A REVIEW ON MULTIPLE SCLEROSIS:
MARKET, MEDICATIONS, AND MICROGLIA

by

ALLISON TROUTEN

Submitted in partial fulfillment of the requirements for the

degree of Masters of Science

Department of Biology

CASE WESTERN RESERVE UNIVERSITY

May, 2018
Case Western Reserve University  
School of Graduate Studies  

We hereby approve the thesis/dissertation of  

Allison Trouten  
candidate for the Master of Science degree*.  

Committee Chair  
**Michael Benard, PhD**  

Committee Member  
**Christopher Cullis, PhD**  

Committee Member  
**Jean Welter, PhD**  

Committee Member  
**Vivek Shenoy, PhD**  

Date of Defense  

**26 March, 2018**  

*We also certify that written approval has been obtained for any proprietary material contained therein.*
# Table of Contents

Introduction ............................................................................................................. 1  
Multiple Sclerosis Epidemiology ........................................................................... 2  
Cellular Mechanisms at Work in Autoimmunity ............................................... 10  
Microglia’s Role in Multiple Sclerosis ................................................................. 15  
Animals Models of Multiple Sclerosis ................................................................. 17  
Proposed Genetic Causes of Multiple Sclerosis ............................................... 18  
Current Treatments for Multiple Sclerosis ......................................................... 25  
Developmental Treatments for Multiple Sclerosis .............................................. 33  
Drug Development .................................................................................................. 39  
Specialized FDA Programs .................................................................................... 44  
The Cost of Innovation .......................................................................................... 47  
Major Players in Biopharmaceutical Development ............................................ 49  
The Future of Drug Development .......................................................................... 51  
Contract Research Organizations .......................................................................... 53  
Renovo Neural: A boutique neuroscience CRO .................................................... 54  

*In situ* hybridization using RNAscope® ............................................................ 58  

  *Probe Design:* ..................................................................................................... 60  
  *Methods:* ........................................................................................................... 61  
  *Results:* ............................................................................................................ 63  
  *Discussion:* ...................................................................................................... 63  

Conclusion .............................................................................................................. 70  
Bibliography ............................................................................................................ 73
List of Tables

Annual cost of common MS drugs ........................................ 32
Efficacy comparison of MS drugs ........................................ 32
Experimental breakdown of probes ................................. 65
List of Figures

Occurrence of typical symptoms of MS ......................................................... 8
Global disease prevalence of MS ................................................................. 9
Most common MS diagnostic criteria used by region .......................... 10
Stimulation of T cell by MHC and T cell receptor interaction .......... 13
Clonal deletion in the thymus ................................................................. 14
Utilizing SNPs to uncover disease susceptibility genes ............. 24
RNAscope®’s Double Z probe technology ............................................. 62
Positive control probes on control liver tissue validate staining process .................................................................................. 66
Positive control probes on brain tissue validate tissue preparation ................................................................................................. 67
Experimental microglial probes on control liver tissue validate probes .......................................................................................... 68
Experimental microglial probes on anterior commisure brain tissue .............................................................................................. 69
Experimental microglial probes on corpus callosum brain tissue .............................................................................................. 70
Many people have helped this thesis come to fruition. I would like to thank the team at Harrington Discovery Institute for giving me my first experience in the biotechnology field and for teaching me the ins and outs of drug discovery. I would like to thank the team at Renovo Neural who have given me all the resources to make this thesis and its research possible. Special thanks to Simon Lunn, Chris Ryan, Paul Harris, Luther Loose and Kelly Simmerman for their work on my RNAscope® research and development project at Renovo. I would like to thank the fine educators at Case Western Reserve University for their inspiring teaching, especially those classes that gave me the knowledge to carry out this research. My thanks to Ed Caner, who kindly introduced me to both my internships. I’d like to thank my family and friends who have supported me through my education. Lastly, I’d like to thank Dr. Bernard, Dr. Cullis, Dr. Welter, and Dr. Shenoy for participating on my committee and guiding me through the process.
A Review on Multiple Sclerosis: Market, Medications, and Microglia

By ALLISON TROUTEN

Abstract

Multiple sclerosis (MS) is a debilitating neurodegenerative disease characterized by the immune-mediated destruction of myelin in the central nervous system (CNS). Microglia are unique surveyors of the CNS and serve as first-responders to protect the brain from attack. These cells have multiple phases which can be neuroprotective or neurodestructive in nature, making them an interesting cell to investigate in MS pathogenesis. Renovo Neural, a Cleveland-based contract research organization, is currently validating effective RNA probes for these cell subtypes on the RNAscope® platform, an *in situ* hybridization assay that allows for identification of an RNA sequence of interest within a fixed tissue or cells. Once able to visualize these distinct subtypes, Renovo will be able to test the effect of drugs that target the microglia populations.
Introduction

Multiple sclerosis is an autoimmune neurological disorder that causes a loss of myelin in the brain and spinal cord, leading to disruption of nerve impulses. Since its formal classification in 1868, MS has proven to be a conundrum to patients and researchers alike (89). A specific problem that needs to be addressed is the lack of reliable methods to tag neural cells that play integral roles in disease pathology, namely microglia. Microglia are phagocytic cells that constantly patrol the CNS parenchyma. Depending on which phase these cells are in, they work to repair and clear debris from the CNS or contribute to its destruction and initiate the inflammatory response. Given their unusual dual nature, microglia serve as an interesting cell to track through the progression of neurodegenerative diseases.

Renovo Neural, a preclinical contract research organization (CRO), has been doing their part to create a cure for MS and other neurodegenerative diseases since its founding in 2008. Renovo is constantly developing new assays to provide clients with the proper tools to investigate this complex field of research. Histology is a widely utilized assay in research but is only as valuable as the reliability of the molecular tags for a protein of interest. Unfortunately, histological probes for M1 and M2 microglia have proven unreliable and irreproducible (15). Consequently, Renovo has chosen to tag these microglia using the RNAscope® in situ hybridization platform. RNAscope® works through the binding of unique RNA probes to a target sequence, followed by amplification of the signal and binding of fluorescent labeling molecules to allow for
visualization (41). This assay allows for the quantitative measurement of RNA levels for virtually any target sequence of interest. Here we discuss the validation of probes for microglial subtypes, the genetics behind MS, current treatments for the disease, as well as CRO’s essential role in advancing a potential drug from the laboratory bench to patient bedside.

**Multiple Sclerosis Epidemiology**

MS is a neurological disorder that involves the immune system’s degradation of self-myelin, that is the patient’s myelin. Without the insulation of myelin, electrical impulses traveling within the CNS are disrupted, leading to a variety of symptoms. Common symptoms of MS include fatigue, numbness, gait difficulty, spasticity, weakness, vision problems (specifically Uhthoff’s syndrome), dizziness/vertigo, tremors, bladder/bowel problems, sexual problems, pseudobulbar affect, pain, cognitive or emotional changes, and depression, although some patients display little to no symptomatology (6, 57, 58). Less common symptoms associated with the disease are trouble speaking or swallowing, breathing problems, itching, headache, and hearing loss (57) (Figure 1). The disease was fittingly named for the many lesions or scar tissue, also called “sclerosis”, seen in the white matter of patients (106).

The National Multiple Sclerosis Society estimates that there are at least 2.3 million people living with MS worldwide (56). MS was first characterized in 1868 by Jean Martin Charcot, who then attempted to treat the disease with various metals,
chemicals, and electrical therapy (89). An epidemiological review conducted in the mid-1990s found clear patterns in the geographic distribution of the MS prevalence. In the United States, residents of the northern states have a risk factor above the national mean, while the southern states are comparatively lower risk (48, 106). In the United States, about 95% of MS sufferers are Caucasian, 5% are African American, and less than 1% are Hispanic (106). People from Scandinavian and Finnish descent demonstrate a significantly higher risk factor than those of other ethnicities (48). MS also has a gender bias towards women and interestingly, a child who moves from one area to another adopts the risk level of their new residence but if the relocation occurs post-puberty, the young adult maintains the risk level of their original residence (48). This may suggest some sort of hormonal involvement and genetic implications in disease pathogenesis.

In 2008, the World Health Organization published an “MS Atlas,” which included more epidemiological data as well as the social and economic impact of MS (6). The study pulled data via questionnaire from nearly 90% of the countries in the world. The data was analyzed and countries were grouped by geographical location as well as gross national income (6). The MS Atlas states the prevalence, or proportion of the world’s population with MS, is currently 30 per 100,000 and regions with the highest prevalence are as follows, in descending order: Europe, Eastern Mediterranean, Americas, Western Pacific, Southeast Asia, and Africa (6). Individual countries with prevalence above the world-average include: Hungary, Slovenia, Germany, the United States of America, Canada, Czech Republic, Norway, Denmark, Poland, and Cyprus (6) (Figure 2). The global incidence of MS, or rate of new cases, is 2.5 per 100,000 and countries with notably
increased incidence compared to the global incidence include: Croatia, Iceland, Hungary, Slovakia, Costa Rica, United Kingdom, Lithuania, Denmark, Norway, and Switzerland (6). Generally speaking, the frequency of the disease increases with increasing distance from the equator in both hemispheres, but more markedly in the Northern hemisphere (6).

The study indicated the number of MS cases worldwide to be about 1.3 million, however their data collection was not exhaustive and excluded Russia (6), leading one to believe the estimate of 2.3 million by the National MS Society is closer to the actual statistic (56). The MS Atlas agreed with an earlier study conducted in the 1990s in that MS affects women nearly twice as often as men (6, 48) and determined the average age of onset is 29.2 years globally (6). Interestingly, the study found that higher income areas had a higher incidence and prevalence of the disease (6), although this could be a misleading statistic as MS can be misdiagnosed in lower-income countries with fewer neurologists specializing in the disease (6). Although all countries surveyed had access to magnetic resonance imaging (MRI) and most had access to a spinal tap and evoked potential diagnostics, the authors stressed that availability is not the same as accessibility (6).

Beyond statistics on disease prevalence, incidence, and diagnosis, the “MS Atlas” also collected data on the resources available to MS patients and their families or caretakers, including printed information, support groups, transportation services, respite care, and programs to keep patients in the workforce (6). In addition to the socio-economic impact, there are psychological ramifications for not only the patient, but family and caregivers as well. Given that the average onset of the disease is before
30 years of age, healthcare workers and caregivers must not merely manage the
disease, but also be cognizant of the patient’s altered lifestyle. Most patients are of the
age to be active in the workforce, but this chronic disease may prevent them, and often
their family members/caregivers, from remaining in the workforce (6). This fact stresses
the importance of government-funded respite care, transportation services to and from
work, as well as a flexible work environment for patients (6).

A multitude of proposed causes have been researched, from family size to
Epstein-Barr virus (48) to smoking, but no studies on the cause of MS are conclusive. A
notably interesting theory is vitamin D deficiency. Vitamin D is a fat-soluble vitamin and
is most significantly sourced indirectly from sunlight. The 7-dehydrocholesterol in skin is
converted to pre-vitamin D3 upon exposure to ultraviolet radiation, which is then
transported to the liver by specialized proteins in the blood (4). Following multiple
hydroxylations, vitamin D is created and utilized by the body (4). Vitamin D deficiency is
one of the most heavily studied causes, as most high-risk countries are in areas of low
sunlight exposure. Studies have been conducted using the cuprizone model with vitamin
D3 as a treatment, resulting in a decrease in demyelination in the white matter
supposedly due to decreased activation of microglia (98).

