TOBACCO SMOKE EXPOSURE AND PEDIATRIC MULTIPLE SCLEROSIS

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ABSTRACT

Tobacco Smoke Exposure and Pediatric Multiple Sclerosis

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Introduction: Multiple sclerosis (MS) is a chronic inflammatory disease which affects approximately 2.5 million people worldwide, including approximately 7,000 children. The etiology of MS is unclear, although researchers generally agree that both environmental and genetic factors are involved. It is also unclear why some patients may only have one demyelinating event (acquired demyelinating syndrome, or ADS) and others develop chronic demyelinating disease (MS). Recent evidence suggests an association between smoking and multiple sclerosis (MS) in adults. A question remains if there is a similar association between secondhand tobacco smoke exposure and MS in children. The purpose of this study is to explore the association between tobacco smoke exposure (TSE) and MS risk in a cohort of children with demyelinating disease.

Methods: Data was obtained from the Canadian National Demyelinating Disease Study. This study included two disease groups, which are distinguished by a single (ADS) versus chronic demyelinating attacks (MS). Parents' self-report of their child's exposure to smoke in the home, as well as biomarker verification by serum cotinine, classified a child as exposed or not exposed. Logistic regression models were created to determine the association between TSE and the odds of MS compared to healthy controls, the odds of ADS compared to healthy controls, and the odds of MS compared to patients with

ADS. In order to determine factors and exposures which distinguish MS from ADS, an assessment of interaction was performed to examine the relationship between TSE and MS risk genes, TSE and serum vitamin D levels, and TSE and prior Epstein Barr Virus exposure on the odds for developing MS compared to ADS patients.. Finally, serum cotinine levels were compared to neurologic functional scores in order to assess if a dose response mechanism exists creating impaired function for pediatric MS. **Results:** TSE was not significantly associated with increased odds for MS compared to healthy controls (OR= 1.84; 95%CI 0.86, 3.95) but was significantly associated with higher odds of monophasic ADS compared to healthy controls (OR=2.24; 95%CI 1.08, 4.63). TSE alone was not associated with increased odds for MS compared to ADS; however, the presence of both TSE and HLA alleles increased the odds for MS by 3.2 (95%CI 1.04, 9.79) when compared to ADS patients. An additive effect was also found between TSE and lower vitamin D, which together increased the odds for MS compared to patients with monophasic ADS (OR=2.89; 95%CI 1.21, 7.46). EBV was individually associated with MS compared to ADS (OR=4.12; 95%CI 1.62, 10.9) and odds for MS appeared to increase further with the addition of TSE (OR=5.13; 95%CI 1.79, 14.9), however sample size limited interpretation of the interaction analysis. TSE had minimal impact on neurological functional score measures, although long-term follow up with regard to exposure could not be properly assessed. **Conclusion:** Exposure to tobacco smoke through secondhand sources was not related to MS but TSE may increase the odds of monophasic demyelinating disease occurrence (ADS). The finding of additive effects between TSE and other disease modifying factors (HLA, vitamin D) may provide

valuable insight into why some children have only one demyelinating attack (monophasic ADS) while others have multiple attacks and are diagnosed with MS. These effects should be further explored in a larger population of pediatric patients and compared to healthy children. Intervention methods should be tailored to help explain to parents the benefits of reducing their child's exposures to environmental tobacco smoke.

DEDICATION

This work is dedicated to my husband Nathan and my loving family. Without their constant support, completing this degree would not have been possible. Nathan, I cannot thank you enough for helping me through the stress of school, always encouraging me to pursue the next challenge, and having patience with me while I dedicated a lot of my time to my studies. Thank you to my mom and dad, Mark and Lilli Hansen, for always providing positive inspiration and reassuring me through the most difficult times. Thank you to Gail and Patrick Lavery for encouraging me to keep up with the program and providing positive encouragement throughout. And finally, thank you to my sister Codie and the rest of my family, friends, and co-workers who were the rocks that kept me steady through this whole process.

