outcome, but no standard of care have been established regarding timing \(^{12}\). Contusive injury to the spinal cord is one in which the structures are disrupted usually because of a penetrating injury. In the case of a contusive SCI stabilization surgery can be used to remove any bone fragments and realign the spinal cord vertebrae. Spinal fusion is also another type of surgery that is used in the treatment of SCI to stabilize the spine at the injury site \(^{13}\).

Rehabilitation is the only mandatory and longest lasting step in the treatment process for SCI. Early rehabilitation is crucial to prevent potential complications, such as loss of muscle strength and bone density and joint contractures, as well as to ensure normal function of the respiratory and digestive systems. Consistent passive exercises and
stretching are recommended in the acute and subacute stages of SCI with special attention to positioning of the joints to prevent rigidity and maintain maximum strength of upper extremities. The intermediate phase usually marks the transition out of bed for patients with SCI, and thus, rehabilitation during this stage focuses on facilitating this process. Strengthening exercises are continued in addition to balance and mobility training based on ability to sit-up. During the chronic rehabilitation phase the most important goal is to achieve independent mobilization for the patient and for them to return home and be able to resume life.

The goal in treatment of spinal cord injury for decades has been to attain significant recovery of function to the individual, yet MPSS, surgery, and rehabilitation have not proven sufficient to meet this goal. Therapeutic hypothermia has been previously researched in other types of CNS injury including traumatic brain injury and hemorrhagic stroke so as early as 1967 scientists began experimenting with localized cooling of a spinal cord injury in animal models. By 1993, researchers at Parkwood hospital were inducing mild systemic hypothermia in patients with compressive or contusive SCI. Experimental and clinical studies have continued to advance, identifying the specific mechanisms by which hypothermia improves function after SCI such as increased preservation of white and grey matter, increased survival of neurons and axons, and decrease in oxidative stress and inflammatory cell activity. The effects on these mechanisms resulted in improved locomotion and decreased tissue damage in animal models. Most recently a comprehensive, multicenter phase II/III clinical trial termed the ARCTIC (Acute Rapid Cooling for Traumatic Injuries of the Cord) trial has been designed to flesh out more
specifics on the efficacy and safety of using moderate induced hypothermia to improve the ASIA score in patients with SCI 16.

Despite the progress in SCI research the cure for paralysis has not yet been realized. Therefore, a considerable amount of research energy has been directed specifically at improving function after SCI through cell-based therapies aimed at regeneration. MSCs (mesenchymal stem cells) have recently come into the spotlight as a promising source for cellular repair after SCI as they are self-renewing multipotent progenitor cells and have the capacity to differentiate into multiple unique lineages. They are easy to isolate, preserve, differentiate, and have low immunoreactivity. Bone marrow and umbilical cord blood are enriched sources of MSCs so they are also accessible 17. In 2005, Park and colleagues demonstrated significant motor improvements in humans given autologous bone marrow cell transplant along with a macrophage stimulating factor showing the therapeutic efficacy of the treatment 18. Now, at least 7 phase I, II, III clinical trials are active around the world using MSCs or bone marrow stem cells in chronic and acute SCI. The goal of using stem cells in SCI is to prevent apoptosis of the cells, regenerate new axons and remyelinate spared ones by replenishing lost populations such as oligodendrocytes. These clinical trials will help to establish safety and efficacy as well as help address some remaining challenges, such as ethical and regulatory concerns, modes of delivery and transplantation, and the most efficient time frames for intervention. Each traumatic SCI is unique but can cause truly life-altering effects for the person and their family which are immediate and can be life-long. Major consequences of SCI include permanent loss of strength, sensation, and function below the injury site.
Secondary Organ and Systemic Dysfunction

Unfortunately, in addition to direct effects, the injury also leads to a host of secondary effects, which can begin manifesting as soon as 1 hour after the injury and can last the life of the patient. These secondary interrelated side effects are broad and vary in severity from patient to patient but constitute a major factor in the medical care of persons with SCI. As an example, about 30% of people with traumatic SCI end-up being re-hospitalized following injury. This hospitalization lasts an average of 3 weeks and is usually caused by a secondary condition, not the initial lesion. Further, the leading causes of death of people with SCI are pneumonia and septicemia, which both pertain to immune suppression in the body, not the injury itself. Since the spinal cord plays a crucial role in coordinating body-wide events, an injury to the spinal cord can cause failure or dysfunction to multiple organs in addition to disruption of the autonomic nervous system. These serious secondary consequences include, systemic inflammation, immune suppression, and pulmonary, metabolic, autonomic, urinary, and gastrointestinal dysfunctions. Each has its own associated risks but more importantly nearly all these affects quality of life for the injured individual, as well as their families and caretakers.

In addition to inflammation at the site of injury, SCI can also cause systemic inflammation and epidemiological studies have linked this inflammation to an increase in post-injury complications namely SIRS (Systemic Inflammatory Response Syndrome). It is hypothesized that SCI propagates chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis thereby encouraging the inflammation through changes in neuroendocrine regulation and neuroimmune cells of the central nervous system \(^{19}\). Although seemingly counter-intuitive, SCI can also cause immune depression syndrome
SCI-IDS), which has been shown to decrease both motor function and neurological outcome. The exact mechanism of SCI-IDS is unknown, but it is identified by a decrease in circulating immune cell types including T and B lymphocytes and monocytes\(^4\).

Pulmonary complications are common, especially pneumonia, and represent a significant cause of mortality for patients with SCI. Usually increasing in severity with higher levels of injury, SCI can cause respiratory muscle weakness, decreased lung volume, and ineffective cough and the best management for these complications is breathing exercises and posture adjustments\(^20\).

Metabolic dysfunction in individuals with SCI is not fully characterized but is usually related to changes in mass distribution and liver function. Altered fat distribution and infiltration into intramuscular and visceral sites along with abnormal metabolic profiles are thought to be the cause of the high prevalence of type II diabetes and cardiovascular dysfunction in people with SCI\(^21\).

Dysfunction of the nervous system is the most logical after an injury to the spinal cord, but due to complexities and variation in severity, it is still not well understood. Autonomic Dysreflexia is the most common and a life-threatening complication of autonomic nervous system dysfunction which can occur after SCI and is considered a medical emergency\(^22\). After SCI, the balance of the sympathetic and parasympathetic signals is disrupted, especially if the lesion is above T6. Put simply, when the descending parasympathetic signals are not balanced by ascending sympathetic signals the result is a sudden and uncontrollable change in heart rate and increase in blood pressure which is fatal if not immediately treated. Dysfunction of the autonomic nervous system in SCI is also thought to be associated with immune dysfunction as a decrease in spleen size and function
has been shown to accompany increased risk of infection especially in high thoracic injuries.

Patients with SCI are at an elevated risk for urinary tract infections (UTI, average of 1.82 episodes per year) because the injury usually alters the dynamics of voiding, a dysfunction termed neurogenic bladder. Bladder function can vary in individuals with SCI based on the level of the injury so indwelling urethral catheters are used in almost all cases for some amount of time. In common animal models of SCI, UTI’s are also a problem with up to 100% of animals showing some signs of infections after surgery. There is significant research to be done in this area since a different pattern of neurogenic bladder requires unique management, but the current standard is administration of a course of antibiotics. However, it is now scientifically accepted that antibiotic treatment can also contribute to the occurrence of UTI and this is also the case in spinal cord injury.

Similar as in the urinary system, SCI is hypothesized to alter the way the gastrointestinal (GI) tract works resulting in symptoms such as abdominal pain, constipation, bloating, difficulty emptying, incontinence, and increased colonic transport. This GI dysfunction, termed neurogenic bowel, is common and occurs in up to 60% of patients. The symptoms can vary greatly in severity, and unlike neurogenic bladder, they are less level dependent making a standard of care even more difficult to achieve. To accompany the range in severity is an equally broad range of bowel management programs ranging from use of suppositories to surgical stimulation of the sacral nerve. Non-invasive interventions for bowel management in patients with SCI are only successful in 67% of those with symptoms. Many treatments for symptoms such as dietary fiber for constipation, are contraindicated and ineffective in these populations. Understandably, GI dysfunction
causes a great burden to patients with SCI and effects their quality of life more than sexual or bladder dysfunction. Bowel management can take a patient with spinal cord injury hours, which seriously hinders their independence and confidence. On top of this, very little research has investigated the mechanisms and extent of GI dysfunction in SCI, let alone potential interventions. Only recently has this been a topic of active research due to increasing attention on the gut microbiome. GI dysfunction is very common in patients with SCI. The 2 main types of neurogenic bowel and upper motor neuron (UMN) bowel syndrome and lower motor neuron (LMN) bowel syndrome. Neurogenic bowel in SCI can also be classified into 3 patterns but only for patients with a complete injury. Pattern A describes any patients with an injury above T7 who do not have voluntary control of their abdominal muscles, but maintain sacral reflexes. Patients with SCI below T7 with control of abdominal muscles and preserves sacral reflexes form pattern B and those without preserved sacral reflex, pattern C. While this is a useful classification for management, it does not include incomplete injuries and no research has yet been done to determine any mechanisms for these patterns.

In the past spinal cord injury was an injury not to be treated even once scientific accomplishments had reached a point where patients with SCI could be treated operatively or non-operatively and survive past the initial acute injury with a stabilized spinal column. This attitude continued into the early 1900’s when Dr. Donald Munro took the unique approach of being fully responsible for his patients. He embodied a commitment to be more than just a neurosurgeon to provide for these patients who had problems with multiple organ systems not just neurological. He incorporated into his practice coordinated rehabilitation efforts directed at improving self-care, mobility, and assimilation into
society, and educational pursuits. Several other physicians followed in the footsteps of Munro, advocating for all the needs of the patient even those outside their specialty but up until the 90’s, most of the medical community believe SCI was not to be treated. In the last 30 years, spinal cord injury research has finally evolved to not only increase our understanding of injuries to the spinal nerves, but also begin to include comprehensive care for maximum overall quality of life for the patient. \(^{30}\) Now, the major funding source in the US, the Department of Defense, encourages grant submissions on the full scope of interrelated dysfunctions such as sexual, bladder, bowel, autonomic, sensory, and respiratory as well as a range of rehabilitation related interventions. SCI is being increasingly treated as a disease affecting the whole body and the medical and research communities are starting to prioritize Quality-of-Life (QoL) for the patients. Most individuals who experience a spinal cord injury also experience some level of GI dysfunction. Despite extensive clinical documentation there is inconsistent success and no standard of care for these issues. Further complications of Gastrointestinal dysfunction can be very dangerous and even deadly, often resulting in the need of a colostomy bag or surgeries. Now research is underway to get answers with respect to the scope and characterization of this dysfunction to develop interventions with the potential to vastly improve quality of life for patients with SCI.