Multiple sclerosis can be broken down into four distinct subtypes following the
initial clinically isolated syndrome (CIS): relapsing-remitting MS (RRMS), primary
progressive MS (PPMS), secondary progressive MS (SPMS), and progressive relapsing MS
(PRMS) (92, 106). CIS refers to an incidence in which some symptoms of demyelination
occur, but not enough to support a definitive MS diagnosis (92). RRMS constitutes the
majority of MS cases and is characterized by periods of neurological deterioration, known as relapses, followed by a phase of temporary recovery (92). The amount of time the relapse or recovery lasts is unique to each case (92). A relapse is defined as the occurrence of a new neurological manifestation, such as weakness in the extremities or vision changes, that persists for over 24 hours and has no other probable cause (24). PPMS is identified by constant neurological decline without any remission/recovery and is the second most common subclass of MS (92). SPMS can be viewed as a fusion of relapsing MS and progressive MS – as it first goes through alternating relapse and remission phases characteristic of the former, before showing signs of a steady gradual decline characteristic of the latter (92). About 40% of patients initially diagnosed with RRMS will later be diagnosed with the secondary progressive form of the disease (106). Interestingly, SPMS is more common in males which is contradictory to the general epidemiology of the disease (106). Similarly, PRMS involves a continuous decline interspersed with relapses, but the neurological deterioration is more constant when compared to SPMS (106).

Diagnostic tools for MS include MRI, cerebrospinal fluid (CSF) analysis, and evoked potential tests (21). The most telling and widely used diagnostic is MRI, which allows physicians to visualize the brain and spinal lesions characteristic of the disease (22). With advances in MRI technology, these lesions can be quantitatively assessed to track disease progression (22). Elevated levels of certain antibodies such as IgG, other immunoglobulins associated with oligoclonal bands, and components of myelin in the CSF, which nourishes and protects the brain and spinal cord, is also indicative of MS
Evoked potential tests assess fine changes in the electrical activity of the brain while the patient is subject to visual, auditory, or sensory stimuli (22). Lastly, in-depth neurological exams are used to rate patients on a scale of 1.0-10.0 on the Expanded Disability Status Scale, which is used to track physical disability (106). Although these diagnostic tools are highly effective, none of these tests are entirely conclusive.

Some discrepancy exists amongst clinicians on the criteria that constitutes a MS diagnosis. When Charcot first characterized the disease, he used what is known as “Charcot’s Triad” to diagnose his patients based on the development of double vision, slurred or garbled speech, and balance problems (106). Since then, multiple diagnostic standards including the McDonald, Poser, and Schumacher criteria have been proposed (6). These criteria models have evolved with advances in clinical technology. The most recent revision of the McDonald criteria, currently considered to be the most reliable method of diagnosis, requires a certain number of clinical attacks and confirmed lesions via MRI, sometimes requiring additional diagnostics such as positive CSF analysis (90). Prior to the development of MRI technology, the Poser criteria was used to diagnosis the disease. This criterion categorizes a case as “clinically definite MS,” “laboratory-supported definite MS,” “clinically probable MS,” or “laboratory-supported probable MS,” based on results of evoked potential tests to measure the electrical activity of the brain or assessment of oligobands in cerebrospinal fluid (21). The first criteria for diagnosing the disease was known as the Schumacher criteria. The Schumacher criteria was based solely on clinical observations, such as patient age, multiple attacks, clinically apparent damage of the white matter via inflammatory markers in cerebrospinal fluid,
and irregular neurological behavior (21, 106). 66% of the global population relies on the McDonald criteria for MS diagnoses, followed by 31% and 3% utilizing Poser and Schumacher criteria, respectively (Figure 3) (6).

Figure 1. Occurrence of typical symptoms of MS. The disease can cause a multitude of symptoms, but results show that motor function or weakness is the most common worldwide (Atlas MS, WHO, 2008, pg 31).
Figure 2. Global disease prevalence of MS. World map shows color-coded prevalence per 100,000 people. This map shows a higher prevalence in countries in the Northern hemisphere. Areas in white indicate countries where no information could be collected (Atlas MS, WHO, 2008, pg 15).
Figure 3. Most common MS diagnostic criteria used by region. The bar chart above compares global usage of the McDonald (red), Poser (light blue), and Schumacher (navy). Results show that the McDonald criteria is the most popular in all regions except for the Western Pacific. This may be linked to MRI access, which is necessary to assess disease by the McDonald criteria. (Atlas MS, WHO, 2008, pg 21).

Cellular Mechanisms at Work in Autoimmunity

A disease is classified as autoimmune when there exists a specialized adaptive immune response to a non-foreign or self-antigen (46). Antigens are proteins or fragments of proteins displayed to immune cells so that the appropriate response can be triggered. In autoimmune disorders, the immune system mistakenly interprets the
patient’s tissue as alien and thus triggers a response to rid the body of the antigen. Since this is endogenous tissue that cannot be removed, the patient’s immune system is constantly in an active inflammatory state. Autoimmune diseases can be systemic or tissue specific and are often driven by antigen-specific T cells (46).

Autoimmunity is often driven by particular major histocompatibility complex, or MHC, genotypes (46). MHCs play a role in an antigen presenting cell’s ability to display certain antigens. Without a T cell’s recognition of an antigen displayed on the proper MHC, an immune response cannot be triggered (Figure 4). There are two classes of MHC: MHC I, which is expressed on all nucleated cells and interacts with receptors on cytotoxic/CD8+ T cells, and MHC II, which is also expressed on antigen presenting cells (APCs) and interacted with helper T/CD4+ T cells. The interaction between MHC and a T cell receptor is what stimulates proliferation of T cells primed for a specific antigen.

Early on in development, a process known as clonal deletion occurs in the thymus. This process begins when immature double negative T cells enter the thymus via the bloodstream after their genesis in the bone marrow and develop into double positive cells (Figure 5). These immature double positive, meaning CD4+ and CD8+, T cells enter the thymic cortex where they are exposed to cortical thymic epithelial cells (cTECs) (51). cTECs express a variety of MHC molecules, both class I and class II. If a T cell can weakly bind to class MHC I, it becomes a CD8+ cytotoxic T cell. In contrast, if a T cell weakly binds to MHC II, it is destined to be CD4+ helper T cell (51). Any T cells demonstrating strong MHC bonds as well as any unbound T cells are killed via apoptosis, as these cells will be too reactive or unhelpful in immune response, respectively (51).
person’s unique MHC may not bind above threshold affinity to T cells to cause deletion at this phase and thus the T cells that recognize this MHC survive. If this MHC is able to present self-antigen to the mature T cells, autoimmunity will occur (46).

Single positive T cells leave the cortex to enter the medulla of the thymus where the encounter cells known as medullary thymic epithelial cells (mTECs), which express a specialized autoimmune regulator gene that confers them the ability to present these immature T cells with a variety of tissue-specific antigens in the context of MHC, educating the T cells on what proteins are endogenous to the body (51). If a T cell binds with high affinity to a self-antigen, the T cell is eliminated; if a T cell makes no contact with an antigen, it undergoes death by neglect; if a T cell binds with low affinity, the T cell survives. Errors in this process can lead to autoreactive T cells (46).

Immune cell transfer experiments further substantiate the role of MHCs and T cells in the development of autoimmune disease. The EAE model of MS, which uses a myelin protein plus adjuvant to mount an immune response against the animal’s myelin, is a prime example (46). EAE can be transferred to an unaffected mouse by exposing the naïve mouse to the infected mouse’s purified T cells so long as the two animals are MHC compatible (46). This demonstrates the importance of appropriate T cell reactivity and the T cell’s critical role in immune response.
Figure 4. Stimulation of T cell by MHC and T cell receptor interaction. APCs are phagocytic cells whose main function is to display antigen on its surface with either MHC I or II (yellow). The APC first engulfs the protein (green), breaks it down, and displays a portion of it on its surface. In order to activate a T cell to proliferate, the T cell receptor (light blue) must be able to interact with this MHC-antigen complex. Costimulatory molecules and cytokines associated with T cell activation are not shown for simplicity.
Figure 5. Clonal deletion in the thymus. Immature double negative T cells enter the thymic cortex from the bloodstream and develop into double positive cells. cTECs expose these immature T cells to various types of MHC and those that bind with low affinity develop into single positive T cells, which migrate to the medulla of the thymus to interact with mTECs. mTECs are specialized APCs that are able to display a variety of self-antigens on their surface. T cells with high affinity interactions or no interactions with the mTECs are destroyed and the low affinity cells mature into immunocompetent T cells. Light brown indicates the medulla region of the thymus and the darker brown area represents the cortex.
Microglia’s Role in Multiple Sclerosis

Researchers have diligently been searching for the cell responsible for the neurological damage associated with MS for decades. Astrocytes, T cells, macrophages and many more have been tested as the culprit for the disease with inconclusive results. An interesting target candidate is microglia, which are glial cells that constantly patrol the parenchyma for any indication of damage (15). They act as the first responders to assaults to the brain or spinal cord and serve as APCs to other immune cells in the central nervous system (74). Microglia have multiple phases with distinct functions, although some argue if these represent distinct cell types rather than distinct phases of the same cell (73). Inactivated patrolling microglia have long processes which retract inward once they are activated (74).

Although debated among the research community, the literature supports that there are four distinct subtypes of microglia: M1, M2a, M2b, and M2c (97). The M1 phase is said to be “classically activated” and characterized by their reactive oxygen species production in response to proinflammatory cytokines, such as TNFα, IL-6, IL-1β, interferon-γ, as well as chemokines (97, 15). Classically activated microglia are associated with a Th1 response, a response known to promote phagocytosis, cell-mediated immunity, and stimulation of macrophages (78). This subtype of microglia is thought to further neuroinflammation and tissue destruction.

M2a microglia are “alternatively activated” and are responsive to IL-4 and IL-13. They secrete anti-inflammatory cytokines and growth factors, leading to repair of the damaged axons via phagocytosis of damaged cells and tissue (97). M2b microglia are
“type II alternatively activated” and M2c microglia have “acquired deactivation”, but still act in a neuro-reparative nature (97). The M2b subtype is activated by the stimulation of certain immunoglobulin Fc receptors, while the M2c phenotype can be activated via IL-10 and glucocorticoids (97). M2 microglia and its associated subtypes all stimulate a Th2 response, which involves an increase in humoral response/antibody generation, stimulation of eosinophils, and decreased macrophage functionality (78). Although there are many markers for microglia, reliable markers with the ability to detect microglia subtypes have yet to be developed. Microglia’s dual functionality could make it a very effective target if researchers are able to push these resident CNS cells to their neuroprotective phenotype.