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CHAPTER 1

INTRODUCTION

Multiple Sclerosis

Multiple sclerosis (MS) is an immune-mediated attack on the central nervous system, resulting in chronic disability (Reeves & Swenson, 2008; Weinshenker et al., 1989). Immune cells incorrectly target healthy tissue in the brain called myelin, which is the protective covering of axons. These events are termed "demyelinating" attacks (Reeves & Swenson, 2008). A first attack of demyelination is called an acquired demyelinating syndrome (ADS or monophasic ADS) which includes clinically isolated syndromes such as optic neuritis (inflammation of the optic nerve due to demyelination) and transverse myelitis (inflammation of the spinal cord due to demyelination)(Reeves & Swenson, 2008). During an attack, inflammation in the brain and sometimes the spinal cord causes neurologic symptoms and often transient disability (Swanson, 2011). For some people these events only happen once and their diagnosis remains as monophasic ADS, but others undergo several of these attacks over their lifetime leaving the conduction of brain signals impaired. The occurrence of multiple attacks is the main criterion that distinguishes MS patients from monophasic ADS patients.

The criterion for diagnosing multiple sclerosis has changed over the last several decades, but with improved technology, consensus definitions have been reached (2005 and 2010 McDonald Criteria) (Polman et al., 2005, 2011). These criteria include laboratory assessments (cerebral spinal fluid markers) as well as magnetic resonance imaging (MRI). The MRI assessments include the need to demonstrate new lesions

appearing over time following the initial attack and new lesions occurring in typical CNS regions, which confirms the chronicity of CNS inflammation in the central nervous system thereby excluding monophasic syndromes (Polman et al., 2011; Sadaka et al., 2012).

Symptoms of Multiple Sclerosis

Damage to myelin results in abnormal conduction of signaling in the brain, and often causes further damage to axons beneath these protective coverings. Symptoms from demyelinating attacks include weakness in the limbs, tingling or burning sensations, balance difficulty, blurry or double vision, and cognitive impairment (Thompson et al., 2013; Venkateswaran & Banwell, 2010). Some patients may also experience persistent fatigue and sometimes accompanying psychological symptoms such as depression (Banwell, 2014). Disease presentation and progression varies widely between patients. When presenting with symptoms to a hospital, patients generally receive intravenous steroids to decrease the inflammation in the CNS (Pohl et al., 2007). This can mollify symptoms transiently, but over time, some patients accumulate disability impacting mobility, coordination, and control over bodily functions, resulting in decreased quality of life (Banwell, 2014). In addition to the inflammatory phase of disease, MS patients also undergo a neurodegeneration process independent of attacks, resulting in permanent disability (Peterson & Fujinami, 2007).

Prevalence of Multiple Sclerosis

The MS International Federation estimates that over 2 million people around the world have MS, translating to 33 per 100,000 people (Thompson et al., 2013). However, developing countries are not always able to report on cases due to limitations in diagnostic equipment (MRIs) and shortage of neurology specialists (Thompson et al., 2013). Over the past several decades, researchers have noted differences in the prevalence of MS by gender and geographic location (Ebers, Sadovnick, & Risch, 1995; Marrie, 2004). The ratio of females to males with MS is about 3:1 indicating a higher risk for development of disease in females. The risk of MS was also thought to increase with greater distance from the equator; however, with the ability to migrate more easily than in the past, this effect has not been seen in more recent studies (Alonso & Hernán, 2008). People are generally diagnosed with the disease between the ages of 20-50, however, in the past decade MS has been increasingly recognized in people under the age of 18 (Banwell, 2014). The following section describes variations of MS occurring in the pediatric population.

Pediatric Multiple Sclerosis

Although cases have been identified and confirmed as far back as 1922 (Wechsler, 1922), ADS and MS in children is still considered rare but with increasing prevalence (Lavery et al., submitted manuscript). The exact prevalence and incidence rates of pediatric MS are unclear, but recent studies have estimated that around 3-10% of adult patients with MS had disease onset before the age of 18 (Banwell, 2014; Yeh,

Chitnis, Krupp, & Al., 2009). The Atlas of MS 2013 estimates that 7,000 children and adolescents around the world currently have MS with a pooled prevalence of 0.63 per 100,000, although they acknowledge that this is likely an underestimate because of the countries unable to provide data (Thompson et al., 2013). A German study reported a similar incidence rate of around 0.64 per 100,000 person years. They also reported that the rate clearly increased by age group from 0.09/100,000 in children 10 years and under to 2.64/100,000 in children 14-15 years old (Reinhardt, Weiss, Rosenbauer, Gärtner, & von Kries, 2014). Likewise, a study in the Netherlands reported an ADS incidence rate of 0.66/100,000 per year, of which 23% of patients went on to be diagnosed with MS (Ketelslegers et al., 2012).