1.2 The Gut Microbiome

History

The human microbiome is defined as all the bacterial genes that make up the microbiota living on and inside a human being. The gut microbiota constitutes the gut
microbiome, which resides in the gastrointestinal tract and is the largest and most diverse group of bacteria in the body. In fact, the number of bacteria in the gut outnumber the sum of all human cells in the body by ten to one. For clarity, “gut microbiota” refers to the bacteria themselves, and “gut microbiome” technically refers to the genetic components which make up these bacteria. In this body of work these two terms will be used interchangeably as the research method used to detect the presence of these bacteria is based on genetic sequence 31. These bacteria are now being heavily researched for their influential capabilities in health and disease. We now know that the human gut microbiota can have a symbiotic or dysbiotic relationship with the host based at least partially on their composition and abundance. When the gut microbiome and the host are symbiotic the bacteria aid the host in performing crucial functions such as production of neurotransmitters, protect the host by educating the immune system, and create an environment for the GI tract to function properly via production of various acids and other compounds that have not yet been discovered. Despite the leaps and bounds made in human microbiome research, a multitude of questions remain pertaining to even its most basic functions. In fact, in the last decade of research it has become clear that modern technology and methods will be necessary to probe the depth and complexity of the human microbiome.

The study of human-associated bacteria dates to the 1680’s when Antonie van Leeuwenhoek, inventor of the microscope, compared his own oral and fecal microbiota. In the 18th and 19th centuries, the most common medical causes of death were infectious diseases and as such most of bacterial research was focused on infectious pathogenic bacteria. Identification of bacteria by microscope requires a living and intact sample of the
bacteria so that it can be observed visually. This led to methods of culturing bacteria for identification, but the understanding of anaerobic bacteria did not come about until 1892, facilitated by Louis Pasteur. By 1977, we knew that bacteria outnumbered human cells in the body 10 to 1. The term “microbiome” was first used in 1988 a book on fungi in biological control systems to define “a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physiochemical properties. The term, thus not only refers to the microorganisms involved but also encompasses their theatre of activity. Studying bacteria with improvements in anaerobic culture techniques, proved useful until the realization that still, only 10-25% of the microbiota of the gut were identified. This was due to difficulty differentiating anaerobic bacteria from each other, and the time-consuming nature of the culture methods. For these reasons, discoveries surrounding these bacteria came to a sputtering halt. Until 2001, when the term “microbiome” appeared in “Ome Sweet ‘Omics- A Genealogic Treasury of Words” as” the collective genome of our indigenous microbes (microflora)” and the publication of the human genome sequence. In response to the publication of the complete human genome, researcher Julian Davies published a comment on it suggesting that this information concerning the host was not incredibly useful without an understanding of the microbes which colonize it. Davies and others requested a “second human genome project” that “would entail a comprehensive inventory of microbial genes and genomes at the four major sites of microbial colonization in the human body: mouth, gut, vagina, and skin.” With new sequencing technology this huge undertaking of identifying the hundreds of various kinds of bacteria would actually be possible. The Human Intestinal Metagenome Initiative (HIMI, Later MetaHIT) was born from an international meeting hosted by the French
Institute for Agricultural Research (INRA) in 2005, with a purpose of more completely defining the human intestinal microbiome in health and disease. Following this meeting the NIH decided to sponsor the Human Microbiome Project to study the human microbiome by examining at least the four body sites that had been suggested by Davies. In 2010 the MetaHIT Consortium published “A human gut microbial gene catalogue established by metagenomic sequencing”. By 2012, the Human Microbiome Consortium published 1000 bacterial genomes associated with the human microbiome and somewhat of a definition for the normal bacterial makeup of the body. In the past decade, research on the human microbiome in health and disease has exploded. The expansion has been greatly facilitated by the introduction of metagenomic sequencing as a method of identifying species. Multiple methods including pyrosequencing, shotgun sequencing, and targeted sequencing are now used but the common is next generation sequencing, specifically 16s rRNA sequencing as a method of identifying many of the bacterial species in the gut. This sequencing technique identifies unique bacteria at 98% accuracy by a specific region (usually V4, V5, or V6) of their ribosomal RNA where they differ from other bacteria. Since the method is sequence and not culture-based it has allowed us to identify the majority of bacteria commonly found in and around the human body whether they are anaerobic or not. Once the bacterial genomes were published, the scientific community realized, that despite accomplishing the huge feat of identification, we really did not know anything about these bacteria other than their names, and without more information we could not determine their role in health or disease. Multi-omics approaches are now being developed to further learn the function of these microbes to understand their role and develop novel microbiome-based treatment strategies.
After all this sequencing, the scientific community is finally starting to come to a consensus on what a “normal” gut microbiome might look like, and its function. This is a challenge because each persons’ microbiome varies significantly and are more different than the same as any other person. Further, not all individual bacteria can be defined as “good” or “bad” as the whole community functions together and some bacteria can offset effects of others. Bacteria also undergo distinct phases wherein they may be pathogenic depending on their environment. Different parts of the gastrointestinal tract contain different concentrations of bacteria with the highest concentration present in the colon. There are an estimated $10^3$ bacteria/mL of content in the stomach, $10^4$/mL in the duodenum and jejunum (upper small intestine), $10^8$/mL in the ileum (lower small intestine), and $10^{11}$/mL in the colon (large intestine). The gut microbiota is primarily comprised of Firmicutes and Bacteriodetes but also contain Actinobacteria and Verrucomicrobia phyla with Streptococcus being dominant in the esophagus and Helicobacter pylori dominant in the stomach. The large intestine contains the most diversity with over 70% of the gut microbes being present here. When fecal samples are acquired they more directly reflect the diversity of colonic microbiome. There is also a difference in association within the colon wherein specific bacteria are associated with the luminal space (easily identified in fecal), and others are predominantly associated with the mucus layer. Since there is such variability between individuals, another method of classification was presented by the MetaHIT consortium. There are groups of species that cluster into symbiotic states and are stable of geography and gender which have been termed Enterotypes. Enterotype 1 has a high abundance of Bacteroides, Enterotype 2 has a high abundance of Prevotella, and Enterotype 3 has a high abundance of Ruminococcus. Each of these Enterotypes have a
unique gene set and have specific metabolic functions, but this method of classification does not explain relative distribution in the diverse groups between individuals.

Quorum Sensing Communication

Despite more consistent changes in mice models studying the gut microbiome, the mouse microbiome contains a sizeable number of genera not found in humans and is not similar enough to the human gut microbiome to make meaningful conclusions or predictions. This, combined with our very limited knowledge of how these bacteria function has led to challenges with interpreting microbiome changes. A potential solution to this problem is to focus on interactions between bacteria and human cells to find mechanistic patterns that can then be associated with either individual bacteria or groups of genera that may have similar functions. By taking a functional approach we can start to see exactly which changes in behavior of the bacteria are affecting the host. Quorum sensing (QS) is the mechanism by which bacteria communicate with and sense their environment. Bacteria produce, and release small molecules called quorum sensing molecules (QSMs). QSMs are secreted by the bacteria and detected by neighboring cells enabling them to sense how many bacteria are around them. This is crucial because bacteria are small and many of their activities are population dependent. There are many types of QS systems but only three general classes of known QSMs, acyl homoserine lactones (AHLs), recognized by Gram (-) bacteria, Autoinducing peptides recognized by Gram (+) bacteria, and Autoinducer-2 (AI-2) recognized by both Gram (+) and (-) bacteria. Many of these systems also function as a positive feedback loop, enabling an entire community to turn a gene on or off at once. In the LuxIR signalling pathway of Vibrio fischeri, for example, two genes, LuxI and LuxR control expression of a luciferase
operon (luxICDABE) which can produce bioluminescence. LuxI codes for the gene producing the synthase protein that produces the autoinducer and LuxR is the receptor and DNA-binding transcriptional activator. Once the autoinducer is produced, it remains in and around the cell as its concentration builds. At the critical threshold concentration, it binds LuxR inducing expression of the luciferase and producing even more autoinducer forming the positive feedback loop in that, and other neighboring cells resulting in light 40.

Many bacteria, including commensal gut bacteria, use these QSMs to coordinate community-wide transcriptional regulation of processes such as biofilm formation, virulence, and other social activities. Essentially, they can determine when the minimum threshold concentration of the QSM has been reached and alter gene expression in response 41. A recent study has also shown that human neurotransmitter serotonin can interact with and influence bacteria that can colonize the GI tract by activation of quorum sensing and virulence indicating the possibility of QSM-mediate host-microbiome interactions 42. Further, since bacteria detect these molecules for genetic regulation, modulation of QSMs could be used to induce specific actions in bacteria. In fact, manipulation of QSM Autoinducer 2 (AI-2) has been shown to promote the growth of specific members of the gut microbiome, as well as to aid the recovery of the microbiome after antibiotic treatment. While QS has most commonly been used to study pathogenic or ecologically relevant bacteria these QS mechanisms and signals could greatly inform the field of microbiome research as potential biomarkers for changes in bacterial diversity, gut health, or disease severity. Further, since bacteria detect these molecules for genetic regulation, modulation of QSMs could be used to induce specific actions in bacteria which, in turn, would have beneficial and long-lasting effects on the host 43.
The human microbiome, especially the gut microbiota, is considered an essential organ. We now know these bacteria collectively carry several times more genes than the human genome and are involved in human biological processes such as regulating metabolism, influencing innate immunity, and regulating epithelial development \(^4^4\).