It is evident that cytokines play a pivotal role in signaling microglia to shift phases, as well as a role in their function once their cell fate is determined. Experiments utilizing a disease model of MS in IL-4 knockout mice showed lower clinical scores than wild type mice. When IL-4 was restored in knockout mice via viral transduction, clinical scores improved (15). In a stroke model experiment using IL-4 deficient mice, the knockout mice had a greater area of infarct or dead tissue when compared to IL-4⁺ mice (15). These experiments simply and elegantly display the M2 microglia’s role in neurorepair.
Animals Models of Multiple Sclerosis

Animal models have played a crucial role in furthering our knowledge of MS, especially rodent models. One of the most common rodent models of MS is experimental allergic encephalomyelitis, better known as EAE. This model was the first to be developed for MS and works by immunizing the animal with myelin fragments, such as myelin oligodendrocyte glycoprotein, myelin basic protein, and proteolipid protein (74, 89). These proteins are mixed with an adjuvant and this emulsification is co-administered with pertussis toxin during induction (74). In doing so, the animal’s CD4+ T “helper” cells are primed with a myelin antigen and thus the immune system is triggered to attack the animal’s native myelin. EAE induces MS-like lesions and inflammation in the brain and spinal cord. Post-induction, animals are monitored and scored based on the degree of disability, followed by post mortem analysis such as histology. Importantly, EAE studies have been used to demonstrate microglia’s dual function as a neuroprotective and neuro-destructive cell type (74).

Another useful rodent animal model of MS is the cuprizone model. Cuprizone, administered via cuprizone-laced chow, binds to copper and is noxious to oligodendrocytes, the cells responsible for myelination in the CNS. Inhibiting the oligodendrocytes leads to demyelination in many areas of the brain, including the corpus callosum and the cerebral cortex (74). Once rodents are weaned off of cuprizone-laced chow, remyelination can be observed (74).

The lysolecithin demyelinating model involves direct stereotactic injection of the detergent lysophosphatidylcholine into the white matter of the brain, such as the...
corpus callosum and the thoracic and lumbar spinal cord funiculi (74). Demyelination is
evident almost immediately and lasts for about three weeks (74). The lysolecithin model
is unique in that it does not activate T cells as the EAE and cuprizone models do (74).
This model is time-consuming and expensive as compared to the two other
aforementioned animal models, as it requires surgical injection of chemicals.

Proposed Genetic Causes of Multiple Sclerosis

A goodly amount of research has been dedicated to identifying disease
susceptibility genes and how environmental factors can epigenetically influence disease
risk. According to the National Institute of Health’s Genetics Home Reference, certain
mutations in the **HLA-DRB1** and **IL7R** genes have been linked to an increased risk of MS
(29, 59). The human leukocyte antigen gene family, which **HLA-DRB1** belongs to, allows
for the immune system to distinguish between self and foreign proteins, while **IL7R**
codes for a part of a receptor found within the membranes of multiple immune cells,
notably T cells, that is known to impact their proliferation (59). **HLA-DRB1** codes for the
beta chain of a heterodimer receptor that is involved in tissue recognition, as it is the
humanized adaptation of the well-known MHC (38). **IL7RA** has been proven to influence
V(D)J recombination, a complex shuffling and excision of genes in somatic cells that
ultimately leads to the diverse repertoire of B and T cell receptors (52, 95). MS has no
clear mode of inheritance, but the disease does tend to recur through generations within a pedigree (59).

Investigating susceptibility genes typically involves surveying single nucleotide polymorphisms, or SNPs. SNPs occur when one nucleotide within a particular gene differs from the nucleotide found in the general population. These unique nucleotide swaps occur roughly 1 in 300 nucleotides and can be utilized to flag genes thought to be linked to an illness or defect (99). A single SNP is often not very informative, so researchers look at the haplotype of a subject, which consists of a combination of SNPs within a defined region of DNA (Figure 6) (54). When attempting to identify gene variants associated with a disease, researchers observe and compare haplotypes within genes with established roles in disease pathogenesis (54). The DNA flanking a gene of interest is tagged using SNPs as well, as some of these polymorphisms occur in the regulatory region or on a seemingly unrelated gene that may modulate the function of the gene of interest (54, 99). Studies examining susceptibility genes must look at a large population so that all common haplotypes are included and correlations between the genes and disorder can be statistically sound.

A study by A. Alcina et al investigates an established risk variant of \texttt{HLA-DRB1}, \texttt{HLA-DRB1*1501}, and its relation to expression levels of the \texttt{DRB1} gene (3). Expression quantitative trait loci (eQTLs), that is, loci that regulate the expression of mRNA of another gene, within the HLA region were observed (3). The HLA gene family, all adjacent to one another in a long stretch of DNA, has strong linkage disequilibrium, suggesting that these genes are linked (3). Using a database of known and documented
SNPs from the HapMap project, the researchers identified six SNPs associated with DRB1 expression that were found in all human populations regardless of ethnicity (3). This publication performed statistical analysis on expression level data via microarray and RNA-Seq. Microarray is performed by the attachment of DNA of interest to a multi-well plate, which then hybridize to fluorescently labeled cDNA probe that was derived from sample mRNA. RNA-Seq technology also involves synthesizing cDNA from sample RNA followed by fragmentation (76) Flanking “adaptor” sequences are bound to the cDNA fragments, followed by local amplification of single cDNA molecules on flow cells and sequencing (76).

The results of this study showed that carriers of the T allele had higher expression of DRB1 than those without the allele, suggesting a dominant effect (3). To assess whether the eQTLs that affect DRB1 expression overlap with the SNPs related to an increase in MS risk, researchers compared genome wide association study data from the International Multiple Sclerosis Genetic Consortium and concluded that three SNPs were linked to the eQTLs of DRB1 (3). When investigators evaluated the eQTLs of other genes in the HLA family and once again crossed this data with data from the Consortium, they found that the most robust MS association genes were eQTLs for DRBS5, DRB1 and DQB1 amongst Caucasians (3). Other SNPs linked to autoimmune disorders such as inflammatory bowel disease and type 1 diabetes were found to be eQTLs for other HLA class II genes (3). Although many believe the link to HLA indicates an involvement in antigen presentation, the results of this study indicate that expression
levels of other genes may add complexity to the mechanism of this susceptibility gene (3).

A study by S. Caillier et al aimed to identify the telomeric border of the HLA class II gene region and to determine which gene within HLA region is truly leading to increased risk of MS, as it is difficult to parse individual SNPs within a haplotype due to linkage disequilibrium (14). Researchers used an African American dataset as a representative low-risk population and assessed $HLA-DRB5$ and seven SNPs marking $MICA$, $AGER$, $BTNL2$, and $HLA-DRA$ genes within the MHC region of the genome (14). Over 1,500 African Americans and over 400 Caucasians, both MS patients and healthy controls, were genotyped by PCR and then statistically assessed (14). After comparison of haplotypes with various combinations of $HLA-DRB1$ and $HLA-DRB5$, results showed $HLA-DRB1$ as the main susceptibility gene and $AGER$ as a lesser yet distinct susceptibility gene (14). Additionally, subjects possessing the $DRB5^{\ast\text{null}}$ mutation had an elevated risk of SPMS (14).

Other researchers considered genes that may be influenced heavily by environment. Vitamin D has been arguably the most researched environmental factor for MS susceptibility, as it would align with the higher incidence and prevalence observed at regions further from the equator where there is less sunlight exposure. Research conducted by S. Ramagopalan revealed that vitamin D response element or VDRE, located within the promoter region of $HLA-DRB1$, can modulate the expression of $HLA-DRB1$ (72). The study involved electrophoretic mobility shift assay, chromatin immunoprecipitation, and transfected cell cultures in its investigation.
The electrophoretic mobility shift assay uses the concept of separating cellular proteins by their size to determine which have been bound by a labeled DNA probe (44). In this case the protein was vitamin D receptor and retinoic acid receptor beta, a requirement for VDRE binding, and VDRE acted as the probe (72). Chromatin immunoprecipitation is also based on DNA-protein interaction and begins with the crosslinking of these bonds, followed by random fragmentation of the exposed DNA (16). Specialized antibodies are then used to tag the protein-bound DNA fragments, crosslinks are released, and PCR is performed to sequence the DNA that had been bound to the probe (16).

Although HLA-DRB1 is the most well characterized MS susceptibility gene, research indicates that other gene variants may predispose a person to the disease. A. Traboulsee et al indicated that IL7R and IL2RA are also linked to an increased risk of MS (91). The study involved genotyping thousands of diseased and healthy subjects and then performing a variety of statistical analyses on the data (91). IL2RA did not have a significant association with MS in the whole population sample, however there was an association found in patients with no prior family history of the disease (91). Similarly, IL7R did not show a statistically significant link to the composite group, but a robust link was found between the gene and patients with progressive MS (91). This may suggest that susceptibility genes may be specific to MS disease subtypes as well as hereditary versus spontaneous cases.

A gene’s presence within a genome does not necessitate that the gene’s functional protein will be expressed, as a given gene undergoes many modifications as it
is converted to a mature protein. G. Galarza-Muñoz et al were interested in DEAD Box Polypeptide 39B’s, better known as DDX39B, impact on the splicing of IL7R and how this impacts the protein load as well as the form of the protein (28). The most marked experiment displaying this was cell cultures whose DDX39B had been silenced by small interfering RNA, leading to the skipping of exon 6 during splicing (28). This splicing pattern was shown to lead to increased levels of soluble IL7R protein, which has been shown to worsen clinical scores in the EAE model (28).

When a topic is heavily researched, it is often effective to pool data from many studies and perform meta-analysis. H. Liu and colleagues did just that, merging data from 27 studies to investigate four SNP variants of IL7A, three of which were found to be linked to increased risk of MS (52). Other studies directly collected tissue from patients to analyze expression of a suspect gene, like the publication by F. Lundmark et al. Following up on previous studies that identified SNPs and haplotypes within the IL7R gene that were found to be associated with increased disease risk, researchers collected blood and CSF from currently untreated MS patients, healthy controls, and patients with other noninflammatory neurological diseases to measure gene expression of these proposed susceptibility genes clinically (53). Although no significant difference in IL7R expression in blood samples from the three groups was observed, there was an increased expression of IL7R and IL7 in MS patients compared to the other disease group, suggesting this may be an MS-specific gene target (53).
Figure 6. Utilizing SNPs to uncover disease susceptibility genes. A large population is genotyped and common SNPs are identified. In this example, position five within the gene is either an A or a G. A solitary SNP is often not very informative so researchers combine multiple SNPs to create haplotypes. The example haplotype here has SNPs at positions five, eleven, and fourteen. These particular combinations of SNPs are studied in a large population to determine if certain haplotypes are more prone to a phenotype.
Current Treatments for Multiple Sclerosis

Although no effective cure for MS has been developed, 15 disease-modifying medications have been approved by the Federal Drug Administration, or FDA, for the treatment of relapses associated with the disease, decreases in the lesion growth, and lessening the degree of disability (24). The National MS Society stresses that these drugs can have a profound impact on disease progression and thus these medications must be administered early on in diagnosis (24). The treatments are often categorized by method of administration (intramuscular/subcutaneous injection, oral, etc.) and pharmaceutical giant Biogen accounts for a third of the treatments on the market (24). There are a wide range of common side effects associated with these medications, which include: headache, flu-like symptoms, skin irritation near injection location, decreased white blood cell count, chest pains, trouble breathing, flushing, depression, throat pain, upper respiratory infections, raised liver enzymes, swollen lymph nodes, colds, bronchitis, nausea, diarrhea, thinning of hair, pain in the abdomen, extremities, and back, sinusitis, trouble sleeping, fever, fungal/viral infections, vomiting, nasal congestion, cardiac arrhythmia, constipation, discolored urine, urinary tract infection, changes in menstruation, increased risk of certain cancers, depression, vaginitis, and gastroenteritis (24). Injectable medications include Biogen’s Avonex®, Bayer’s Betaseron®, Teva’s Copaxone®, Novartis’s Extavia®, Sandoz’s Glatopa™, Biogen’s Plegridy®, Biogen’s Zinbryta® and EMD Serono and Pfizer’s collaborative Rebif® (24). Oral treatments include Aubagio® from Sanofi Genzyme, Gilenya® from Novartis, and Tecfidera® from Biogen (24). MS medications administered via intravenous infusion
include Sanofi Genzyme’s Lemtrada®, Novantrone®, Genentech’s Ocrevus®, and Biogen’s Tysabri® (24). There are three divisions of MS drugs including those that relieve the symptoms of the disease, treatments to manage relapses, and disease-modifying medications that lessen the frequency and severity of relapses and reverse the advancement of the disease (106).