Pediatric MS is similar to adult MS with some notable differences. Children have much higher relapse rates in the first two years of their disease compared to adults, which generally follows by a length of time in remission (Bigi & Banwell, 2012). They also have more defined symptoms of depression and fatigue compared to adults (Bigi & Banwell, 2012). The population of pediatric MS patients is also more ethnically diverse than the adult population and gender differences are only present in children over the age of 11 (Banwell et al., 2011). Interestingly, in adult patients about 20% of patients have a family history of MS compared to only about 7% in pediatric patients (Banwell, 2008; Yeh et al., 2009). This difference also led researchers to explore environmental factors that may be contributing to MS.

Unknown Etiology for Multiple Sclerosis

Two main questions still exist with how MS occurs. The first question involves the crossing of an inflammatory response through the blood brain barrier (BBB), which is a tight junction that regulates what can enter into the central nervous system (Minagar & Alexander, 2003; Weiss, Miller, Cazaubon, & Couraud, 2009). It is unusual for this to happen once as seen in patients with acquired demyelinating syndrome, but even more unusual for these attacks to occur over and over as seen in multiple sclerosis patients. This leads to a second question which considers why some people only have one demyelinating attack, but others go on to have multiple attacks. Several risk factors have been studied to help answer these questions and are described in the following section.

Risk Factors for Multiple Sclerosis

The cause(s) of the demyelinating attacks are unknown, although many risk factors have been suggested, including a combination of genetic and environmental components. Family members of persons with MS are more likely to develop the disease, although family history of MS is more common in adult MS patients than in pediatric MS patients (Banwell, 2008; Yeh et al., 2009). The concordance for monozygotic twins is about 30%, and the risk of MS increases by 3-4% in first degree relatives, which suggests that there is a genetic component to the disease. Since the genetic association with MS is not 100%, other environmental factors are likely contributing to the disease as well (Ebers et al., 1995; Marrie, 2004).

Several exposures have been examined to help determine the cause of MS, but no single infectious agent or environmental exposure has been determined. Associations have been noted with vitamin D intake, sunlight exposure, and infectious agents, which are described in more detail in the next chapter (Alotaibi, Kennedy, Tellier, Stephens, & Banwell, 2004; Banwell et al., 2011; Hanwell & Banwell, 2011; Langer-Gould, Brara, Beaber, & Koebnick, 2013). As mentioned previously, research has noted a variation in those who have MS by gender and geographic location (Banwell et al., 2007). Race was also thought to be a component of MS risk as the disease tends to be more prevalent in Caucasian people of northern European heritage. Differences in some of these risk factors have also been noted between MS patients and those with ADS who do not develop further symptoms following one demyelinating event (Banwell et al., 2009; van Pelt et al., 2013).

More recently, an association between active smoking and MS has been established (Ramagopalan et al., 2013; Riise, Nortvedt, & Ascherio, 2003; Sundström, Nyström, & Hallmans, 2008). Research examining the effects of smoking on MS risk has shown that adult smokers are more likely to be diagnosed with MS (Hedström et al., 2013b; O'Gorman et al., 2014), have greater disability after diagnosis with MS (Di Pauli et al., 2008; Pittas et al., 2009; Weiland et al., 2014), and have a shorter life span and decreased quality of life compared to those who did not smoke (Weiland et al., 2014). Population studies have found that the rate ratio for developing MS in a population is increased by 81% for smokers compared to non-smokers (RR=1.81; 95%CI 1.1, 2.9) (Riise et al., 2003). The associations seen in the adult studies are significant, yet few

studies have addressed the association with passive smoke exposure.

Only one population-based case-control study has examined the relationship between secondhand smoke exposure and pediatric multiple sclerosis. This study found that children exposed to tobacco smoke at home were twice as likely to develop MS compared to children not exposed to tobacco smoke (Mikaeloff et al., 2007). A more recent study has also shown that non-smoking adults who were exposed to tobacco smoke from secondhand sources who also have specific genetic factors (Human Leukocyte Antigen Complex or HLA genes) have an increased risk of MS by almost 8-fold (Hedstrom et al., 2014). These findings highlight the need for more studies to confirm the relation between TSE and MS in another pediatric population.