The symbiotic relationship between gut microbiota and the host impart significant metabolic, immunological, and protective functions crucial for health guiding the research focus from diversity and identification of these bacteria, to their functional characteristics. The gut microbiome has at least three major functions in its symbiotic state; immunomodulation, nutrient metabolism, and maintenance of the gut barrier. Gut microbiota play a role in both the innate and adaptive immune system through the gut associated lymphoid tissues (GALT) in the GI tract. This tissue contains vital immune cells such as effector and regulatory T cells, B cells, and resident macrophages, the proper development of which is at least partially dependent on the microbiome \(^3^5\). Gut microbiota ferment carbohydrates that are indigestible to make short chain fatty acids (SCFA) such as butyrate, propionate, and acetate, which are a rich source of energy to the host in addition to creating the acid environment in the gut that discourages colonization of pathogenic bacteria. Further, gut microbiota possesses efficient protein metabolizing machinery that function in tandem with human proteinases in addition to contributions to the synthesis of vitamin K and components of vitamin B. A considerable body of evidence has accumulated showing the gut microbiomes contribution to structural development and integrity of the gut mucosa barrier which is crucial to the proper function of the gastrointestinal tract. Mechanisms underlying the function of the GI tract have even been identified, such as the maintenance of tight junctions by bacterial stimulation of TLR2 signaling \(^4^4\).
The above research and other studies have demonstrated the overall importance of a properly balanced microbiome for human health and, it has now been implicated in many diseases involving multiple organs systems. As SCI also results in systemic remote organ dysfunctions, a brief background on those of relevance to SCI and its secondary effects will be discussed here. There has been evidence that the microbiome is associated with chronic diseases such as obesity, inflammatory bowel disease (IBD), Diabetes type II, and non alcoholic fatty liver disease (NAFLD), among many others. Interestingly, all the above listed illnesses often are co-morbidities associated with chronic SCI.

Microbiome in metabolic disease

As demonstrated by their ability to produce short chain fatty acids (SCFA), gut bacteria play a role in energy metabolism in the host, and therefore likely also play a role in metabolic disease. Indeed, both in vivo and human studies have repeatedly indicated that host-microbiome interactions may be crucial factors in metabolic disease such as obesity and diabetes. Microbiota from an obese twin can produce a similar phenotype of adiposity in the lean twin. This indicates an amazingly sensitive response of the gut microbiome to diet. Differences in infant microbiomes have also been related to a higher chance of being obese later in life. In animals, a difference in the ratio of relative abundance of Firmicutes to Bacteroides has been observed, as well as decreased bacterial diversity in obese cases.

Different microbiota compositions have been identified in individuals with insulin resistance and type II diabetes and evidence is building of a gut microbiota contribution to the pathogenesis of these diseases. The largest human study to date, of 345 people with type II diabetes (T2D) and controls showed a decrease in Faecalibacterium prausnitzii,
Roseburia intestinalis, Roseburia inulinivorans, and Eubacterium rectale, and an increase in Bacteroides caccae, Clostridium, and Akkermansia muciniphila. Some of these changes have also been observed in chronic inflammatory diseases, such as IBD, further evidencing low-grade inflammation in metabolic disease. In general, members of Bacteroidetes have been particularly associated with metabolic diseases in humans, but not consistently between studies indicating the great diversity within just one phyla \(^{47}\). Despite these results, so far relationships between the gut microbiome and these diseases have been correlative and the challenge now is proving causality to develop some meaningful interventions. Dietary interventions are of great interest, because of their non-invasive nature, but not enough is known about the specific functions of the bacteria to formulate a standard diet for gut microbiota-modulated control of metabolic disease \(^{48}\). However, since diet has already been used to control metabolic disease, the real value, is in learning how to utilize the microbiome to maximize and accelerate the effects of dietary changes \(^{49}\).

**Microbiome in chronic inflammatory diseases**

Given the role of the gut microbiome in maintaining intestinal barrier integrity and immune system development, it is no surprise that there is an increasing body of evidence suggesting connections between the gut microbiome and chronic inflammatory diseases. Since spinal cord injury invokes a large inflammatory reaction that persists well into the chronic stage, some of these changes in microbiota and their metabolites may be relevant. Inflammatory bowel disease (IBD) is perhaps the most commonly studied for interactions with the gut microbiome. IBD is a group of inflammatory disorders of the gastrointestinal tract wherein the lining of the intestine is chronically inflamed for which there is no cure and the mechanisms of which are poorly understood \(^{50}\). Different animal models of IBD
have shown certain bacteria such as Akkermansia muciniphila, Clostridium ramosum, and Bacteroides to be enriched associated with inflammation. Conversely, other microbes, namely SCFA producers, have been associated with a less severe disease state such as Faecalibacterium prausnitzii, Odoribacter splachnii, and Roseburia. Roseburia and F. prausnitzii produce butyrate, an energy source for the host, and induce differentiation of T_{reg} cells via the GPR43 receptor. In humans with IBD, an overall trend of decrease in bacterial diversity, increase in Bacteroides and Enterobacteriaceae, and decrease in Firmicutes have been observed compared to healthy controls. More research is underway to understand the mechanism of these bacterial effects in IBD. The gut microbiome has also been studied with respect to systemic inflammatory arthritis diseases. For example, studies have shown enriched abundance of Prevotella copri and decrease in Bacteroides in humans with new onset rheumatoid arthritis (NORA). Animal studies with spondyloarthritis, another group of systematic inflammatory arthritis, have shown higher abundance of Paraprevotella and lower abundance of Rikenellaceae. The fact that all these are chronic inflammatory diseases yet have very different gut microbiome changes both in mouse models and humans points out to the complex nature of both the microbiome and inflammation.

1.3. The Gut Mycobiome

Introduction to Fungi

The mycobiome is the fungal component of the human microbiome but has been largely understudied. Through great advances curtesy of next generation sequencing we now know that different body sites have specific fungal populations and that these can be associated with disease as well. The field of human mycobiome research is still in its
infancy, and even the term “mycobiome” itself was only first used to refer to the fungal microbiome in 2010. Like bacteria, fungi research was historically limited by available culture methods until sequencing technology became readily available as unculturable fungi comprise the largest portion of the human mycobiome. So far, we know very little about the role of fungi in health as most of research has been done on disease states, however evidence has shown that mycobiomes at different sites can potentially interact with each other. For example, a gut disturbance by C. albicans can influence A. fumigatus in the lung mycobiome. Fungi in the gut have also been shown to interact with bacteria. Candida albicans, is an example of a fungal pathobiont meaning it is usually present in the host and its harmless in normal conditions but does have pathogenic potential.53

Interactions of Fungi with the Microbiome

Next generation sequencing has led to the discovery of diverse communities of fungi all over the human body including the gut. Numerous studies have identified anywhere from 16-75 species with common phyla being Ascomycota, Basidiomycota, and Zygomycota.54 There are a host of factors influencing gut microbiome/mycobiome interactions including microbial metabolites, host immunity, extra-gastrointestinal organs, disease susceptibility, and diet. Like the microbiota, changes in diversity of fungi have also been shown in inflammatory bowel disease (IBD) suggesting that the mycobiome and microbiome can influence each other in a disease state.53 In colorectal cancer, different fungal microbiota at the mucosa correlated to tumor size and disease state. Diet also influences the gut fungal community perhaps as much as bacteria. In 2013, Hoffmann and colleagues reported a link between consumed foods and abundance of fungi in the gut where C. albicans is associated with intake of carbohydrates compared to protein.55

In
obese humans, a decrease in fungal biodiversity was observed, and an increase in relative abundance of at least 1 genera, Mucor, with diet-induced weight loss. Correlations have also been found between high-density lipoprotein (HDL)-cholesterol and relative abundance of the family Aspergillaceae, however, it is unknown how these variations and associations would influence metabolism. Since gut bacteria play a role in immune regulation it makes sense that fungi would have some sort of role as well, however, this research has been more directed at opportunistic fungal pathogens rather than in the symbiotic state. This symbiotic state is reliant on production of extracellular substances by gut bacteria that can inhibit growth of fungi such as SCFAs and medium-chain fatty acids (MCF), bacteriocins, antimicrobial peptides, and secondary bile acids. For example, butyric acid, a SCFA has been demonstrated to inhibit the yeast-hyphal (Y-H) transition in C. albicans, which is a necessary step in its conversion to a pathogenic state wherein it can invade human tissues \(^{55}\). Future studies will be able to delineate more detail as to the composition of the human gut mycobiome, as well as to inform mycobiome/microbiome interactions and the implications of these interactions on the host in health and disease. Mycologists and Microbiologists are now pushing for co-sequencing of both fungi and bacteria from the identification of gut fungal changes alongside changes to the microbiome helps to further understand the players in the pathophysiology of GI dysfunction in people with SCI \(^{54}\).

Chronic SCI causes interrelated secondary complications that significantly compromise health and quality of life after injury, including pathological dysfunction in the autonomic nervous system, immune system, and gastrointestinal tract. As discussed in the introduction, chronic GI dysfunction is a serious secondary complication of SCI. GI
dysfunction affects close to 60% of the SCI population and is ongoing which greatly decreases the quality of life. Further, this dysfunction is related to autonomic dysreflexia, a potentially life-threatening medical complication. The mechanism of GI dysfunction in SCI is not known and current treatments, such as dietary fiber increase, have been ineffective and sometimes even contraindicated in this population. This study aimed to further characterize the state of the GI tract and GI dysfunction in the presence of chronic spinal cord injury. We hypothesized that SCI leads to a chronic inflammatory state inducing changes in gut bacteria and fungi causing downstream changes evidenced by GI dysfunction in the host. In this thesis, experiments were conducted in a rat model of SCI at two levels and severities and human fecal samples analyzed. While more research is needed, this data helps to lay the groundwork for understanding GI dysfunction within the context of the human micro- and myco- biomes in SCI to develop interventions to improve quality of life for this population.
Chapter 2. ANALYSES OF GASTROINTESTINAL CHANGES IN A RAT MODEL OF C5 MODERATE CONTUSIVE SPINAL CORD INJURY

As introduced above, SCI leads to serious remote organ effects in addition to the injury itself, and many of these effects are incompletely understood. Rats are 90% genetically identical to humans and thus are a common model in biomedical and translational research. In spinal cord injury research, rats and mice are a great resource because human occurrence of these injuries is not incredibly high, and the most useful methods are highly invasive. In this set of experiments, we aimed to characterize gastrointestinal changes using a rat model of moderate contusive spinal cord injury at vertebra five. This model is very relevant as SCIs in humans are commonly cervical or thoracic and incomplete. Utilizing this model allowed us to evaluate changes in gastrointestinal function by examining gastrointestinal transit and histological analysis of gastrointestinal tissue.