A typical MS patient in the United States spends about $30,000 annually on medication, while drug prices in other countries are substantially lower and their annual cost is about $14,000 (106). With an estimated 90% of patients in the United States and Europe being administered a disease-modifying medication, the MS market is a very lucrative one and anticipated to be valued at $14 billion globally in 2018 according to BBC (106). The majority of medications on the market are for the treatment of RRMS, the most common subtype of the disease and thus representing the largest submarket (106). The progressive forms of the disease have fewer treatment options and represent an essentially underserved market.

Avonex®, a weekly intramuscular injection of interferon β-1a, was introduced to the United States market in 1996 (106). Although the drugs exact mechanism of action is unknown, this cytokine seems to modulate an anti-inflammatory environment in the brain and spinal cord leading to a decrease in relapses and active lesions, as well as the prevention of further physical impairment (60, 100). Avonex® has three routes of administration including: a prefilled syringe, a pen injector, or a powder that is ultimately converted into its injectable form (60). The joint venture Rebif®, approved in the United States in 2002, also contains interferon β-1a (75) and Plegridy®’s, FDA-
approved in 2014, active component is a pegylated version of this cytokine (68). Rebif® is taken three times weekly (75), while Plegridy® is dosed once every two weeks (68).

Betaseron® is very similar to Anovex®, its active ingredient being interferon β-1b.

This medication, which was the first disease-modifying drug to be approved for relapsing MS in 1993, is administered via an autoinjector pen every 48 hours and each dosing can be tracked on a phone application, which shares the information with a patient’s healthcare providers (61, 84, 106). Comparable to the above, Betaseron® is thought to function by decreasing pro-inflammatory molecules, increasing anti-inflammatory molecules, and possibly by inhibiting certain immune cells from traversing the blood brain barrier, although there is no confirmed mechanism of action (28). This drug was proven to reduce the recurrence of relapses, increase the time between relapses, and decrease the amount of new brain lesions (61, 84). Extavia®, FDA-approved in 2009 (24), has an identical active ingredient and thus functions in the same manner and has the same dosing frequency as Betaseron® (33).

Copaxone® is another injectable relapsing MS drug that was approved by the FDA in 1996 whose active compound is glatiramer acetate. This ingredient’s exact mechanism of action is not clear, but it is believed to be immunomodulatory (70). Its effectiveness was shown in its ability to prevent disease-like pathology in EAE-induced mice. Copaxone® is an injectable formulation that can result in reduction in the number of relapses, as well as a decrease in the quantity lesions (70). Glatopa™ is a generic medication for relapsing MS that also contains glatiramer acetate and was approved in
the United States in 2015 (31). Both of these drugs are taken three times weekly (70, 31).

*Aubagio®* is a daily oral prescription that functions by restricting the production of pyrimidines by blocking dihydroorotate dehydrogenase. It is thought that in blocking this enzyme’s function, active T and B cells are unable to reproduce and cytokine release is suppressed, while resting lymphocytes are unaffected (7, 8, 106). The relapsing MS drug, FDA approved in 2012, has been proven to reduce new lesions, assuage relapses, and reduce the degree of a patient’s physical disability (8). Its active ingredient is teriflunomide (7, 8).

*Gilenya®,* chemically fingolimod, is a once-a-day oral medication that claims to decrease the number of patient relapses by half (30). Although the mechanism of action is not exactly understood, the drug is believed to decrease the number of circulating lymphocytes (30). Fingolimod is broken down into its metabolite, which binds and blocks a variety of sphingosine 1-phosphate receptors, trapping the leukocytes in the lymph nodes (34). This relapsing MS treatment was approved by the FDA in 2010 (106).

*Tecfidera®,* FDA-approved in 2013, is one of the most popular relapsing MS medications and contains dimethyl fumarate as its active ingredient (36, 106). The oral drug, taken twice a day, has been proven to reduce the number of relapses, prevent advancement of physical disability, and decelerate the development of lesions (36, 86). *Tecfidera®*’s exact mechanism of action is not understood, but the drug may possess anti-oxidant characteristics that help shield the brain and spinal cord from attack, as
well as inhibit certain immune cells (85). Dimethyl fumarate and its corresponding metabolite are known to impact the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway which helps mediates reactions to oxidative damage (36).

Lemtrada® contains alemtuzumab as an active ingredient and is only prescribed to patients who have tried at least two other forms of treatment with no relief from symptoms due to an abundance of autoimmune side effects associated with the drug (50). This relapsing MS medication, FDA-approved in 2014, is given in eight separate intravenous infusion treatments over a span of two years (24, 50). Lemtrada® is an antibody thought to bind to overactive B and T cells responsible for the myelin damage via CD52 and destroys them, although the drug has no distinct mechanism of action (50). Months after treatment, the body will replenish these cells, which will hopefully no longer target self-tissue (50).

Novantrone® was initially created by a subsidiary of Amgen, but was sold to EMD Serono who ultimately discontinued the drug (62). This medication was approved by the FDA in 2000 (62) and was the only FDA-approved drug for the treatment of SPMS and PRMS (106). Novantrone® and its generics have mitoxantrone hydrochloride as their active ingredient are thought to function by intercalating between the bases of DNA, resulting in breakage of the genetic material, as well as hindering the function of topoisomerase II which is involved in the unwinding of DNA that is necessary for replication and transcription to occur (63). This prevents the growth the B cell, T cell, and macrophage populations (63). The drug, which is administered by intravenous
infusion every three months, may also lessen the creation of pro-inflammatory cytokines and inhibit antigen presentation (63).

Ocrevus™ is an intravenously infused drug approved by the FDA in 2017 and is the only treatment approved for both relapsing MS and PPMS (35, 64). The drug’s active ingredient is ocrelizumab, a humanized antibody against CD20, which was shown to decrease physical disability progression as well as lesion volume in clinical studies, although a mechanism of action is not completely understood (35, 64, 106). CD20 is a surface marker found on B cells that is vital for B cell activation and growth (106). The first two low-dose infusions are performed two weeks apart, followed by high dose infusions twice annually (35).

Tysabri®, another MS win for Biogen, is a once monthly drug shown to halt the advancement of physical disabilities, prevent the occurrence of new lesions, and prevent relapse (93). The medication, FDA-approved in 2006 (24), has natalizumab as its active ingredient and is administered via intravenous infusion every 28 days (93). This recombinant humanized IgG4κ monoclonal antibody serves as a VLA-4 antagonist (37, 106). VLA-4 is necessary to leukocytes to cross the blood brain barrier and preventing this molecule’s binding works as a blockade to prevent many leukocytes from entering the CNS (37, 106). By blocking these leukocytes’ entry into the brain, the myelin is not attacked and an inflammatory response is not triggered.

Zinbryta® was a monthly relapsing MS medication with daclizumab as its active ingredient (104). It was believed to regulate IL-2 response, a key signaling molecule in the immune response recognizing self and foreign tissue, by binding to the IL2
receptor’s CD25 subunit receptors (104, 106). By blocking this receptor, T cell activity
would be diminished as well as inflammation (106). The drug was approved in 2016, but
in March 2018, Zinbryta® was voluntarily pulled from the market by joint marketers
Biogen and AbbVie due to concerns with brain inflammation (49).

With the approval of each successive drug, the hurdle for a new drug to enter
the market place becomes greater. Pricing must remain competitive while considering
unprecedented improvements such as enhanced efficacy or diminished adverse side
effects. For example, Gilenya is the most expensive MS drug (Table 1) but importantly it
also has the lowest annualized relapse rate (Table 2). Dosing regimen will impact total
annual cost to patients as well. Drug developers must consider dosing even in the early
stages of development, as this will affect patient compliance, and thus success of the
drug in the future. Although the aforementioned medications help treat and alleviate
the symptoms, none of these are curative.
WHOLESALE ACQUISITION COST OF SELECTED THERAPIES FOR MS, 2013
($ MILLIONS)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Estimated Annual Cost Per Patient*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-1a (Avonex, Rebif, Cinnovex)</td>
<td>13,000 - 30,000</td>
</tr>
<tr>
<td>Copaxone (glatiramer acetate)</td>
<td>10,000 - 30,000</td>
</tr>
<tr>
<td>Interferon-1b (Betaseron, Extavia)</td>
<td>10,000 - 30,000</td>
</tr>
<tr>
<td>Tysabri (natalizumab)</td>
<td>20,000 - 45,000</td>
</tr>
<tr>
<td>Gilenya (fingolimod)</td>
<td>25,000 - 60,000</td>
</tr>
<tr>
<td>Ampyra (fampridine)</td>
<td>25,000 - 30,000</td>
</tr>
<tr>
<td>Tafidera (dimethyl fumarate)</td>
<td>25,000 - 55,000</td>
</tr>
<tr>
<td>Aubagio (teriflunomide)</td>
<td>20,000 - 45,000</td>
</tr>
</tbody>
</table>

*For an annual course of therapy around the world. Cost in dollars will vary based on exchange rates, which is why these figures are only approximate.

Table 1. Annual cost of common MS drugs. The table provides a range of yearly drug costs to the patient. This table does not include all currently FDA-approved MS medications. (*Multiple Sclerosis (MS) Drugs and Biologics: Technologies and Global Markets, BBC, 2014, pg 18*)

EFFICACY RESULTS OF MS DISEASE-MODIFYING THERAPIES

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Annualized Relapse Rate</th>
<th>Annualized Relapse Rate Reduction % versus Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avonex (interferon beta-1a)</td>
<td>0.67</td>
<td>18-32</td>
</tr>
<tr>
<td>Betaseron, (interferon beta-1b)</td>
<td>0.16-0.42</td>
<td>33-43</td>
</tr>
<tr>
<td>Copaxone (glatiramer acetate)</td>
<td>0.30-0.59</td>
<td>29-75</td>
</tr>
<tr>
<td>Rebif (interferon beta-1a)</td>
<td>0.84-1.82</td>
<td>8-34</td>
</tr>
<tr>
<td>Tysabri (natalizumab)</td>
<td>0.22</td>
<td>67</td>
</tr>
<tr>
<td>Novantrone (mitoxantrone)</td>
<td>0.70</td>
<td>77</td>
</tr>
<tr>
<td>Gilenia (fingolimod, FTY720)</td>
<td>0.16-0.36</td>
<td>38-55</td>
</tr>
<tr>
<td>Aubagio (teriflunomide)</td>
<td>n/a</td>
<td>32.6-57.9</td>
</tr>
<tr>
<td>Tecfidera (dimethyl fumarate)</td>
<td>n/a</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 2. Efficacy comparison of MS drugs. The table above reports the yearly rates of relapse for some currently marketed MS medications, as well the reduction in relapses when compared to placebo. This table does not include all currently FDA-approved MS medications. (*Multiple Sclerosis (MS) Drugs and Biologics: Technologies and Global Markets, BBC, 2014, pg 25*)
Developmental Treatments for Multiple Sclerosis

Despite the multitude of FDA-approved drugs for MS, researchers are constantly looking for treatments to provide improved outcomes for longer periods of time or ultimately a cure. Many of these radical treatments involve removal of one’s own tissue to purify stem cells. Generally speaking, stem cells possess a unique ability to differentiate into a multitude of different cell types depending on the cellular signals received. There are different strains of stem cells including hematopoietic stem cells, mesenchymal stem cells, neural stem cells, induced pluripotent stem cells, and human embryonic stem cells, each with their own intrinsic capabilities (82). Despite the ethical controversy over stem cells, these cells can be harvested from a variety of non-fetal tissues including adipose, skin, bone marrow, and blood cells (82).