Study Purpose

MS is a debilitating disease that affects millions of people around the world. Although research has been conducted to delineate potential causal factors of the disease, no single risk factor has been identified. Identifying environmental factors in a young population provides the opportunity to examine more temporal relationships between these exposures and MS onset. The effect of active smoking on the increased risk of MS in the adult population, as well as findings from two small studies examining passive tobacco smoke exposure and MS risk, suggest that smoke exposure in any form may be an important risk factor for MS. Furthermore, smoke exposure in the presence of certain genetic factors, may be a mechanism behind which MS occurs (Hedstrom et al., 2014; Hedström et al., 2011). Literature is lacking with regard to how TSE might contribute to

the risk of MS in children and the consequences that this exposure might have on the child's disease burden. Information on TSE and genetic interactions has not been studied in children, particularly how these interactions may influence the risk of MS versus ADS. The purpose of this study is to examine the association between secondhand TSE and pediatric MS. Furthermore, this study aims to expand on the findings from the only other pediatric study addressing TSE and MS risk by assessing the relationship between TSE and other environmental risk factors for MS. Findings from the proposed study will aid in recognition of risks for this specific population, but may also provide potential insight into risk factors for other inflammatory or autoimmune disorders.

CHAPTER 2

PRELIMINARY STUDIES

Research suggests that demyelinating disease is likely caused by a combination of genetic and environmental factors (Ebers et al., 1995; Marrie, 2004). No single risk factor has been identified, but several potential exposures have been examined. Additionally, only a few studies have assessed risk factors that may influence the risk of MS compared to patients with only one demyelinating attack (ADS patients). The following sections describe prior research on potential genetic risk factors for MS as well as potential environmental influences to help ascertain why this disease occurs.

Risk Factors for Multiple Sclerosis

Potential Genetic Factors

Family members of persons with MS are more likely to develop the disease, although concordance rate among identical twins is only 30% (Ebers et al., 1995). Several potential genes have been studied and linked to MS progression, most commonly within the Human Leukocyte Antigen (HLA) complex, which is the locus of genes responsible for immune system regulation (Caillier et al., 2008; De Jager et al., 2008; Disanto et al., 2011; Lincoln et al., 2005). The HLA complex helps the immune system distinguish foreign particles (bacteria, viruses, etc.) from the body's healthy tissue (NIH, 2015). The gene complex consists of three classes of genes that provide different roles in immune system function, although the roles of some of the genes are not well understood.

The Class I genes (HLA-A, HLA-B, and HLA-C) are responsible for displaying proteins on the surface of most of the cells in the body so that the immune system knows to pass over them if they are normal body tissues (NIH, 2015). If the cell is part of a foreign body, then the immune system will know to attack the foreign invader due to lack of the displayed proteins. The Class II genes (DPA1, DPB1, DQA1, DQB1, DRA, and DRB1) provide instructions to the cells on how to make the proteins that are displayed to the immune system. The function of the Class III genes is thought to be related to inflammation and other immune functions, though the full purpose of this Class are still relatively unknown (NIH, 2015).

The International Multiple Sclerosis Genetics Consortium (IMSGC) investigated the role of the HLA genes on the risk of MS using a Genome-Wide Association Study to compare a large number of MS cases to controls (N=9,772 cases and 17,376 controls) (Sawcer et al., 2012). The presence of HLA-DRB1*15:01 (a component of the Class II HLA genes) was found to have the strongest association with MS. The IMSGC further identified several places for mutations that may be linked to MS, including 29 susceptible loci.

Replicating these findings in the pediatric population, Disanto et al. (2011) found that children with the HLA-DRB1*15 allele present were almost 3 times more likely to have been diagnosed with MS ($p<0.001$) (Disanto et al., 2011). Interestingly, patients with monophasic ADS (only a single demyelinating event) did not have the same risk, indicating that perhaps the absence of this allele distinguishes patients who have one demyelinating event from those who have multiple events and are diagnosed with MS.

Similarly, van Pelt et al. (2013) found that several single nucleotide polymorphisms (DNA sequence variations) significantly differed between patients with pediatric-onset MS and healthy controls, and between patients with MS and ADS (van Pelt et al., 2013). They hypothesized that disease onset during childhood may represent a heightened genetic susceptibility or greater exposure source. Masterman et al. (2000) expanded upon this and theorized that carriers of the HLA-DRB1*15 allele are more likely to develop MS at a younger age compared to non-carriers of the allele (Masterman et al., 2000).