2.1 Methods

Moderate C5 SCI rodent model

Adult female fisher rats were housed according to the NIH guidelines and the guide for the Care and Use of Animals. Significant differences in locomotor performance and preserved spinal cord matter have been observed in male vs. female rats, so all females were chosen to decrease variations. All procedures have been approved by the University of Miami Miller School of Medicine Institutional Animal Care and Use Committee. All samples collected from the animals were blinded before being transferred to the laboratory personnel for analysis. Animals were handled by a core facility until sacrifice. Before surgery, animals were weighed and anesthetized with a mixture of 2%
isoflurane and 40% oxygen and prepared for surgery as described by Datto et al. An appropriate level of anesthesia was determined by monitoring corneal and hindlimb withdrawal reflexes. The backs of the rats were then shaved and aseptically prepared with chlorhexidine and lacrilube ophthalmic ointment applied to the eyes to prevent drying. During the procedure animals were kept on a warming pad to maintain body temperature which was assessed by rectal probe. The rats were then subjected to a moderate contusion injury using the MASCIS weight drop device (NYU Impactor) (2). A laminectomy at cervical vertebra C4 was produced exposing the dura mater. Stabilization clamps were placed to support the column and the exposed spinal cord injured (C5) by dropping a 10 g rod from a height of 12.5 mm. After the injury the muscles were sutured and the skin closed with metal clips. The animals were allowed to recover in warmed cages (approximately 30°C) with water and food easily accessible for 24 hours. Standard rodent diet was made available with sterilized water and animals kept on Alpha-Dri® bedding in pairs. Rats were then kept at 24°C on 12 hour on/off light cycles. All rats, both SCI and sham operated controls, were given and antibiotic treatment of 5 mg/kg Gentamicin for the first 7 days after surgery. This treatment was to prevent additional infections specifically UTI’s (Urinary tract infection) which is a serious and common occurrence in both rodents and humans with SCI. An analgesic, Buprenex, was also administered at 0.03 mg/kg daily for 2 days. All animals had access to food and water and their bladders were manually expressed twice a day until normal function returned. Sham operated animals underwent all surgical procedures except for weight-drop to injure the cord to serve as controls. Pairs were assigned after surgery resulting in co-housing of injured and uninjured animals. This study comprised four groups of animals: sham animals, who were sacrificed at 4 weeks,
SCI animals sacrificed at 4 weeks, sham animals sacrificed at 8 weeks and SCI animals sacrificed at 8 weeks. The 8-week time point represents a more chronic injury whereas 4 weeks is around the time of maximum observed functional recovery of the animals. All 4 groups were sampled for each experiment unless otherwise noted.

Gastrointestinal transit assay

All animals were orally gavaged with 25 mg Congo red dye in 1 mL sterilized water 1 hour prior to sacrifice. All animals from each of the 4 groups were sacrificed 1 hour after gavage by a terminal injection of ketamine followed by overdose of isoflurane. The entire gastrointestinal tract excluding the stomach was removed from all animals and immediately placed on ice. The whole tract containing the intestinal content was divided into 5 sections; proximal small intestine (duodenum), distal small intestine (jejunum) cecum, proximal colon, distal colon. Sections were comparable in length for all animals. Representative content sample from each section was transferred to an eppendorf tube and kept on ice. Samples were sufficiently diluted (1:5 or 1:10) in sterilized water, mixed thoroughly and absorbance measured at 497 nm. Absorbance values from the same section from each group were then averaged and the SCI group compared to the control at 4 and 8 weeks respectively.

Histological analysis of colonic tissue

After all content was removed tissue sections from the proximal and distal colon were washed thoroughly with cold PBS and then transferred to 4% Paraformaldehyde at 4°C overnight. After fixing, the tissue was rinsed and placed in cold PBS and transferred to the histological Core at the University of Miami Miller School of Medicine, Miami
Project to Cure Paralysis. The tissue was embedded in paraffin, sliced, mounted, then stained by Hematoxylin and Eosin and imaged on a Nikon Ts100 microscope. Slide images were evaluated by a histology specialist at the Crohn’s and Colitis Center at University of Miami Miller School of Medicine.

2.2 Results

Gastrointestinal transit

Changes to gastrointestinal transit were analyzed by measuring the changes in Congo Red absorbance in different portions of the GI tract in SCI animals compared to control a set time after gavage. There was an overall change in pattern of transit at 8 weeks compared to 4 weeks post-injury, but no significant changes seen between control and injured animals (Figure 2.2.1).

Histological analysis of colonic tissue

Proximal and distal colon tissue was excised from SCI and control animals 4 (figure 2.2.2) or 8 (figure 2.2.3) weeks post-injury to determine physiological damage. Tissue sections were fixed, paraffin embedded, mounted, and H&E stained. Tissue was poorly sliced, and most samples show signs of tissue sheering and shredding making it very difficult to determine damage. Further, tissue was sliced horizontally rather than vertically, so intestinal crypts were not clearly visible and could not be analyzed for damage. Slides of colon sections from both SCI and control were analyzed blindly and no difference was seen in morphology in sections without mechanical damage.
2.3 Discussion

An important first step was to evaluate a common SCI rat model for GI dysfunction and gastrointestinal damage. The main goal was to confirm the usefulness of the impactor SCI animal model to study GI function. If this model demonstrated secondary organ affects it could be used to probe the mechanisms and test potential intervention strategies before bringing them to the clinic. We wanted to use the moderate C5 injury since the severe
Figure 2.2.2 - Gastrointestinal histology. H&E histology stain of proximal (left) and distal (right) colon tissue of control (top) and C5 injured (bottom) rats at 4 weeks post-injury.

Figure 2.2.3 - Gastrointestinal histology. H&E histology stain of proximal (left) and distal (right) colon tissue of control (top) and C5 injured (bottom) rats at 8 weeks post-injury.
thoracic model is more common, and some gastrointestinal changes have already been observed in that model. However, since cervical spinal cord injuries are more common in patients with SCI, we aimed to determine if the GI dysfunction could be observed at different levels and severities of injury.

The purpose of the gastrointestinal transit assay was to determine differences in gastrointestinal transit time. If animals with an SCI had slowed GI transit, the Congo red would not go as far through the GI in a set time when compared to the control group. While there was a difference in transit pattern there was no significant difference in transit time. This would indicate that a change in GI pattern may be unrelated to the injury and that tie after injury may play a more significant role. Another possibility is that this group did not have a severe enough injury to manifest an effect on transit time. This is likely because the injury was moderate, and animals recovered even limb and motor function by about 4 weeks. This question could be answered by conducting a study with both a moderate and severe injury in parallel for multiple time points and repeating the experiments to delineate the contributions of time since injury as well as injury severity. The gastrointestinal permeability assay and colon tissue histology were used to determine the integrity of the gut barrier. Histological analysis is the primary and crucial method of determinizing damage to the GI mucosa. However, it usually requires sacrifice of the animal, so it is difficult to use histology alone to monitor GI state and, hence, this method is usually combined with functional studies like the permeability assay discussed above. Hematoxylin and Eosin (H&E) is an acidic dye that is used to visualize tissue samples. Hematoxylin is a basic dye that stains acidic structures such as DNA purple whereas Eosin is an acidic dye that stains basic components such as muscle cells and intracellular...
membranes pink. Therefore, this stain allows one to distinguish damaged cellular structures such as membranes and other signs of tissue damage\textsuperscript{57}. In the gut, changes to crypt depth and structure, mucosal thickness, and increased presence of macrophages can indicate tissue damage. To best analyze colon tissue the sample should be embedded and sliced vertically to visualize the crypt structure. Unfortunately, all samples in this experiment were sliced horizontally giving a helicopter view of the crypts. From this viewpoint it is difficult to distinguish some structures. Despite this, the sections from control and injured animals were comparable with no damage observed, no changes in macrophage density, or disruption of membrane structures. Since histological changes have previously been reported in animal models of SCI there are two main reasons why no changes were observed here. 1.) This was a moderate cervical injury and previous studies were conducted in severe thoracic models meaning the animals may not have been injured enough to manifest GI changes. 2.) It is possible that damage did exist but could not be visualized from hematoxylin and eosin stain on horizontally sliced tissue. In future experiments, in addition to repeating experiments with various levels and severities, further histological analysis could be conducted. For example, disruption of tight junctions is also a hallmark of GI mucosal damage and can be histologically visualized by staining tight junction proteins such as ZO-1 and occludin\textsuperscript{58}. These methods have previously been used in other disease states to visualize GI damage but not in a rat model of SCI.
Chapter 3. ANALYSIS OF MICROBIAL AND GASTROINTESTINAL CHANGES IN A RAT MODEL OF T9 MODERATELY SEVERE CONTUSIVE SPINAL CORD INJURY

A thoracic contusion at the 9th vertebrae is one of the most commonly used rodent models for spinal cord injury research, even though it is not the most prevalent in humans. As discussed above in Secondary Organ and Systemic Dysfunction, UTIs occur in both rodents and humans with an SCI, with 100% of rodents showing signs of a UTI after surgery. Although SCIs in humans are usually cervical or high thoracic in nature, the higher injury level can increase both the severity of the UTIs and chances of autonomic dysreflexia in the model. The T9 contusion provides the opportunity to study the effect of a thoracic injury without increased loss of life. We utilized this model of T9 moderately severe contusive SCI to look at gastrointestinal changes in a more severe SCI with minimal loss of life from autonomic dysreflexia and UTI infection. The model allowed us to examine changes to bacterial communication, bacterial composition, and proinflammatory cytokines between sham control and SCI animals at two different time points.

3.1 Methods

Moderately Severe T9 SCI rodent model

Adult female Fischer rats were housed and prepared as described above in 1.1. Rats underwent a laminectomy at T9 to expose the dura mater. Upon stabilization the animals were then subjected to a moderately severe contusion by using the same MASCIS weight drop device from a height of 25mm. Post-operative care was the same as described previously with antibiotic and analgesic administration and recovery conditions. This set of animals also contained the same 4 groups (Sham and SCI, 4 and 8 weeks) with the only difference being the level and severity of the injury.