A radical MS treatment that has been conducted in clinical trials is autologous hematopoietic stem cell transplantation, or aHSCT. This therapy begins by harvesting a patient’s hematopoietic stem cells, followed by the suppression of the immune system by high-dose chemotherapeutics. The previously isolated stem cells are then reinfused into the body, fundamentally reprogramming the immune system (83). Although this procedure can be done with donor stem cells as well, autologous transplantations carry a smaller risk of rejection. Long-term clinical studies reported aHSCT patients, who had not been taking any disease-modifying medications since the treatment, had sustained remission and in some cases improved disease prognosis in a five-year follow-up (83). A Canadian phase II clinical trial for aHSCT showed no new lesions, decreased rate of brain atrophy, and a 95% survival rate in a 13-year follow-up (5).
Despite its effectiveness, aHSCT is very dangerous procedure reserved only for patients that have not exhibited any symptom relief from standard MS medications, as the ablation of immune cells via chemotherapy put a patient at very high risk of infection (83). Mesenchymal stem cells (MSCs) are known to infiltrate to the site of inflammation exceedingly well and to impede autoreactive T cell growth, making them an attractive to candidate for the treatment of MS lesions (26, 17). To be classified as an MSC, cells must adhere to a plastic culture dish, express certain epitopes, and be able to differentiate into bone, cartilage, or fat in the proper growing conditions (79). Research in this field has shown that introduction of previously isolated MSCs reduces demyelination and improves clinical scores in the EAE model (26). MSCs have the capacity to shift the local cytokine profile from pro-inflammatory Th1, to anti-inflammatory Th2 (79). Interestingly, J. Zhang et al found that this effect is seen even in xenogenic models where EAE-induced mice were treated with human bone marrow-derived MSCs (102).

While MSCs have proven to be effective independently, studies involving genetically-modified MSCs offer even greater advantages. IL-10 is an anti-inflammatory cytokine with a well-known influence on MS and its disease models (67). Experiments conducted by N. Payne et al involved transduction of a \textit{IL-10} carrying lentiviral vector into human adipose-derived MSCs (67). These engineered stem cells were injected into EAE-induced mice and control animals were treated with an inert enhanced green fluorescent protein-expressing vector (67). IL-10 and enhanced green fluorescent protein levels were measured by enzyme-linked immunosorbent assay and flow
cytometry. The enzyme-linked immunosorbent assay involves the binding of a variety of antigens of interest to a multi-well plate. This antigen is then bound by a specific primary antibody, often followed by an enzyme-linked secondary antibody that allows for the detection of the antigen-antibody complex (66). Flow cytometry begins with fluorescently labeling of cells of interest in a sample. Different fluorescent tags allow for the detection of multiple cell types within the same sample. A sophisticated flow cytometer utilizes pressure to analyze cells individually by a laser optics system and the data is then read as a computer output, allowing for precise quantification of the level of RNA or protein in a given sample (39). Both engineered MSC-dosed groups demonstrated inhibition of myelin-oligodendrocyte glycoprotein, the inducing agent of the EAE model, -specific T cells (67). Results also showed that the IL-10 expressing MSCs secreted significantly more of the anti-inflammatory cytokines than controls and demonstrated superior impediment of EAE progression, corroborated by histological analysis revealing that the increase in IL-10 led to almost a complete absence of demyelination, axon damage, and inflammatory cells in the brain (67).

Research led by Y. Zhang et al considered neurotrophin-3’s role in treating ethidium bromide-induced demyelination in rats (103). This gene, which is known to be involved in oligodendrocyte development and maturation, was inserted into an adenoviral vector, which was then transfected into MSCS (103). Administration of the neurotrophin 3-expressing MSCs led to enhanced coordination and recovery of nerve electrical impulses, assessed by beam behavioral tests and evoked potentials tests, respectively, when compared to controls (103). Immunohistochemical analysis showed a
significant increase in the quantity of remyelinated axons in the genetically modified MSCs, offering confirmation at the cellular level that oligodendrocytes were more active as a result of neurotrophin 3 expression (103). To confirm cell morphology, samples were viewed by transmission electron microscopy, which showed cells with an atypical morphology thought to be immature oligodendrocytes (103).

Another study by M. Cobo et al focused on engineering MSCs to express vasoactive intestinal peptide to treat EAE-induced mice (17). Vasoactive intestinal peptide is known to act as a neuroprotectant in its hinderance of glial cell activation as well as an immunomodulator in its diminishing of T cell reactivity (17). The team tested to see if the lentiviral vector alone could carry the gene of interest, but their results indicate that only when the MSCs expressed vasoactive intestinal peptide via viral transduction could the protein cross the blood brain barrier into the CNS and help alleviate EAE symptoms (17). Vasoactive intestinal protein abundance was measured by enzyme-linked immunosorbent assay and Western blot. Western blots separate proteins by gel electrophoresis and then transfer the protein to a membrane which is treated with primary antibody and enzyme-linked secondary antibody to allow for visualization of bands of protein once exposed to the proper substrate. Additionally, decreased quantities of proinflammatory cytokines measured by quantitative PCR were noted in the vasoactive intestinal peptide-expressing MSCs as well as the unmodified MSCs (17).

Apart from the formulation of the therapeutic itself, one must also consider the route of administration of a given drug. Research from F. Yousefi et al compared
intraperitoneal to intravenous injections of adipose-derived MSCs (101). The peritoneum offers a point of access into the blood and lymph systems concurrently, whereas intravenous injection enters only the blood vessels. Although these administration routes showed no significant difference in EAE clinical scores or their levels of pro-inflammatory cytokine secretion, flow cytometry and enzyme-linked immunosorbent assay were used to show differences in T regulatory cells (101). T regulatory cells play a key role in development of tolerance to self-antigens and act as a second line of defense to downregulate autoreactive T cells that escaped clonal deletion. Investigators reported a higher percentage of splenic CD4+ CD25+ FOXP3+ T regulatory cells in the intraperitoneal injection group compared to the intravenous injection group (101). Additionally, intraperitoneal injection of MSCs was shown to decrease the number of leukocytes in the CNS (101).

While MSCs have shown clear benefits, neural stem cells, or NSCs, present another promising therapeutic route with their ability to differentiate into a variety of neural cell types (oligodendrocytes, astrocytes, etc.) and a neuro-reparative function (82). It has been shown that MSCs are able to secrete a variety of growth factors, chemokines, and cytokines that greatly impact the surrounding cell environment (79). J. Jadasz et al considered p57kip2 protein’s role in modulating OPC differentiation in adult rat NSCs as well as human MSC factors ability to promote oligodendrogenesis across species was investigated (45). Rat NSCs were transduced with vectors carrying the gene of interest or control vectors (45). In situ experiments consisted of performing immunohistochemistry on sagittal brain sections following exposure to the control
NSCs, showing p57kip2 nuclear localization differences in the hippocampus and dentate gyrus (45). Next, NSCs were cultured with bone marrow-derived human MSC factors which resulted in the majority of cells expressing increased myelin basic protein after culture, providing support of remyelination (45). Early p57kip2 expression was observed in the nucleus followed by more universal expression after some time (45). NSCs containing vectors with mutated nuclear localization signals within p57kip2 yielded decreased oligodendrogenesis, highlighting the importance of the protein’s cellular location (45). These results mirror what was found when rat NSCs were exposed to rat-derived MSC factors, indicating a conservation of MSC factors across species.

Researchers also tested NSCs’ ability to myelinate synthetic nanofibers when incubated in either mouse or human MSC factors, resulting in nearly equal efficiency in myelination in both groups (45).

A small clinical trial assessing use of MSCs as a therapeutic for multiple sclerosis was conducted to observe the treatment’s effect on visual symptoms associated with the disease. Ten patients with SPMS associated with vision impairment were enrolled and treated with intravenous injection of autologous MSCs and then assessed by eye exam, MRI, and visual evoked response tests (19). Ten-month follow-up showed signs of decreased disability, improved eye exam scores, decreased latency in visual evoked response, and an increased optic nerve era (19). Visual evoked response tests involve a patient staring at a checkboard pattern on a screen while electrodes on the scalp shielding the occipital lobe create readouts that give insight into the functionality of the visual pathway (96). MRI scans did not show a significant decrease in lesion size,
indicating this treatment may improve function but not necessarily the physical aspects of the disease (19).

MSCs offer a very promising treatment route with their ability to help protect a drug molecule from degradation and shuttle it across the blood brain barrier. It appears that the location of injection of these stem cells is critical for their effectiveness. Both genetically engineered and non-engineered MSCs have the ability to traffic to the site of inflammation and lead to a decrease in inflammation. It should be noted that these cells may not be good candidates for treatments of non-inflammatory disease models or disorders (79). More experimentation be done before MSCs become an approved treatment for MS and other inflammatory diseases.

Drug Development

The drug development process is a costly, time-consuming endeavor broken up into many phases of increasing rigor. The FDA is a federal agency whose purpose is to ensure that products such as food, tobacco, dietary supplements, cosmetics, and both prescribed and over the counter therapeutics are safe for human use. Although the FDA is concerned with efficacy as well, it is mainly the burden of the drug sponsor to prove a drug’s effectiveness in order for the venture to be worth pursuing. Prior to a drug’s review by the FDA for approval to test in humans, years of discovery and preclinical work are conducted.
It is difficult to imagine a time when therapeutics were not scrupulously tested as they are today, but the humble beginnings of the FDA in the 1906 Pure Food and Drugs Act only gave the organization the right to penalize or seize control of drugs that they deemed to be misbranded (27). Prior to 1938, a drug patent covered merely the labeling for a medication and there existed no regulations for marketing a therapeutic (27). Testing of treatments has occurred for centuries, but the formal and organized process of evaluating efficacy and safety is a relatively new concept. The predecessor to the FDA was the American Medical Association’s Council of Pharmacy and Chemistry, which would assess drug quality and grant a seal of approval as well as advertisement opportunities in their journal to those that met their standards (27).

Michigan was the first state to approve the consented, controlled clinical investigation of a drug with limited liability for its sponsors in 1935 (27). A couple years later, it was found that the marketed drug sulfanilamide, used in the treatments of streptococcal infections, contained a poisonous vehicle (27). This spurred the 1938 Food, Drug, and Cosmetic Act, which required that all drug sponsors submit an NDA, or New Drug Application, a thorough report of all pertinent information of a potential therapeutic (27). At this time, preclinical animal studies were encouraged but not required and the FDA had no authority in regard to product labeling (27). It wasn’t until another medical disaster that the organization would further tighten regulations.