Potential Environment Factors

Because genetic factors do not account for all the variability associated with MS risk, environmental factors are likely to contribute to risk as well (Baranzini, Srinivasan, & Khankhanian, 2010; Dyment, Ebers, & Sadovnick, 2004; Ebers et al., 1995). Several environmental factors have been examined to help determine the etiology of MS. Most attention in this area has focused on vitamin D intake and sunlight exposure and prior exposure to infectious agents such as Epstein Barr Virus (Ascherio & Munger, 2010; Bäärnhielm et al., 2012; Langer-Gould et al., 2013; Mowry et al., 2010).

Low vitamin D has been suspected as a risk factor for MS because prior studies have shown an increasing prevalence of MS with increasing distance from the equator (Ebers et al., 1995). It was suspected that people further from the equator have less sun exposure and therefore less circulating vitamin D levels. More recently it has been suspected as a risk factor for MS because of the anti-inflammatory effects it produces in the body (Singh et al., 2015). Munger et al. reported some protective effects with

consuming vitamin D supplements in a cohort of adults with MS (Munger et al., 2004). Patients who consumed more than 400 IU/d of vitamin D supplements were less likely to develop MS (RR= 0.58, 95%CI 0.35-0.96), although the findings were not significant when looking at vitamin D in the diet alone (Munger et al., 2004). In pediatrics, studies have confirmed this association, noting higher risk for MS with lower concentrations of 25-hydroxyvitamin D. Banwell et al. (2011) found that children with more than 74 nmol/L concentration of vitamin D in their blood serum were less likely to develop MS compared to children with very low serum concentrations of vitamin D (less than 50 nmol/L) (RR= 0.35, 95%CI 0.15-0.79).

Coinciding with the impact of Vitamin D, sunlight exposure has also been considered a risk factor for MS. Sunlight, specifically UVB rays, help convert cholecalciferol in the skin to a form of Vitamin D that can be absorbed in the body (NIH, 2016). Van der Mei et al. showed that 2-3 more hours per day of sunlight during the summer decreased the risk of MS by 60% (OR 0.32, 95%CI 0.16-0.59) (van der Mei et al., 2003). They also found that high exposure to sunlight during early childhood reduced the risk of MS. Another study confirmed this finding and reported that people with the lowest amounts of sunlight had the highest risk for MS (OR=2.2, 95%CI 1.5-3.3) (Bäärnhielm et al., 2012).

Research on prior exposures to infectious agents has shown conflicting results in the MS population. Some studies have shown that patients with pediatric MS are more likely to have been exposed to Epstein-Barr virus, which has shown to chronically activate B-cells, a lymphocyte primarily responsible for producing antibodies (Alotaibi et

al., 2004; Banwell et al., 2011). In a similar study, 86% of children with MS were found to be seropositive for Epstein-Barr virus while only 64% of controls were seropositive ($p=0.03$) (Banwell, Krupp, et al., 2007). The authors suggested that exposure to EBV could cause a heightened immune response, since those exposed to EBV were 3 times as likely to develop MS. Vaccinations were initially thought to trigger an immune response that lead to demyelination, but further research has found no correlation between vaccine administration and development of MS or related disorders (Langer-Gould et al., 2014; Mikaeloff, Caridade, Suissa, & Tardieu, 2009; Roos & Eckerman, 2002).

The common biological mechanism across the studies cited is the potential to increase inflammatory response given the exposures, thereby compromising immune function/regulation. More recent research has explored associations with tobacco smoke and MS risk because of the influence of tobacco smoke on inflammation (Johannsen, Susin, & Gustafsson, 2014). Smoking has also previously been linked to increased risk for various cancers, heart disease, lung disease, and birth defects (premature birth, low birth weight) (Groner, Huang, Nagaraja, Kuck, & Bauer, 2015; Mullen, 1999; Rajkumar et al., 2014; Wong, Malaison, Hammond, & Leatherdale, 2013). Many adult studies have found a link between active smoking and MS or smoking and disability progression with MS (Etemadifar, Afzali, Tabrizi, & Hosseini, 2012; Manouchehrinia et al., 2013; Pittas et al., 2009). While long-term smoking may lead to chronic inflammatory disease, the mechanism is not completely understood. The following sections discuss evidence for a link between smoking and tobacco smoke exposure on risk for MS, and further relate potential biological mechanisms behind increased risk.