16s rRNA bacterial sequencing
The intestinal content from both groups of animals sacrificed at 8 weeks was collected and sequenced by 16s V4 rRNA gene sequencing on the Illumina MiSeq platform. Operational taxonomic units (OTUs) were considered significant if the FDR-corrected P value was less than or equal to 1. Data processing and multivariate statistical analyses were conducted using PhyCA-StatsTM analysis software. Probe intensities were background subtracted and scaled to Control Mix. Hybridization scores were then calculated as log2 (mean probe fluorescence intensity) x1000. OTUs were defined as high 16s rRNA gene sequencing similarity, with the majority having >99% intra-OTU concordance. Before classification analysis, data reduction was conducted by multiple filtering steps and taxa-sample intersections calculated using abundance (AT) and binary matrices (BT). Together AT and BT dissimilarity scores were computed using Unifrac and weighted Unifrac distance metrics. Weighted Unifrac metric considers OTU abundance in addition to phylogenetic distance between OTUs. Hierarchical clustering via average-neighbor (HW-AN) and principal coordinate analysis (PCoA) were used to graphically summarize inter-sample relationships according to AT and BT dissimilarity. OTUs with most significant differences in abundance between groups were identified by unsupervised classification using the nearest shrunken centroid method. A randomization/ Montecarlo permutation-based test (Adonis test) was employed to test for significant differences between discrete and continuous variables.

Detection of Quorum Sensing molecules in intestinal content

Biosensors based on Escherichia coli living cells containing plasmids that encode for proteins of the bacterial communication machinery have been used previously to detect
changes in quorum sensing communication in bacteria. Specifically, E. coli cells containing plasmids of genes involved in regulation of short chain (pSB406) and long chain (pSB1075) AHL systems were employed in this study as described previously. In this system, transcriptional regulation genes combined with a bioluminescent reporter gene are used to visualize changes in these quorum sensing communication pathways. When quorum sensing molecules are present, transcription of the regulatory protein of the operon and the reporter protein is initiated. As the concentration of QSMs increases, the emission of bioluminescent light by the reporter protein increases in a directly proportional manner. Calibration curves can be then constructed relating the concentration of QSMs with the intensity of the light generated. In addition to these biosensors, another, based on Vibrio harveyi (MM32) to detect changes in autoinducer 2 (AI-2) was also used as described by Raut et al. Overnight cultures of the cells are refreshed in LB broth (pSB406 and pSB1075) or AB media (MM32) and grown to OD600 of 0.45 (pSB406 and pSB1075) and 0.02 (MM32). Dose-response curves were generated to determine the response from each sensors’ cognitive analyte; C6-AHL for pSB406, C12-AHL for pSB1075, and AI-2 for MM32. 10µL of analyte or intestinal content (homogenized and diluted to 1% weight/volume in distilled deionized water) was added to 90µL of sensor and incubated for 2 hours before measuring bioluminescence. The AI-2 biosensors are based on the same principle and also function in the same manner as those for AHLs.

Detection of pro-inflammatory cytokines in intestinal tissue homogenate

Changes in pro-inflammatory cytokines IL-1β, IL-12, MIP-2 (macrophage inflammatory protein 2), TNF-α, and serotonin were determined by measuring the concentrations of
small intestinal homogenates from all 4 animal groups. Whole intestines were collected at sacrifice and washed thoroughly in cold PBS. Tissue from the small intestine was homogenized by Dounce homogenizer in an equal amount (weight/volume) of distilled deionized water. Commercial ELISA kits from Life Technologies (Carlsbad, CA) were used per the manufacturer’s instructions to determine concentrations.

Serotonin levels

Intestinal content from SCI and control animals at 4 and 8 weeks post-injury was analyzed for serotonin. Serotonin was measured using a calprotectin ELISA kit from Life Technologies (Carlsbad, CA) per manufacturer’s instructions.

3.2 Results

Microbiota sequencing Intestinal content from 8-week post-SCI and control animal groups was sequenced via 16S V4 rRNA gene sequencing using the Illumina MiSeq platform. A total of 5,048,814 reads were obtained from 23 (20 sample, 3 control) samples with a mean of 219,513 reads per sample. There was similar richness and read diversity between groups, shown by alpha diversity and Shannon analysis (figure 3.2.1). Rarefaction curve analysis indicated that the samples were sequenced to adequate depth to capture microbiome composition. A difference in microbiota beta diversity was observed between SCI and control animals at 8 weeks post-injury at the family, genus, and species level. Lactobacillaceae, Turibacteraceae, and Bifidobacteriaceae were the most abundant
Figure 3.2.1- Diversity of OTUs in 8-week intestinal content. A.) Alpha diversity of OTUs in intestinal content of 8-week sham control and SCI rats: observed diversity based on number of OTUs present in each sample and Shannon diversity index to account for richness and evenness of OTUs within a sample. B.) Dimensional reduction of Bray-Curtis distance between samples using the PCoA ordination method.
families with Bifidobacteriaceae and Clostridiaceae significantly more abundant in the SCI group compared to control (Figure 3.2.2, Table 3.2.1).

![Figure 3.2.2 - Family level differences in Microbiota. Most abundant taxa in intestinal content of sham control and SCI rats at the family level at 8 weeks post-injury.](image)

<table>
<thead>
<tr>
<th>Family</th>
<th>Chi-square</th>
<th>P-value</th>
<th>SCI Mean</th>
<th>Control Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillaceae</td>
<td>0.0993</td>
<td>0.75</td>
<td>43.8</td>
<td>41.7</td>
</tr>
<tr>
<td>Turicibacteraceae</td>
<td>0.5404</td>
<td>0.46</td>
<td>9.54</td>
<td>22.8</td>
</tr>
<tr>
<td><strong>Bifidobacteriaceae</strong></td>
<td><strong>11.2941</strong></td>
<td><strong>&lt;0.001</strong></td>
<td><strong>19.3</strong></td>
<td><strong>1.2</strong></td>
</tr>
<tr>
<td>Peptostreptococcaceae</td>
<td>0</td>
<td>1</td>
<td>7.26</td>
<td>7.74</td>
</tr>
<tr>
<td>Clostridiaceae</td>
<td>5.8346</td>
<td>0.02</td>
<td>12.5</td>
<td>0.961</td>
</tr>
<tr>
<td>Streptococcaceae</td>
<td>0.5404</td>
<td>0.46</td>
<td>1.2</td>
<td>10.7</td>
</tr>
<tr>
<td>Micrococcaceae</td>
<td>3.1875</td>
<td>0.07</td>
<td>0.535</td>
<td>2.31</td>
</tr>
<tr>
<td>Coriobacteriaceae</td>
<td>0.0441</td>
<td>0.83</td>
<td>1.41</td>
<td>1.43</td>
</tr>
</tbody>
</table>

*Table 3.2.1* - Most abundant bacterial families. The top 8 most abundant bacterial families in samples 8-weeks post-SCI or sham surgery by Kruskal-Wallis rank sum test including percent relative abundance of each family.
From a total of 785 classifiable OTUs, 59 were significantly different in the SCI group (figure 3.2.3), and of those 59, 35 OTUs were enriched in the SCI group. At the species level Lactobacillus intestinalis, Clostridium disporicum, and Bifidobacterium choerinum were found to be enriched in the SCI group whereas Clostridium saccharogumia was decreased in the SCI group. Interestingly, Lactobacillus Intestinalis accounted for 15.5% of all sequences (Table 3.2.2).

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Interpretation</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus intestinalis</td>
<td>Enriched in case</td>
<td>9.87 x 10^{-7}</td>
</tr>
<tr>
<td>Clostridium disporicum</td>
<td>Enriched in case</td>
<td>2.69 x 10^{-7}</td>
</tr>
<tr>
<td>Bifidobacterium choerinum</td>
<td>Enriched in case</td>
<td>1.39 x 10^{-11}</td>
</tr>
<tr>
<td>Clostridium saccharogumia</td>
<td>Enriched in control</td>
<td>2.35 x 10^{-4}</td>
</tr>
</tbody>
</table>

Table 3.2.2- Species level identification. OTU, species level identification, and adjusted p-value for the 4 species found differentially abundant in SCI animals compared to the sham control.
Pro-inflammatory cytokine levels

Small intestines were extracted from both SCI and control animals at 4 or 8-weeks post-injury and homogenized. Intestinal tissue homogenate was assessed for proinflammatory cytokines IL-1β, IL-12, MIP2, and TNF-α (Figure 3.2.4).

IL-1β and IL-12 were increased in the SCI group at 4 weeks post-injury and returned to basal levels at 8 weeks. Both MIP2 and TNF-α levels were higher in the SCI group compared to at 8 weeks. To determine if there were any associations between inflammation and the microbiota changes we conducted PERMANOVA analysis to determine covariate significance. We discovered that three of the pro-inflammatory cytokine variables contributed significantly to the beta diversity of the samples. Cytokines IL-1β, IL-12, and
MIP-2 were found to be significantly correlated with changes to the microbiota diversity in SCI animals. For IL-1β, 23 OTUs were significantly correlated by a log 2-fold change per RL unit, eight of which were identified at species level and all negatively associated including *Streptococcus acidominimus*, *Clostridium* sp.40, *Ruminococcus bromii*, *Faecalibacterium prausnitzii*, *Gemmiger formicillis*, *Ruminococcus obeum*, *Dorea longicatena*, and *Corynebacterium mastitidis* (Table 3.2.3).

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Log&lt;sub&gt;2&lt;/sub&gt; Fold Change</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus acidominimus</em></td>
<td>-4.41</td>
<td>0.0217</td>
</tr>
<tr>
<td><em>Clostridium</em> sp. 40</td>
<td>-4.02</td>
<td>0.0279</td>
</tr>
<tr>
<td><em>Ruminococcus bromii</em></td>
<td>-4.72</td>
<td>0.0212</td>
</tr>
<tr>
<td><em>Faecalibacterium prausnitzii</em></td>
<td>-4.82</td>
<td>0.024</td>
</tr>
<tr>
<td><em>Gemmiger formicillis</em></td>
<td>-3.99</td>
<td>0.0279</td>
</tr>
<tr>
<td><em>Ruminococcus obeum</em></td>
<td>-4.98</td>
<td>0.0148</td>
</tr>
<tr>
<td><em>Dorea longicatena</em></td>
<td>-6.55</td>
<td>0.00387</td>
</tr>
<tr>
<td><em>Corynebacterium mastitidis</em></td>
<td>-7.23</td>
<td>0.00143</td>
</tr>
</tbody>
</table>

*Table 3.2.3- Species correlated with IL-1β. OTU, species information, fold change, and adjusted P-value shown for taxa significantly correlated with changes in IL-1β.*

The second cytokine, IL-12, had 12 OTUs significantly associated, five of which had a positive log 2-fold change per RL unit, and three of which were identified at species level as *Lactobacillus intestinalis*, *Bifidobacterium choerinum* (negative log 2-fold change), and *Clostridium saccharogumia* (positive log 2-fold change). MIP2 was significantly correlated with microbiota diversity and 72 OTUs had an unadjusted P-value of <0.05 but none met the absolute value log 2-fold change threshold of 1.
Quorum sensing molecule levels

To determine changes in bacterial signaling in the gut, intestinal content from both SCI and control groups was collected and analyzed at 4 and 8 weeks post-injury. Short and long-chain N-acyl homoserine lactones (AHLs), signaling molecules for Gram negative communication and Autoinducer 2 (AI-2) used for Gram positive communication were measured in intestinal content. Levels of short and long-chain AHLs remained similar between animals at both 4 and 8 weeks. However, levels of AI-2 were similar at 4 weeks, but significantly elevated at 8 weeks in the SCI group compared to the control group (Figure 3.2.5).