In the 1960s, a European trial for Grünenthal’s thalidomide was underway and included some clinical sites in the United States (27, 25). American doctors were given samples of the drug but not made aware that it was an experimental medication until
after they had prescribed it to many pregnant women to treat morning sickness (27). Thalidomide led to a staggering increase in serious birth defects, resulting in the 1962 Drug Amendments (27). These amendments forbid the testing of a drug in humans until sufficient animal trials have been conducted and implemented the use of investigational new drug applications, or INDs (27). Additionally, the act established the requirement of informed consent for all volunteers, incorporated Good Manufacturing Practices (GMPs), and the requirement of appropriate control groups within a study (27). Further changes were carried out during the AIDS epidemic of the 1980s, triggering the development of the FDA’s expedited programs (27). Jumping forward over 25 years, the FDA now has a notoriously strict regulatory framework, substantiated by the dismal ~12% success rate for drugs that make it to clinical testing (11, 23).

Prior to a drug’s involvement in clinical trials, there are many preclinical assessments that must be completed for the FDA to consider a potential therapy. This begins with target identification and validation, meaning the structure within the body that the potential therapeutic acts upon is selected and corroborated (43, 11). Studies at this phase include both in vitro and in vivo methods. Following target selection and validation, a library of compounds is synthesized to act on this target. High throughput screening is used to identify a manageable number of lead candidates from the hundreds that were originally synthesized (87, 43, 11). These compounds are then subject to various pharmacokinetic and pharmacodynamic studies that determine how a drug is broken down in the body and how the drug impacts the body as a whole,
respectively (11). Additionally, the compound’s toxicity and best mode of administration is assessed (87).

Small molecules deemed promising in the high throughput screening, known as “hits”, are then selected to be further optimized in the hopes of improving the compound’s effectiveness. Dose response curves, which plots the quantity of drug administered against the biological response, are generated for each of the hits (43). Medicinal chemists observe the chemical structures of each of these hits and group them based on structural similarities with the aid of computational analysis, forming a candidate for a lead series (43). These structures are then modified to optimize activity and are meticulously tested, a few being chosen to moving forward to preclinical testing (11). Preclinical testing consists of animal testing as well as cell or tissue-based assays (11). Even at this early stage, it is critical to consider how this drug may be administered to humans and how production could be scaled up to produce the quantities necessary for a clinical trial and the potential market.

Before clinical trials can commence, an IND is submitted to the FDA, detailing all the findings of preclinical work and plans for proposed clinical trials. The FDA has 30 days to review the submission or it is tacitly approved (11). Clinical trial sites’ individual institutional review boards or ethics committees oversee the experiments at each site and are tasked with creating the appropriate documentation for patient informed consent (11). The trial can be suspended or cancelled by the institutional review board or the FDA at any point in the clinical process (11).
Phase I of clinical trials typically involves less than 100 healthy subjects. As such, this phase is mainly to evaluate safety, assessing how the drug is metabolized and any adverse side effects (65, 11). These trials help establish a safe dose range that will be utilized in subsequent phases to determine the therapeutic dose. Phase II trials recruit hundreds of diseased individuals and divides them into a treatment or placebo group to judge a drug’s efficacy. At this phase, dosing is further refined and additional side effects are documented (11). Phase III enlists thousands of diseased subjects to test if the drug is effective and harmless longer term (65). The findings of this trial form the foundation of the labeling of the drug if approved. These studies are typically blinded as to avoid bias from healthcare workers and sponsors conducting the studying (65).

After successful completion of the three phases, the drug sponsor must submit an NDA or Biologics License Application (BLA) depending on if the therapeutic was chemically synthesized or derived from living organisms, respectively (11). These massive applications contain a detailed report of all preclinical and clinical findings as well as information on the safe mass production of the formulation and the safety warnings to be including in product packaging (11). In order to be an FDA-approved pharmaceutical, the manufacturing company must abide by GMPs, which are designed to mitigate risk in the production process of a drug (11). Even after a drug is approved for market, the FDA requires extensive post-approval reports from drug companies to fully analyze long-term side effects of the therapeutic as well as any varied effects across demographics (11).
Therapeutics are often useful in the treatment of more than one disease. Applying for a secondary indication or repurposing a drug eliminates a substantial amount of time and resources spent in the discovery phase and is significantly less risky than developing a drug *de novo*. A drug can be repurposed if it was removed from market for its original indication or if earlier stage research on the molecule was abandoned (9). Repurposing requires a new method-of-use patent as well as a new NDA. Similarly, supplemental NDAs are filed as an addendum to an original NDA when a company would like to have an additional indication approved for their currently marketed drug. Shockingly, the infamous thalidomide is somewhat of a repurposing marvel and was approved in 1998 for the treatment of erythema nodosum leprosum (25). After this, the drug was approved for the treatment of multiple myeloma and refractory anemia through the efforts of Celgene (25). Moreover, the formulation has been further modified to create the second-generation lenalidomide and pamolidomide (25).

**Specialized FDA Programs**

As the FDA and drug development process have evolved, specialized programs have been established to accommodate expedited approval in certain cases. Acceptance into these programs allows for a company’s application to have expedited review, more frequent meetings with the FDA, and use of non-clinical endpoints, to name a few. It
should be noted that acceptance into these expedited programs is extremely subjective. Drug sponsors make their case and negotiate with the FDA in order to receive these designations.

The FDA’s Fast Track can be granted to drugs indicated for serious and/or chronic diseases for which there is no current curative therapy, such as AIDS or diabetes. A therapeutic may be granted this designation if the sponsor has evidence of its significant advantages over currently marketed therapies (11). Priority review designation requires that the FDA must approve or deny an application months earlier than a standard application (11). Approvals for priority review tend to be for diseases that may not be necessarily common in the US, but are of urgent medical need in other countries, such as cholera, malaria, and leprosy (71).

Some FDA classifications allow for the use of surrogate endpoints to appraise a therapeutic’s intended clinical effect. A surrogate endpoint is used in certain cases as an indicator of a potential clinical endpoint, which are outcomes such as increased survival or eradication of the disease (11). Decreased tumor size and its correlation to increased survival rates in cancer patients is an example of the surrogate and clinical endpoint relationship. Another example of a surrogate endpoint in diabetes is HbA1c, a blood marker for glycated hemoglobin indicative of blood glucose levels (40). Both Breakthrough Therapy and Accelerated Approval designations allow for the use of surrogate endpoints to receive expedited approval for their potentially life-saving medications. Breakthrough therapies are granted to medications that prove improved outcomes compared to commercially available drugs (11, 12). This designation has
recently been granted to treatments for Hodgkin's lymphoma and \textit{BRCA} mutation-linked breast cancers (13). Accelerated Approval is permitted to drugs intended to treat chronic debilitating disease for which there currently is no treatment, similar to the Fast Track designation but with the use of surrogate endpoints as a basis for approval (1, 11). Some examples of accepted Accelerated Approval applications are multiple myeloma and chronic lymphocytic leukemia (2).

Given the average 20-year patent life for a medication and the ~7 years spent in clinical trials alone (11, 47), it is often difficult for a pharmaceutical company to recoup the development cost of their medication. This structure discourages the development of drugs for orphan diseases, which affect less than 200,000 people nationally. In order to incentivize entry into this small market, the FDA permits seven years of market exclusivity to the first drug sponsor to develop a treatment for an orphan disease (11). Similarly, if a medication is approved by the FDA for a pediatric indication, the company is granted an additional 6 months of patent exclusivity (11).

Even after a drug is on the market, the Hatch-Waxman Amendments of 1984 allow for the extension of patent life. Drug developers are able to file for this extension to make up for the time lost bringing a drug to market (32). These amendments also set up standardized system for the development of generic therapeutics (32). These laws dramatically impacted the pharmaceutical industry and made life-saving drugs more accessible to all.
The Cost of Innovation

Many studies have been conducted in an effort to find the average cost of bringing a pharmaceutical to market. Two meta analyses of the pharmaceutical industry have attempted to uncover the costs associated with each phase, the amount of time spent in each phase, and any noticeable trends. These studies made a distinction between out-of-pocket cost versus the capitalized cost, which takes into account the time investment or opportunity cost (23, 55).

DiMasi’s 2013 study pulled data from various sources to track over a hundred drug candidates overseen by 10 pharmaceutical companies. Their statistical analysis showed an average out of pocket cost of about $1.4 billion and a capitalized cost of $2.6 billion, representing a 166% and 145% increase since 2003, respectively (23). The study showed that each successive phase was increasingly more expensive, but the likelihood of failure as a drug enters each subsequent phase decreases (23). This analysis calculated an average of 96.8 months from the start of clinical trials to a drug’s market approval and an average time of 31.2 months, 33.1 months, 37.9 months, and 45.1 months spent in prehuman testing, phase I, phase II, and phase III trials, respectively (23). This represented a significant decrease in the time spent in discovery/preclinical, referred to as prehuman, development (23), suggesting that technology has allowed for molecules to be more efficiently screened in these early stages. Moreover, failure tends to happen at these earlier phases than seen in previous studies (23), indicating a “fail fast” method of innovation. A 2011 study conducted by Morgan et al revealed a broad
range of cost to bring a drug to market, spanning from $161 million to $1.8 billion
capitalized, reported in 2009 dollars (55).

As health insurance buyers have more options for treating a given disorder, it is
becoming increasingly more difficult for a hopeful therapeutic to prove its effectiveness.
A publication by Camejo et al develops mathematical equations that attempt to assess
the cost effectiveness and net benefit of a new pharmaceutical venture (18). These
equations include factors such as price of comparators, effectiveness of comparators,
proposed price of a newly developed drug, and the effectiveness of the new compound,
and a threshold constant that represents the amount a buyer will pay for each unit of
effectiveness (18). Although this valuation of a new drug would be extremely helpful in
deciding which compounds to move forward from a portfolio, the validity of these
equations has been questioned due to the subjectivity of some of the factors (18).

The cost of drug development does not show a trend to decrease in the near
future. The current increased spending and lower success rates are driven by the
stringent regulations requiring larger and more complex clinical trials. Additionally, with
each new drug introduced to market the bar is raised and researchers are investigating
more perplexing diseases as technology reveals the possibilities. Although a time-
consuming and expensive process, the life-saving outcomes of the process justify the
investment.
Major Players in Biopharmaceutical Development

Although the name of the final pharmaceutical manufacturer is what consumers see on the pill bottle, there are many contributing institutions in the development of a drug from its beginnings in the discovery phase to its responsible manufacturing including venture capitalists, the US Patent and Trademark Office, academic research institutions, insurance companies, start-ups, the National Institutes of Health, the FDA, biopharmaceutical research companies, clinical research organizations, clinical trial sites, and non-profits (11).

The majority of basic research is carried out in academic labs and industry becomes increasingly involved as the drug gets closer to market (11). The National Institutes of Health is a key funding source for many academic labs and research organizations, whereas venture capital is typically used to support small start-ups. Biopharmaceutical research companies may contract clinical research organizations to perform some of the experimentation if an institution does not have the means to do the experimentation on site or if experimentation on site would be more expensive than outsourcing. Non-profits are often able to provide funding in the form of grants to researchers whose studies align with their mission. One mutually beneficial strategy is larger pharmaceutical companies doing away with much of their internal discovery units and opting to license formulations that have been developed to a certain stage by academic labs or small pharmaceutical companies. This provides the large pharmaceutical company with the flexibility to invest in an array of projects without the
confinement of developing the compound in their own labs, while the originators of the formulation receive critical funding to push the drug through costly clinical trials.