Tobacco Smoking and Multiple Sclerosis

Tobacco smoke contains more than 7,000 chemicals, of which around 70 are cancer-causing (CDC, 2015). In addition to cancer, smoking is a known risk factor for many other diseases including cardiovascular disease and stroke. Most studies exploring links between active tobacco smoking and MS have reported the odds for MS to be higher in smokers compared to non-smokers (Briggs et al., 2014b; Di Pauli et al., 2008; Ramagopalan et al., 2013; Salzer et al., 2013). In a study in Norway, the rate ratio for developing MS in the country was found to be 81% higher for smokers compared to non-smokers (RR=1.81; p-value 0.014) (Lucchinetti et al., 1997). Interestingly, 75% of the MS subjects were either current or past smokers, which was much higher than the percentage of smokers in the general population of Norway (35%). Another population-based study found that odds of MS increased by 30% if the person reported ever smoking, although this study used spouses as the comparison group who were likely to have similar exposures to environmental factors (Ramagopalan et al., 2013).

Similarly, Hedstrom et al. (2013) reported a 50% increase in odds for developing MS in ever smokers compared to never smokers. Those who were current smokers were at a higher risk for developing MS (OR=1.6) compared to people who quit smoking more than 10 years ago (OR=1.0) (Hedström, Hillert, Olsson, & Alfredsson, 2013a). They also found significant trends for increasing risk of MS with increased duration of smoking and increased intensity of smoking (more smoked per day). Palacios et al. (2011) found that the incidence rate ratio for MS was higher in the smoking population, showing that smoking can be attributed to a 40% increase in MS risk on average (Palacios, Alonso,

Brønnum-Hansen, & Ascherio, 2011). Salzer et al. used cotinine (a biomarker for tobacco smoke exposure) to examine risk of MS. Results from the study showed risk for MS increased two-fold in groups that had elevated cotinine levels (OR=2.2, 95%CI 1.3-3.8) (Salzer et al., 2013). Additionally, Hernan et al. found increased risk for MS even in patients who had quit smoking. The odds for MS (compared to never smokers) was 1.3 (95%CI 1-1.7) for those who were classified as ever smokers who had stopped smoking and 1.4 (95%CI 1-1.9) for current smokers.

Tobacco Smoking and Disease Progression

Other studies have examined adult patients with MS to evaluate their disease progression based on exposure to tobacco smoke, generally through active smoking. When MS advances to a more progressive form of the disease (secondary progressive MS), patients suffer from increasing pain and disability. Hernan et al. (2005) examined the odds for disease progression to secondary progressive MS (Hernán et al., 2005). The hazard ratio for conversion to secondary progressive MS was 3.6 (95%CI 1.3-9.9) for ever smokers compared with never smokers, suggesting that smoking influences disease advancement in MS patients. Similarly, Sundstrom et al. (2008) found that ever smokers were more likely to have a progressive disease compared to never smokers (HR 2.1; 1.1-4.0). Moreover, early smoking initiation (and therefore a longer time smoking) lead to an earlier progressive disease onset (Sundström et al., 2008). Healy et al. (2009) observed 1465 MS patients longitudinally for an average of 3.29 years, and reported that smokers converted to progressive MS more quickly than never smokers (OR=2.5; 95%CI 1.4-4.4)

(Healy et al., 2009). Hedstrom et al. (2013) also found that those who quit smoking more than 10 years before the study assessment demonstrated reduced risk for MS progression compared to those who were current smokers, indicating important information that could be used for smoking cessation motivation (Hedström et al., 2013b). The results from these studies suggest a slight linear relationship with smoking and disease progression.