Serotonin levels

Serotonin levels were measured by ELISA in fecal samples from both SCI and control groups at 4 and 8 weeks post-injury. Control samples at both 4 and 8 weeks had similar levels of serotonin. However, in the SCI animals serotonin levels are slightly higher compared to the control at 4 weeks, and significantly decreased at 8 weeks.

3.3 Discussion

In this set of experiments, we aimed to determine the effect of SCI (with antibiotic treatment) on intestinal inflammation and gut microbiota. The experiments utilized an SCI rat model, and in this case, a moderately severe thoracic injury. L. intestinalis, C. disporicum, and B. choerinum were significantly enriched in samples from SCI animals.
Figure 3.2.5- Quorum Sensing Molecules in intestinal content. Levels of A.) Autoinducer-2, B.) short-chain (C6) N-acyl homoserine lactone, and C.) long-chain (C12) N-acyl homoserine lactone in the intestinal content of 4 and 8-week sham control and post-SCI animals. (2-tailed T-test between each of the 4 groups, * = p-value <0.05, ** = p-value <0.001).
whereas C. saccharogumia was significantly depleted (Table 3.2.2.). 16s rRNA sequencing revealed high abundance of Lactobacillaceae, a family of bacteria that ferments carbohydrates into lactic acid. Researchers have shown that probiotic administration of lactic acid producing bacteria increased functional recovery in mice after induction of an SCI. Since decreases in L. intestinalis have also been linked to obesity in rats, the increase in abundance in this case is likely beneficial to the host. Production of AI-2 in the gut has been previously identified as a contributing factor in Lactobacillaceae persistence. We also observed a significant increase in AI-2 levels in intestinal content in 8 week-post-injury SCI animals, therefore, the increase in abundance of L. intestinalis is likely supported by the increase in AI-2 quorum sensing we observed.
Clostridium disporicum was also found to be increased in the SCI animals, but this species is not usually present in the healthy human gut, and its function or role in disease is not known. We were unable to elucidate whether this species has a positive or negative effect on the host, since some members of the Clostridium family are notoriously pathogenic, while others promote GI health. For example, Favier and colleagues recently showed that C. disporicum is one of the first most dominant species to colonize the infant GI tract. This led us to hypothesize that C. disporicum possibly has a positive effect on the intestinal health of the animals after SCI.

The 3rd species found to be increased in the SCI animals was Bifidobacterium choerinum (Table 3.2.2.). Bifidobacteriaceae is now commonly accepted as a commensal gut species present in healthy individuals, that has a positive effect on the host. In a study by Spichalova et al, B. choerinum was found to have probiotic effects and a protective role against Salmonella enterica infection in piglets. This leads us to hypothesize that this species may also have a positive and potentially probiotic effect in the host post-SCI.

Clostridium saccharogumia was the only bacteria identified at species level that was decreased in the SCI animals. According to the literature, this species converts plant lignans in edible material such as flaxseed into enterolactone in vivo. This is significant because enterolactone has been suggested to have various health benefits including anti-cancer effects and improving cardiovascular health. These other reports lead us to believe that the decrease of C. saccharogumia does not exert positive effects on the host post-SCI. It is important to note that none of these four species significantly altered have been previously implicated in SCI or SCI-related bowel dysfunction.
Our study also showed mild intestinal inflammation in the animals at 4-weeks post-SCI by altered levels of pro-inflammatory cytokines IL-1β, IL-12, MIP-2, and TNF-α. This is in agreement with previous research where intestinal damage, increases in mRNA of Toll-like receptors, and decreases in ileum mucosa thickness were observed in mice post-SCI. We additionally determined that IL-1β, IL-12, MIP-2 were significantly correlated with specific microbiota changes. In total, 8 OTUs were significantly correlated with the changes in IL-1β (Table 3.2.3.). These OTUs all correspond to bacteria that produce butyrate in the GI tract, an important short chain fatty acid for gut health. Butyrate has a strong anti-inflammatory effect on macrophages and can suppress ongoing inflammation in the central nervous system. Of these 8 OTUs, Faecalibacterium prausnitzii is of special interest as it has been shown to protect against induced GI states. This means its role in the gut may be significant enough to prevent GI damage and that the decrease in this butyrate producing bacteria may have also then contributed to increased levels of IL-12 in the SCI group.

All the animals in this study, with C5, T9 injuries, and controls, were treated with gentamicin for 7 days after injury to prevent urinary tract infections (UTI). These infections are known to occur in impact contusion models of SCI in rodents. Since the ultimate goal of this study was to lead to a translational treatment option, this antibiotic treatment was included not only to prevent infection of the rats, but also to mimic the fact that patients with SCI have a greatly increased risk for UTIs. In fact, Whiteneck et al. reported an annual incidence of UTI in persons with an SCI, or almost 2 cases per year in catheter-free patients. UTIs also contributed to 10-15% of urinary sepsis induced SCI mortality and broad-spectrum antibiotics are the most common method of treatment. There is, however a
potential for a synergistic effect with the gentamicin that alters the natural course of microbiota repopulation after SCI. As the field transitions toward therapeutics this potential interaction will likely be of greater interest since antibiotic prescription is so common for those with SCI.

Previously, Gungor and colleagues sequenced the microbiome of patients with SCI to investigate any potential differences and highlighted SCI induced changes to gut microbiota after injury. While this clinical study was important, it did not investigate other parameters aside from microbiome composition, yet our findings correlate with theirs including the presence of Faecalibacterium prausnitzii and Bifidobacterium choerinum in subjects with SCI.
Chapter 4. DEVELOPMENT OF A SCI BIOREPOSITORY OF HUMAN PHYSIOLOGICAL SAMPLES OF PATIENTS WITH SCI: PILOT HUMAN STUDIES OF THE MICROBIOME IN PATIENTS WITH SCI

As microbiome changes become a focus of biomedical research in health and disease, an important question remains, how relevant is the rodent model for human microbiome studies? Since each spinal cord injury is different, there is already variability to be considered before taking into account the variability of each individual's microbiome. This means any useful data gained using the animal model would have to be verified in a large number of human samples before clinical interventions could be implemented. Although we observed differences and correlations in rodents the very next step must be to confirm that these changes also occur in humans. Therefore, in order to collect the most clinically relevant information and decrease time between discovery and translation, we have planned a large-scale human sample repository for research of gastrointestinal problems in individuals with SCI. The repository plan includes a minimum of 70 individuals who are neurologically intact or with an SCI. Specifics of the criteria for inclusion or exclusion from the study were carefully selected to minimize bias and capture the full variability of both the GI state and the injury are included below. This human study includes collection of fecal samples, blood samples, SmartPill™ administration, and detailed questionnaire covering diet, lifestyle, and medications. As there is so much variability in microbiomes between individuals, the goal is to collect control samples from the same household. For funding purposes, a smaller, preliminary group of ten individuals were consented and fecal samples obtained, along with a recent diet questionnaire to assess the feasibility of the larger study. A special fecal collection kit was designed using the
Fecotainer© for persons with an SCI as they usually have a unique bowel routine. In these ten individuals (five control, five SCI), we were able to determine bacterial communication and composition, fungal composition, and short chain fatty acid concentration.

4.1 Methods

Inclusion/exclusion criteria the collection of human fecal samples

Under the supervision of Dr. Mark Nash at the Miami Project to Cure Paralysis, a clinical trial was designed to examine how gut bacteria are related to gastrointestinal transit time and assess whether this association is affected by spinal cord injury. To preliminarily establish proof of concept and ability to conduct the full study, a set of ten patients, five with a spinal cord injury and five neurologically intact individuals were evaluated. Each individual was consented, and a stool sample collected and analyzed for fecal bacteria, fungi, SCFAs, fecal calprotectin, QSMs, and blood in stool. Inclusion/exclusion criteria were as follows:

Patients with a spinal cord injury:

Eligibility criteria:

1. Age 18-65
2. Spinal cord injury resulting in Tetraplegia or Paraplegia (C5-T6) and motor complete or incomplete (AIS A-C) impairment
3. ≥ 1 year post-injury
4. Self-reported history of constipation or other gastrointestinal dysfunction (e.g., extended bowel care time or difficulty in bowel emptying)
Exclusionary criteria:

1. Currently hospitalized
2. American Spinal Injury Association (AIS) D-E
3. Self-reported history of Crohn’s disease or diverticulitis, gastric blockage/obstruction, or swallowing disorder, or gastrointestinal surgery ≤ 3 months before the study
4. Implanted cardiac pacemaker, spinal cord stimulator, morphine (pain), or intrathecal pump
5. Concurrent use of surface functional electrical stimulation (“FES”)
6. Women who know or suspect they may be pregnant

Neurologically-intact individuals

Eligibility criteria:

1. Age 18-65
2. Self-reported history of Crohn’s disease or diverticulitis, gastric blockage/obstruction, or swallowing disorder, or gastrointestinal surgery ≤ 3 months before the study
3. Implanted cardiac pacemaker, spinal cord stimulator, morphine (pain), or intrathecal pump

Sample collection and Diet analysis of SCI individuals

Five individuals with SCI and without were consented, given a recent diet questionnaire, and given a FecoTainer fecal sample collection kit with complete instructions (Figure 4.1.1).
DNA extraction, PCR, sequencing, and sequence processing

Samples were placed into a MoBio PowerMagg Soil DNA Isolation Bead Plate. DNA was extracted following MoBio’s instructions on a KingFisher robot. Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region, as per the protocol of Kozich et al. (2013). Amplicons were sequenced with an Illumina MiSeq using the 250-bp paired-end kit (v.2). Sequenced were denoised, taxonomically classifies using Greengenes (v. 13_8) as the reference database, and clustered into 97%-similarity operational taxonomic units (OTU’s) with the mothur software package (v. 1.39.5) (Schloss et al 2009), following the recommended procedure ((https://www.mothur.org/wiki/MiSeq_SOP; accessed Nov 2017).
Quality control

The amplified DNA was co-sequenced from samples and from 4 each of template controls, and extraction kit reagents processed in the same manner. Two positive controls consisting of cloned SUP05 DNA were also included (number of copies=2x10⁶). Operational taxonomic units were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25% of their mean abundance in samples.