I had the opportunity to work for a local non-profit, Harrington Discovery Institute. This disease-agnostic company’s mission was to enable the successful development of therapeutics by helping researchers through the translational “valley of death” which lies between discovery and the filing of an IND. Selected scholars receive not only grant funding, but invaluable guidance in the form of the Innovations Support Center. The Innovations Support Center is a panel of experts specially selected for each project. Importantly, the scholar and their institution maintain all intellectual property throughout this process. Harrington Discovery Institute has developed several specialized programs for rare disease, eye disease, and Alzheimer’s disease, as well as a partnership program with the University of Oxford.

Harrington Discovery Institute acts as a feeder system into a Cleveland-based accelerator company BioMotiv. BioMotiv assesses the scholar projects from Harrington Discovery Institute and selects ones with promise after their two-year term at the institute is complete. BioMotiv is able to provide service such as pharmaceutical expertise, legal support, and funding to create a start-up company around the scholar’s discovery. The goal is to further bridge the gap between discovery and filing for clinical trial approval. Harrington Discovery Institute and BioMotiv’s mutually beneficial relationship is a great example of how multiple entities must collaborate in order for a drug to successfully make it to market.
The Future of Drug Development

It is interesting that an industry as innovative and ever-changing as pharmaceuticals has relied on a relatively static model of drug development. This outdated design makes recouping the cost of developing the pharmaceutical difficult, and that is only if the drug becomes approved and is successful. In fact, only 3 out of 10 approved new medications breaks even or is profitable once research and development costs are considered (47). Drug companies are forced to cut back on research and development spending because of the risks associated with taking a drug to market (47).

One of the biggest threats to pharmaceutical companies is the limited time on their intellectual property protection in the form of patents (47), as a typical patent has a 20-year lifespan from its earliest filing date. A company or researcher may file a provisional patent to serve as a placeholder of sorts for filing date until a formal patent is filed. Although two decades may seem like a considerable amount of time, after considering the 3-6 years of discovery research (11) and average 7 years in clinical testing (11, 47), the remaining time to regain money spent on a project is not substantial. If a patent is ever disputed, trial expenses further add to the total investment of a drug. Once a therapeutic’s patent expires, generic versions of the formulation can legally be created by other companies.

As the pharmaceutical offerings have expanded, health insurance providers and payers have demanded more from a given drug. Providers are becoming more and more
selective about which medications they cover. It is not enough to simply be safe and effective; a drug must cost less and/or be more efficacious than its competitors (47). As the drug offerings have expanded, the depth and length of clinical trials is expanding and researchers are delving into more perplexing and chronic disorders (47). Additionally, stricter requirements for clinical trial volunteers and the need for larger studies make patient recruitment difficult.

An article assessing the state of the drug development process highlighted the importance of cooperative collaboration between all contributing members. The author especially stresses the shifting role of the CRO from simply contracted lab work to a functional service provider (47). Outsourcing entire departments is becoming popular, emphasizing the CRO’s increasingly important role in the evolution of this system. CROs can serve a multitude of purposes such as conducting preclinical animal work if a company does not have a vivarium on site or providing support in coordination of clinical trials.

With the advent of new technology making genotyping faster, easier, and cheaper, many believe that the future of pharmaceutical is in personalized medicine (47). This would involve generating individualized treatment plans based on a patient’s distinct genetic makeup. Its growing popularity is evident in the advent of basket and umbrella clinical studies commonly used in oncology research. Basket studies investigate all diseases associated with a unique genetic mutation, whereas umbrella studies consider all mutations linked to a certain disorder (11).
Contract Research Organizations

CROs have a unique and pivotal role in the development of a drug. These organizations are contracted by pharmaceutical, medical device, or biotechnology companies to conduct research studies that the client cannot perform on-site. The client maintains all intellectual property while having an unbiased party overseeing the study. A CRO’s function within the drug development process is dependent upon the services the company provides and determines if they are categorized as a preclinical or clinical organization (81). These providers can offer a wide array of services including early stage toxicological screening or advise on regulatory requirements for clinical trials.

Although presented as a static stepwise process, the drug development process is a dynamic system and thus CROs must constantly evolve to remain relevant in the process (81). These companies are cultivating niches within the industry for themselves, often focusing on a single indication or specializing in a certain service. Clients must decide if they prefer a “one stop shop” CRO to conduct a study in its entirety or contract the work out to multiple niche CROs to conduct each portion of the study independently (81).

The CRO market consists of several subsets including clinical development, preclinical/drug safety/pre-human, discovery and central lab (80). The compound annual growth rate of the total global CRO market is at 7.6% and is expected to be worth $4.5 billion by 2020 (80). 2017 industry leaders such as LabCorp, Charles River,
PPD, and Envigo accounted for almost 40% of the preclinical market (80), indicating a need to develop novel assays to be able to survive in this competitive market. North America and Europe are the dominant markets in contract research, however Asia is a growing competitor (20). CROs represent a growing market due to pharmaceutical companies outsourcing more and more of their research. Outsourcing offers many benefits to the company including cutting operational costs, refined focus on core competencies, shared liability with their partnered CROs, and the ability to hire experts for specialized services (20). In order to stay competitive, a CRO may often patent a technique or assay or maintain trade secrets (20).

The trend of increased outsourcing of laboratory work has led to a pharmaceutical model where no experimentation is performed on site. For example, SAGE Therapeutics, is known as a virtual biotech company with the mission to develop treatments for CNS diseases. Their lead compounds act on the GABA and NMDA receptors and look to treat postpartum depression, major depressive disorder, and tremors associated with Parkinson’s disease (88). SAGE’s fully outsourced model proves effective, as two of their drug candidates are currently in clinical trials (88).

Renovo Neural: A boutique neuroscience CRO

Renovo Neural Inc., is a niche preclinical CRO founded in 2008. The privately held company is a spin-off of the Cleveland Clinic Foundation and specializes in
neurodegenerative disease, particularly MS. From its humble beginnings, Renovo has grown and currently employs over fifteen people and greatly expanded its offerings. Renovo offers a wide selection of services including: MS rodent models, histology, MRI, multiple in vitro assays, and three-dimensional microscopy (3DEM). Although the company focuses on neurodegeneration, much of the expertise can be utilized to study indications outside of this field.

Renovo offers two types of MS mouse models: cuprizone-rapamycin and EAE, both previously described in the Animal Models of Multiple Sclerosis section. The company has modified the traditional cuprizone model with the addition of co-administration of rapamycin, which impedes spontaneous myelin not associated with a candidate drug. The structure of an animal study is dependent upon if the client’s drug is thought to be neuroprotective or remyelination. For a paradigm of neuroprotection, animals are induced with cuprizone-laced chow for 6 or 12 weeks concomitantly with client-specified dosing of their compound. In a remyelination model, animals are induced for 6 weeks and allowed to recover for 12 weeks. Animals are dosed with drug during the recovery phase. At the end of the animal studies, mice are sacrificed, perfused, and their brains are fixed, allowing them to be examined histologically. Alternatively, mice lesions can be assessed via rodent MRI during the in-life phase, allowing for a longitudinal study.

Histology offers a way to view microscopic cellular structures by exposing the sample with a variety of protein markers in order to visualize features of interest. Renovo offers full histology service, including tissue processing, sectioning, staining,
imaging, and analysis. Tissue processing includes perfusion of the animals, tissue fixation and embedding in a variety of resins or waxes. This CRO offers an array of standard stains such as Nissl, Haematoxylin and Eosin, Tol Blue, and Silver Staining, but offers specialized stains for neural tissue as well. Renovo has immunohistochemistry capabilities, which relies on antibodies linked to enzymes or fluorescent dyes to allow for labeling of cellular structures. Slides can be imaged by a variety of microscopes manually or automatically. Following imaging, Renovo offers analysis services such as cell morphology, overlays, reconstruction, stereology, density, and absolute counts.

Prior to initiating a large-scale animal study, researchers will often perform cell-based assays as a more economical way to screen compounds. Renovo offers in vitro experimentation such as oligodendrocyte progenitor cells (OPC) differentiation, OPC proliferation, and OPC/oligodendrocyte toxicity assays. A homogenous population of myelin-producing OPCs are harvested from PLP-EGFP transgenic mice and then plated onto a 96-well plate. These cells are treated with a client’s test compound at various doses along with positive controls. The OPC differentiation assay evaluates a client compound’s ability to promote the maturation of OPCs into active oligodendrocytes and helps determine the dose at which this compound is most effective. Additionally, this assay can assess a potential drug interaction and determine signaling pathways used to promote OPC differentiation.

Renovo’s proliferation assay utilizes 5-ethynyl-2’-deoxyuridine, or EdU, which intercalates into DNA and serves as an indicator of OPC reproduction. Plates are scanned to calculate the proportion of cells that took up this compound. The OPC and
oligodendrocyte toxicity assay allows for the identification of harmful compounds and/or lethal doses of a client’s drug. This assay calls for the incorporation of specialized fluorescent dyes that allow for the distinction between live and dead cells.

Renovo offers two disparate forms of imaging: MRI and 3DEM. MRI operates by using a magnetic field to align the hydrogen protons found in the water molecules of a subject. A radio frequency pulse is applied and the atoms flip their alignments. After this pulse, the protons return to their native orientation, emitting a signal that can be read by a detector. These signals are interpreted by a computer which creates the readable image. Renovo has a rodent-specific 7T/20 Bruker MRI machine capable of specialized forms of MRI including: anatomical MRI, MT weighted imaging, functional MRI, manganese-enhanced MRI, pharmacological MRI, and diffusion tensor imaging. MRI is useful in collecting data during the in-life phase of experimentation, allowing for longitudinal studies.

3DEM is a high-resolution form of imaging that allows for the visualization of cellular ultrastructure. Samples are fixed in glutaraldehyde, stained with heavy metals, trimmed, and mounted before imaging. This microscope operates by discharging a beam of electrons onto the sample that is continuously cut by an automated diamond knife. The deflection of the electrons is read by a detector and the pattern of the electron back scatter ultimately produces an image. Hundreds of tissue slices can be imaged in a 24-hour period and these image stacks allow for three-dimensional reconstructions and analysis. Renovo’s 3DEM unit is a two-system laboratory with a Zeiss Sigma VP scanning electron microscope system equipped with a Gatan 3View® door and a new FEI
Volumescope. This assay has an exceedingly broad range of applications, from squirrel retinas to fruit fly muscle tissue.

Renovo Neural is an unusual CRO in that it invests 25-30% of its revenue in research and development every year. This money goes to expand the CRO’s offerings to its customers. These research and development efforts include: a nanofiber myelination assay, traumatic brain injury models, regenerative medicine, stroke models, and Parkinson’s disease models. A research and development project that I became very involved with during my internship at Renovo Neural was an in situ hybridization method called RNAscope®, which tags RNA molecules as opposed to protein-tagging that is typical of traditional histological methods. Specifically, we were interested in markers of M1 and M2 microglia activation, as they are believed to play an integral role in the inflammatory response of MS.

**In situ hybridization using RNAscope®**

Advanced Cell Diagnostics’ (ACD Bio) RNAscope® is an innovative in situ hybridization platform that allows for visualization and quantification of an RNA target sequence. Probes are custom created for virtually any target sequence or selected from hundreds of catalog probes. These probes allow for precise amplification of a target as well as background noise suppression (41). Given the ability to customize probes, the high specificity, the reduction in background noise, and the ability to use up to four
probes simultaneously, RNAscope® has clear advantages over conventional in situ hybridization methods.

Briefly, tissue is processed to permeabilize the cells or tissue to be tested, allowing for access to the target RNA (41). The platform’s unique double Z probes are designed in a way so that the binding of two adjacent Z probes creates a joint 28-base pair scaffold for preamplifiers to bind followed by the addition of amplifiers which are then bound by fluorescent label probes (41) (Figure 7). These labels allow for the RNA to be visualized, each probed target represented by a distinct dot (41). This allows for the manual or automated quantification of the target RNA.

Described here is an experimental design to validate probes for M1 and M2 microglia specific RNAs, as outlined in Table 3. For control tissue, we used mouse liver which endogenously expressed all RNA associated with the probes. In addition to microglial probes, ACD Bio also included pre-validated positive control probes. These positive control probes included Polr2a, PP1B, and Hprt, which are all known housekeeping genes that aid in validation of experimental procedure.

Renovo has tracked microglia using Iba1 histological staining at twelve weeks cuprizone-induced demyelination as well as into six weeks of recovery in past studies (10), but more recent experimentation has shown peak Iba1 expression at six weeks induction. Consequently, we utilized the corpus callosum from mice that had undergone six weeks of cuprizone-rapamycin induction. We hypothesize that there will be greater population of M1 microglia as compared to M2, as these animals were sacrificed at the peak of microglia activation after toxic insult. This experiment was
initiated to quantify the ratio M1 to M2 microglia in brain tissue. Once internally validated, Renovo hopes to offer this service to clients as a mean to quantify RNA. Follow-up studies will include tissue from animals in the recovery phase of the cuprizone-rapamycin model, where we would anticipate a greater M2 population.

Probe Design:
Positive probes provided by ACD Bio included Polr2a, PP1B, and Hprt. Polr2a, or RNA polymerase II subunit A, codes for a subunit of RNA polymerase II which is used to transcribe mRNA in all eukaryotes (69). PP1B codes for the beta catalytic subunit of serine/threonine-protein phosphatase which is known to play a role in basic cellular functions, for instance cell division and protein production (94). This enzyme sequence is highly conserved throughout the eukaryote domain, with 100% sequence identity between the mouse and human forms of the gene (94). The Hprt gene encodes hypoxanthine guanine phosphoribosyl transferase, which functions in the genesis of purine nucleotides and is highly conserved in eukaryotes (42). These are reliable control probes given their ubiquitous expression in eukaryotic tissue.

After literature review of various genes involved in M1 and M2 microglial function, three were selected and PubMed was used to determine gene identification numbers. These numbers were used to search through ACD Bio’s probe catalog and select the appropriate experimental probes. When customizing our iNOS probe, we simply provided the gene identification number and ACD Bio carried out customization. For selection of control specimens, we looked at expression patterns of our experimental probes in non-brain tissue.
**Methods:**

Animals were perfused with 4% paraformaldehyde at 6 weeks of cuprizone and rapamycin induction. Control liver and experimental brain samples were fixed in paraformaldehyde in 1x PBS at room temperature. Tissue was then dehydrated in ethanol and embedded in paraffin. Post embedding, all tissue was treated in accordance with ACD Bio’s RNAscope® Multiplex protocol (77).

Tissue was sectioned at 5µm, after which the sections were mounted onto glass slides and allowed to air dry. Tissue was dehydrated by a series of xylene and alcohol washes. Slides then incubated in a HybEZ™ Oven and at 40°C for 30 minutes after which hydrogen peroxide was then added to each section to block endogenous peroxidases. Slides were then washed with distilled water.

For RNA retrieval, slides were incubated in a steamer prepared with the RNAscope® 1x Target Retrieval Reagent and heated to 99°C. After steaming for 15 minutes, slides were rinsed with distilled water and then alcohol before drying at room temperature.

Once dried, RNAscope® Protease Plus was added to each slide. Slides were then allowed to incubate for 30 minutes at 40°C on a humidity control tray in the oven followed by drying in the oven without the use of the humidity tray. The target probes were prepared as a mixture per the protocol and the mixture was added to the slides, which were then baked at 40°C for 2 hours prior to subsequent washing.
For visualization of target probes, each probe was developed sequentially utilizing TSA-based fluorescent markers followed by baking the slides at 40°C for 15 minutes with RNAscope® Mulitplex FLv2 HRP blocker.

Following the development of all three channels, DAPI as a nuclear co-stain was added to each slide and briefly incubated. DAPI was extracted and ProLong Gold Antifade Mountant was added to each slide. Glass coverslips were added and slides were allowed to dry in the dark overnight. Slides were imaged after coverslipping using confocal or compound light fluorescent microscopy.

Figure 7. RNAscope®’s Double Z probe technology. Two adjacent Z probes (green) must bind the span of the target sequence (gray) to provide a 28-base pair sequence (blue) for preamplifiers (orange) to bind. The preamplifiers are then bound by amplifiers (yellow), which create a binding site for fluorescent label probes (pink). The fluorophores allow for the visualization of each probe-bound target RNA molecule as a distinct dot.
Results:

Before utilizing the probes for M1/M2 the differentiation of microglia, internal probes provided by ACD Bio were used to ensure that the staining process worked and that tissue was prepared properly. Positive Polr2a, PP1B, and Hprt staining confirmed successful execution of the protocol.

The staining of control liver tissue with positive control probes showed strong signal with punctuate dots for each of the channels (Figure 8). To confirm tissue integrity, positive control probes were run on experimental brain tissue. This showed positive staining for all three probes as well (Figure 9).

Liver tissue was stained with experimental probes to confirm binding specificity. This showed successful staining for each of the three probes selected for M1, M2, and general microglia. There was particularly high expression of Arg1, shown in red (Figure 10).

To assess microglia ratios in brain tissue, anterior commissure and corpus callosum brain tissue were stained. These images showed a high degree of background (Figure 11, Figure 12). Additionally, negative control staining resulted in some background staining (not shown).

Discussion:

Renovo’s goal was to assess M1/M2 microglial populations in brain tissue of a mouse model of demyelination. We were able to successfully validate these probes in control liver tissue (Figure 10). Positive control probes on liver and brain tissue validated
successful execution of the staining protocol and proper tissue preparation (Figure 8, Figure 9).

*In situ* hybridization with microglial probes of brain tissue resulted in a high degree of background and few punctuate dots. This could be due to low abundancy of RNA or suboptimal RNA target sequence selection. To remedy this, the experiment will be repeated to increase n and new studies will be initiated to assess other microglial probes, such as *TREM2*, a marker for the M2 phenotype.

Further experimentation will need to be performed in order to validate these probes for markers of microglial activation. Renovo intends to stain another cuprizone-rapamycin treated brain as well as brain tissue from cryogenic models of traumatic brain injury that are known to elicit a microglial response. In conclusion, we have validated RNAscope® for use within the cuprizone brain although optimization of microglial probes is ongoing. Quantification of M1 and M2 microglia after acute assault and during recovery will elucidate how these cell populations mediate injury and recovery in MS.
<table>
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**Table 3. Experimental breakdown of probes.** Control and microglia probes are detailed in the above. Each of the probes was tested in control liver tissue and experimental brain tissue.
Figure 8. Positive control probes on control liver tissue validate staining process. Tissue was stained with probes for RNA target sequences Polr2a (green), PP1B (red), and Hprt (magenta) to confirm proper staining protocol was followed. All three probes showed successful staining, highlighted by distinct dots in each channel. Nuclei are stained with DAPI in blue. Photo taken with confocal microscope.
Figure 9. Positive control probes on brain tissue validate tissue preparation. Tissue was stained with probes for RNA target sequences Polr2a (green), PP1B (red), and Hprt (magenta) to confirm proper tissue preparation. All three probes showed successful staining. Nuclei are stained with DAPI in blue. Photo taken with confocal microscope.
Figure 10. Experimental microglial probes on control liver tissue validate probes. Tissue was stained with probes for RNA target sequences *Aif1/Iba1* (green), *Arg1* (red), and *iNOS* (magenta) to validate these experimental probes. Successful staining can be seen with high expression of *Arg1*. Nuclei are stained with DAPI in blue. Photo taken with confocal microscope.
Figure 11. Experimental microglial probes on anterior commisure brain tissue. Tissue was stained with probes for RNA target sequences Aif1/Iba1 (green), Arg1 (red), and iNOS (magenta) to allow for quantification of M1 and M2 microglial populations. A high degree of background can be seen with few distinct puncta. Nuclei are stained with DAPI in blue. Photo taken with compound light fluorescent microscope. A) overlay of all three probes B) iNOS and DAPI C) Arg1 and DAPI D) Aif1/Iba1 and DAPI.
**Figure 12. Experimental microglial probes on corpus callosum brain tissue.** Tissue was stained with probes for RNA target sequences *Aif1/Iba1* (green), *Arg1* (red), and *iNOS* (magenta) to allow for quantification of M1 and M2 microglial populations. As seen in the other brain tissue sample, there was a high degree of background and few distinct puncta. Nuclei are stained with DAPI in blue. Photo taken with compound light fluorescent microscope. A) overlay of all three probes B) *iNOS* and DAPI C) *Arg1* and DAPI D) *Aif1/Iba1* and DAPI

**Conclusion**

Autoimmune diseases occur when one’s immune system attacks healthy self-tissue, resulting in a chronic inflammatory response. One of the most widespread autoimmune disorders affecting over 2.3 million worldwide is MS, which involves the loss of myelin in the CNS, leading to interruptions in the transmission of electrical
impulses. The disease incidence has a peculiar geographical pattern, with higher incidence rates being seen in countries further from the equator, suggesting some environmental impact on disease pathogenesis. Two disease susceptibility genes have been identified for MS and may allow for preventative screening of high-risk patients in the future.

There are over a dozen FDA-approved MS medications currently on the market, many believed to diminish autoreactive T cell populations. Experimental treatments of the disease include MSCs, often carrying vectors expressing genes for certain anti-inflammatory cytokines or other neuroreparative cell signals. With their tendency to relocate to the site of inflammation and their ability to cross the blood brain barrier into the CNS, these stem cells offer an attractive option to halt the destruction of brain and spinal cord myelin that is characteristic of MS.

Due to the number of cells that participate in orchestrating an immune response and the complexity of their relationships, it is difficult to select a target cell for diseases such as MS. An intriguing candidate is microglia, which are some of the first responders during CNS attack. Microglia are bifunctional immune cells that can serve as mediators of tissue destruction or tissue repair, depending on their phase which seems to be driven by local cytokine environment.

Renovo Neural has validated experimental M1 and M2 microglia probes in control liver tissue through ACD Bio’s RNAscope® in situ hybridization platform. Although follow up studies are needed to validate these markers in brain tissue before offering this as a service, these probes could determine the ratio of M1 and M2 microglia which
will allow clients to verify a drug candidate’s impact on microglia populations. With CROs ever-growing role in the drug development, this offering could enable clients to determine the most effective of their candidates in the early research and discovery phases. Through the continued collaboration of academic labs, CROs, pharmaceutical companies, non-profit organizations and other key player in drug development, more life-saving therapeutics can be made available to treat MS and other neurodegenerative diseases.
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