Statistical analysis

Alpha diversity of estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. The significance of diversity differences was tested with an ANOVA. To estimate beta diversity across samples, we excluded OTUs occurring in fewer than 10% of the samples with a count of less than three and computed Bray-Curtis indices. The beta diversity was visualized emphasizing differences across samples and using non-metric multidimensional (NMDS) ordination. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 4,999 permutations for significance testing. All analyses were conducted in the R environment.

Fungal Sequencing, data curation, and sequence processing

ITS2 fungal genes were sequenced on an Illumina MiSeq, Raw FastQ files were quality-filtered and clustered into 97% similarity operational taxonomic units (OTUs) using the mothur software package [http://www.mothur.org]. A total of 6.248 x 10⁴ high quality reads were obtained and the final dataset contained 155 OTUs (including those occurring once with a count of 1) and a read range of 1690 and 3.4125 x 10⁴. High quality
reads were classified using the Warcup (v2) reference database. A consensus taxonomy was obtained for each OTU. The OTU abundances were then aggregated into taxonomies and the most relatively abundant OTUs plotted. In the figure legends, “Other” represents lower-abundance taxa. The abundance of the OTUs was converted into pairwise dissimilarities (Bray-Curtis index) and non-metric multidimensional scaling (NMDS) analysis conducted to visualize microbiome similarities via ordination plots. Official significance testing was done with permutational analyses of variance (Adonis function from vegan package) and alpha diversity calculated using Shannon’s diversity index.

Short-chain fatty acid (SCFA) detection in human fecal samples

Short-chain fatty acid (SCFA) extraction procedure was done from human fecal samples as described by Zhao and colleagues. SCFA were detected using gas chromatography coupled to a flame ionization detector on a “Thermo TG-WAXMS A GC Column, 30 m, 0.32 mm, 0.25 µm.” SCFA data was analyzed on a GC-FID and with Prizm 7.

Detection of fecal calprotectin in human samples

Calprotectin concentration was determined in each fecal sample using the Calprotectin ELISA kit from Eagle Biosciences (Nashua, NH) according to the manufacturer’s instructions.

Detection of Quorum Sensing molecules in human fecal samples

Changes in quorum sensing molecules were detected in human fecal samples using the same method as described in 3.1.
4.2 Results

Sample collection and Diet analysis of SCI individuals

Gender, level of injury, ethnicity, and age of each individual is shown in Table 4.2.1.

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age</th>
<th>Gender</th>
<th>Ethnicity</th>
<th>Injury level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>Male</td>
<td>Hispanic</td>
<td>T2</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>Male</td>
<td>Hispanic</td>
<td>C5.5</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>Male</td>
<td>Hispanic</td>
<td>C5.5</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>Male</td>
<td>Hispanic</td>
<td>C6.5</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>Female</td>
<td>Non-hispanic</td>
<td>C7</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>Female</td>
<td>Non-hispanic</td>
<td>No SCI</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>Male</td>
<td>Non-hispanic</td>
<td>No SCI</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>Male</td>
<td>Hispanic</td>
<td>No SCI</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>Male</td>
<td>Hispanic</td>
<td>No SCI</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>Male</td>
<td>Non-hispanic</td>
<td>No SCI</td>
</tr>
</tbody>
</table>

*Table 4.2.1- Characteristics of human samples. Subject ID, age, gender, ethnicity, and injury level for all participants in the human portion of the study.*

All five SCI individuals had self-reported history of constipation or other GI problems and complete neurological impairment. All individuals in the SCI group also had chronic injuries (>10) and most have developed a functional bowel routine by this point and thus this population may not represent someone who is recently injured or not regularly managing bowel function. Recent diet was collected from each SCI individual for 24-72 hours before sample collection. (Table 4.2.2,4.2.3)

No diet information was collected from the control individuals. Diet logs were then evaluated and converted into a table format to document fat and carbohydrate intake. The
present data was not analyzed in parallel with microbiota and fungi because the diet information was only available for SCI individuals.

<table>
<thead>
<tr>
<th>Participant number</th>
<th>Meat</th>
<th>Dairy</th>
<th>Fruit</th>
<th>Vegetable</th>
<th>Grain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>x</td>
<td>xxx</td>
<td>xxxx</td>
<td>xxx</td>
<td>xxx</td>
</tr>
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<td>x</td>
<td></td>
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<td>xxxxx</td>
<td>x</td>
<td>xxx</td>
<td>xxxxxx</td>
</tr>
<tr>
<td>5</td>
<td>xxxx</td>
<td>xx</td>
<td>xxxxx</td>
<td>xxx</td>
<td>xxx</td>
</tr>
</tbody>
</table>

*Table 4.2.2*—Food groups for diet profile. Breakdown of meat, dairy, fruit, vegetable, and grain consumption for sampled individuals with an SCI.

<table>
<thead>
<tr>
<th>Participant number</th>
<th>Carbohydrate</th>
<th>Fiber</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xxxxxxx</td>
<td>xx</td>
<td>x</td>
</tr>
<tr>
<td>2</td>
<td>xxx</td>
<td>x</td>
<td>xxxxxx</td>
</tr>
<tr>
<td>3</td>
<td>xxx</td>
<td></td>
<td>xx</td>
</tr>
<tr>
<td>4</td>
<td>xxxxxxx</td>
<td>xxx</td>
<td>xxxxxxx</td>
</tr>
<tr>
<td>5</td>
<td>xxxxxxx</td>
<td>xxxxx</td>
<td>xxxxxx</td>
</tr>
</tbody>
</table>

*Table 4.2.3*— Macronutrient consumption. Macronutrient breakdown of carbohydrate, fiber, and protein for sampled individuals with SCI.

Microbiota sequencing

Human fecal samples from 5 patients with SCI and 5 neurologically intact individuals were sequenced via 16S V4 rRNA gene sequencing on the Illumina MiSeq platform. A total of 153,679 high quality reads were obtained with the final dataset consisting of 5268 OTUs including those occurring once. The read range was 12162-21057 and high-quality reads were classified using the Greengenes reference database. A consensus taxonomy was obtained for each OTU and then the OTU abundances aggregated.
and relative abundance of most abundant ones plotted. In the figure “others” represents lower abundance taxa. The abundance of the OTUs was converted into pairwise dissimilarities (Bray-Curtis index) and non-metric multidimensional scaling (NMDS) analysis conducted to visualize microbiome similarities via ordination plots (Figure 4.2.1).

![NMDS Ordination](image)

*Figure 4.2.1- NMDS Ordination of human samples. NMDS Ordination plot of dissimilarity matrix of bacteria diversity in human fecal samples.*

Official significance testing was done with permutational analyses of variance (Adonis function from vegan package) and alpha diversity calculated using Shannons diversity index (Figure 4.2.3). The most abundant genera were Bacteroides, Blautia, Faecalibacterium, Lachnospiraceae, Ruminococcus, Bifidobacterium, Roseburia, Prevotella, Alistipes, and Ruminococcaceae_unclassified (Figure 4.2.2). Despite a lack of statistical significance, there are differences in the percent relative abundance of the most abundance symbiotic species between control and SCI individuals (Table 4.2.4).
The single most abundant genera in both SCI and control groups was Bacteroides at 11% and 16% of the total abundance respectively. A total of 41% of the abundance in the SCI group was contributed to other less abundant OTUs compared with 31% less abundant OTUs in the control group.
There were no significant differences at the phyla or family levels. At the genus level, Roseburia was significantly decreased in the SCI samples and Mogibacterium was significantly increased. Multidimensional scaling analysis revealed that the samples from the control group were more similar to each other than the gender and age matched SCI samples (Figure 4.2.1).

<table>
<thead>
<tr>
<th>Genera</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevotella</td>
<td>0.3903</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Roseburia</strong></td>
<td><strong>0.0493</strong></td>
</tr>
<tr>
<td>Enterobacteriaceae_unchnssed</td>
<td>0.3567</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>0.8809</td>
</tr>
<tr>
<td><strong>Mogibacterium</strong></td>
<td><strong>0.025</strong></td>
</tr>
</tbody>
</table>

Table 4.2.5- Genera of interest. Genera of interest and associated p-values in human fecal samples.

Further, the control samples also had a tighter diversity profile as shown by Shannon diversity index compared to the samples from SCI persons (Figure 4.2.3).
Quorum sensing molecule levels

To determine changes in bacterial signaling in the gut, the fecal samples from SCI individuals and controls were analyzed for changes in quorum sensing molecules as in the animal studies (Figure 4.2.4).

![Figure 4.2.4. Quorum sensing molecules in human samples. Levels of A.) short-chain (C6) N-acyl homoserine lactone, B.) long-chain (C12) N-acyl homoserine lactone, and C.) Autoinducer 2 in human fecal samples.]

Short and long-chain N-acyl homoserine lactones (AHLs), signaling molecules for Gram negative communication and Autoinducer 2 (AI-2) used for Gram positive communication were measured in intestinal content. Levels of short and long-chain AHLs and AI-2 remained similar between individuals with SCI and controls.

Fungal sequencing

To determine abundance of fungal species in the gut, ITS2 fungal genes were sequenced on an Illumina MiSeq and classified using the Warcup (v2) database. A total of $6.248 \times 10^4$ high quality reads were obtained and the final dataset contained 155 OTUs (including those occurring once with a count of 1) and a read range of 1690 and $3.4125 \times$
$10^4$. Fungi were detected in a total of 5 of the 10 individuals. The identified species present included Candida, Ascomycota, Saccharomyces, and Zygosaccharomyces (Figure 4.2.5).

One SCI sample and one control samples contained a high abundance of unidentified fungi. There were no significant differences in fungi abundance or fungal diversity, nor were there any identifiable associations with microbiota diversity.

**SCFA analysis**

Since short chain fatty acids (SCFAs) play an important role in gut function and are produced by gut bacteria concentrations of these molecules were analyzed in both the SCI and control groups. Specifically, each human stool sample was evaluated for acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, hexanoic acid, and heptanoic acid (Figure 4.2.6).
There were no overall significant differences in SCFA concentrations in SCI stool compared to the control groups.

Calprotectin levels

To examine pathophysiological inflammation in the gut in a non-invasive manner, concentration of fecal calprotectin was determined in 5 SCI patients and compared to 5 neurologically intact individuals. All levels of fecal calprotectin were comparable except
for 1 SCI sample and there was no significant difference between SCI and control groups or association with microbiota changes (Figure 4.2.7).

Determination of blood in stool (Hemoccult)

As another non-invasive method of analyzing gut health, a hemoccult assay was conducted to determine the presence of blood in stool in each of the human fecal samples (Figure 4.2.8). Blood was detected in 2 out of 5 SCI individuals. No blood was detected in the stool of any control individuals.

4.3 Discussion

In these experiments, 16s rRNA microbial sequencing was conducted on human fecal samples to determine any changes to the gut microbiome in persons with an SCI.
Although the alpha diversity of the samples was not statistically significant, there was a larger spread of diversity in the SCI group compared to the controls. This difference was further observed via NMDS ordination plot of dissimilarity, where the points corresponding to the control individuals cluster more closely to each other than those of the SCI samples. This likely indicates a difference in microbial composition will be more prevalent with a larger sample size. The top 10 most abundant genera of bacteria comprised 58% and 68% of sequences from SCI and control groups respectively, with the SCI having
a larger number of less abundance genera. Two different genera were found to be statistically different between groups. Mogibacterium was increased and Roseburia was decreased in relative abundance in the SCI.

Mogibacterium is a Gram-positive, strictly anaerobic and non-spore-forming bacterial genus from the family of Eubacteriaceae. Previously detected in the human gut microbiome and found to be enriched in colorectal cancer patients along with decreases in Faecalibacterium and Blautia. Nothing is known about its role in the gut microbiome 69.

Roseburia is a Gram positive anaerobic inhabitant of the human colon. It has been shown to be increased or decreased by several factors. One of the main functions of Roseburia in the GI tract is its ability to produce butyrate, a short chain fatty acid important in colon health. Increase in Roseburia has been associated with weight loss and reduced glucose tolerance whereas decreases in Roseburia abundance have been linked to irritable bowel disease (IBD) and ulcerative colitis (UC). Roseburia is also of interest because its abundance can be increased non-invasively by consuming specific fibers such as beta-glucan and inulin. Interestingly, despite a significant difference in a butyrate producer, Roseburia, SCFA profiling did not reveal a difference in fecal butyrate between patients with SCI and the control group 70.

Several other genera of bacteria were further analyzed for significance as they have been of interest in previous microbiome research and, thus, might prove interesting when evaluated in these conditions. Bacteroides and Prevotella have been identified as enterotype-defining genera when classifying the human gut microbiome and are both affected by diet. No statistical significance in these genera was observed. Bacteroides is a Gram-negative non-spore-forming obligate anaerobic bacteria that plays a role in human
GI health. It is a part of the phyla Bacteroidetes, one of two main colonizers of the human GI tract. Bacteroides participates in carbohydrate fermentation in the gut and lower levels have been related to the development of IBD. Bacteroides is also capable of regulating T cell growth and cytokine expression via expression of polysaccharide A and is hypothesized to be protective against colitis \(^71\). Although it has been classified as a general symbiont, the role of Prevotella in the gut microbiome is unknown. It has been linked to a plant-rich diet but also to inflammatory conditions. So far, it is most well known as a primary marker of the Prevotella dominated enterotype. Prevotella-dominated enterotypes have been found to have a different fiber utilizing capacity meaning that individuals with more Prevotella may be affected differently by dietary fiber intake \(^72\). This may be important in understanding the GI dysfunction in patients with SCI, as fiber has been contraindicated in this population, and the relative abundance of Prevotella was 3 times higher in the control population than in those with an SCI.

Enterobacteriaceae is a very large family of bacteria including many harmless symbionts as well as familiar pathogens such as shigella. In other mammals, Enterobacteriaceae diversity in the gut is reported as highly variable from individual to individual. Faecalibacterium is a genus of bacteria commonly found in the gut of healthy individuals. The only identified species of this genera, Faecalibacterium prausnitzii, plays a key role in gut health as it is a producer of butyrate from dietary fiber \(^73\). Lower levels of this species had also been correlated with Crohn’s disease, obesity, asthma, and major depressive disorder \(^74\). The appearance of this genera in both the rat model and Gungor’s study in humans, combined with its role in intestinal health makes it species of interest after SCI.
Fecal calprotectin levels were also evaluated. Fecal calprotectin has emerged as a stool marker for intestinal inflammation. Stool markers are desirable for detection of intestinal inflammation because the methods are non-invasive and inexpensive. Calprotectin is a small calcium-binding protein consisting of 2 heavy and 1 light polypeptide chains. The main sources of calprotectin are neutrophils and monocytes. In active IBD, more neutrophils migrate from circulation into the mucosa resulting in the leaking of pro-inflammatory proteins like calprotectin which can be detected in stool. The concentration of fecal calprotectin is directly proportional to the intensity of the neutrophilic infiltration in the gut mucosa. No statistical significance was observed between the SCI and the control groups. However, 2 of the 5 SCI samples displayed blood in the stool. Even though there was no significant difference in calprotectin levels one SCI sample had almost double the concentration. Interestingly, this sample also had blood present in the stool, however, the other blood positive sample had normal calprotectin concentration. A larger sample size would be needed to ascertain if there is a difference in subset of SCI individuals with GI dysfunction not identifiable by hemoccult assay.

Fungi was only detected in five of the ten samples independent of control or SCI status. Further, of these five, two samples from two different individuals comprised mostly of unclassified fungi. This indicates that the pool of identified fungi associated with the human gut may not yet be large enough to meaningfully evaluate fungal composition in the gut. Fungal presence in the gut appears to be largely dependent on recent diet. For example, Candida has been positively associated with a high carbohydrate diet but not amino acids, protein, or fatty acids. Given proper sample size and complete data collection, fungal abundance could be correlated to the diet profile of SCI individuals and compared
to a control population. Further, co-occurrences of fungi such as Candida with bacterial
dysbiosis have been linked with other inflammatory bowel conditions, a link which could
be examined in this population as well.
Chapter 5. CONCLUSIONS AND FUTURE PERSPECTIVES

The initial experiments in animals revealed the importance of different severities and levels of injuries to the spinal cord. The fact that intestinal damage was not observed with the moderate injury indicates that severity of injury may play a role in the extent of the dysfunction and that this should be further researched. Despite observations of significantly different bacterial composition in the samples from SCI injured animals vs controls, it is difficult to correlate these changes without evidence of GI dysfunction in the model. Another important possibility to consider is the fact that, especially with regards to studying a secondary effect like GI dysfunction, verifiable evidence of damage is crucial to evaluate dysfunction in an animal model. Overall, no considerable damage or GI dysfunction was observed in the SCI group via these experiments. It is likely that the injury was not severe enough to result in severe damage to the GI tract which could be determined by conducting future studies with a more severe injury. These experiments could then be repeated with ZO-1 and occludin staining, and vertical histology analysis to determine. SCI is a complex injury that can also change over time. Therefore, a more temporal profile of GI state would be useful in determining the time when GI dysfunction occurs. For example, it is possible that dysfunction and damage are present in the acute phase only and then resolve even though that is not the case in humans. For our purposes, no repeat studies were done to further assess these results to continue to move toward studies with humans. It may be useful in the future to return to the rat SCI model, once more is known about the profile of GI dysfunction in humans with SCI. The animal model could serve as a tool to understand the mechanisms of gastrointestinal damage after SCI and gain knowledge on how to modulate the GI dysfunction after SCI.
The more severe injury model underlined the importance of time of intestinal inflammation also within the context of microbiota changes. By assessing the GI state at 4 and 8 weeks we determined temporary increases in inflammation, but by only sequencing 8-week samples we were unable to compare the inflammation profile with changes in microbiota over time. Conducting all experiments including sequencing at 2-3 time points would allow for better visualization of associations between cytokine levels and microbiota changes as well as GI damage. The complexity of SCI is compounded by additional factors such as gender and antibiotic administration post-injury. By using only female rats we eliminate potential complications with gender-based differential healing but introduce bias toward sex hormones, which have been implicated in SCI recovery in both mice and humans. Another complication is the potential that microbiota variability within each group could be enhanced by cage coprophagia across groups and synergistic effects with gentamicin. While, the experiment could be repeated with a group that did not receive antibiotics, all animals show signs of a UTI post-surgery which can result in a serious infection. It may be necessary to determine the exact effects of the gentamicin in this model if it is used directly for treatment development in later steps. As far as cage coprophagia, animals could be potentially kept in separate cages however this results in an additional financial burden. Coprophagia is a normal process for rodents that plays a vital role in nutrient absorption and completely separating the animals may also have some detrimental mental effects as depression is also been correlated with SCI.

The final set of experiments in humans continued to highlight the significance of a temporal profile in analyzing microbiota changes in diseases states. Further, while
similarities can be seen between our human SCI microbiota results and that of the previous study, a much larger sample number is needed to confidently determine significance of relationships with specific bacteria and fungi, inflammatory state, and GI dysfunction. Assessing microbiota alongside with short chain fatty acids (SCFAs) allowed us to determine if changes in SCFA producing bacteria would correlate to changes in concentrations of the SCFAs themselves.

Additional investigation is needed to fully translate this work to treatment development in humans, but these observations and analysis helped to start understanding the effects of SCI on the gut microbiome and to highlight the significance of correlations between the microbiome in SCI murine models and human patients. While limited by the accuracy of the rodent model, time points post-SCI injury, gender bias, and antibiotic treatment, this knowledge is critical in the design and development of potential microbiome-based therapeutic interventions post-SCI. As far as translation to humans, several bacteria continue to be of interest including those producing short chain fatty acids and correlations with quorum sensing communication. Moving forward, the human repository remain crucial in understanding the breadth and depth of gastrointestinal changes that occur in this population and the animal model will likely become a second source primarily used to test interventions strategies. Continued research into gut microbiota and mycobiota changes associated with SCI will aid in the long-term goal of improving bowel function to enhance quality of life for patients with an SCI.
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