Neuroimaging of the brain and spinal cord through the use of magnetic resonance imaging (MRI) is often used to monitor MS progression. Although symptoms do not necessarily correlate with images seen on MRIs, these measures are often used as surrogates for disease progression (Lavery, Verhey, & Waldman, 2014). Brain inflammation is visualized on an MRI scan as areas of signal hyperintensity using a T2-weighted scan (Verhey et al., 2011, 2013). These hyperintense areas are revealed as brightened spots on the MRI sequences which can be quantified and total lesion volume can be calculated. In addition, MRI scans are qualitatively assessed by determining new versus old lesions and further assessed using gadolinium contrast agents (Lavery et al., 2014; Verhey et al., 2013; Verhey et al., 2011). Gadolinium is typically administered intravenously, and when it is visualized in the brain it represents an area of blood-brain barrier breakdown. Such enhancement is typically present for 30 days, therefore enhancement may be used to determine recent activity (Banwell, Shroff, et al., 2007; Callen et al., 2009). Generally, increasing number of lesions, increased lesion volume and decreasing brain volume are noted as markers of disease progression (Thomas et al., 2012; Verhey et al., 2011, 2013).

Healy et al. (2009) examined neuroimaging factors such as lesion volume and brain parenchymal fraction (BPF) as a marker for disease progression. BPF is a calculated ratio of grey and white matter volumes compared to total intracranial volume. BPF was significantly decreased in current smokers compared to never smokers and T2 lesion volume was significantly greater in current and ex-smokers compared to non-smokers (p-value = 0.002), indicating greater disease burden (Healy et al., 2009). Arikanoglu et al. (2013) also measured white matter lesions and T2-weighted lesions in the brain. Subjects were followed for 3, 6, and 12 months following their first demyelinating attack to determine relapses and further lesion development. Results suggest that white matter lesions were more detectable in smoking patients versus non-smoking patients (Arikanoglu, Shugaiv, Tuzun, & Eraksoy, 2013). Zivadinov et al. (2009) also found that tobacco smoking was associated with increased lesion burden and increased brain atrophy in MS patients (Zivadinov et al., 2009).

Importantly, evidence of more profound structural degeneration (more lesions and brain atrophy) among smokers versus nonsmokers is also observed through increased disability and disease burden. Nicotine, which increases microvascular blood flow, may be raising the influx of permeable solutes across the blood-brain-barrier. This influx is thought to initiate the development of MS (Zivadinov et al., 2009) and is discussed more in the next section. These previous studies have shown that risk of disease, as well as MS progression is associated with active tobacco smoking. All of the previous research on active smoke exposure has involved adult patients.

Tobacco Smoking and Burden of Disease

MS relapse rate, number of relapses, or time to first MS relapse are often included as outcome measures in MS treatment trials because they are easy to quantify and relapses most significantly impact the patient (Lavery et al., 2014). A relapse often leads to hospitalization for several days, which disrupts school, work, and other daily activities for families. Even following a hospital stay, a patient can suffer from visual impairment, paresthesia, fatigue, dizziness, and decreased movement (Banwell, 2004). More relapses generally indicate a more active disease and further degradation of myelin in the brain and spinal cord (Hahn, Shroff, Blaser, & Banwell, 2004; Thomas et al., 2012; L. Verhey et al., 2011). Few studies have been able to observe the effects of smoking on relapse rate due to the length of follow up time needed for these observations; however the few have had significant findings. For example, Di Pauli et al. (2008) used hazard models to determine the time to relapse in patients with MS. The hazard ratios for relapses were significantly increased in smokers compared to nonsmokers (HR 1.83, 95%CI 1.17-2.84) (Di Pauli et al., 2008). This study was able to adjust for many variables such as age, gender, and interferon treatment and still showed an increased relapse risk due to smoking. A similar study also found that those who had quit smoking more than 10 years ago had significantly decreased disease activity compared to people who quit smoking in the last year (Weiland et al., 2014).

Patients with MS develop varying degrees of visual, cognitive, physical, and psychological disability. These symptoms can be measured through neurological functional scales to determine the effects of MS on individual functional outcomes.

Additionally, a global scale was also created using scores from 8 functional exams to better qualify a patient's disability. This score, the Expanded Disability Status Scale (EDSS), ranges from 0 to 10 with 0 representing no disability to 10 representing death from MS and varying degrees of disability in between (Kurtzke & Al., 1983). This measure is commonly used in clinical trials and observational studies, but it is not very sensitive to change and lacks a clinically defined relevant change (McMillan & Moore, 2006). Clinical trials have generally defined disability progression, such as an increase in the EDSS of 0.5-1.0 points, but this has been challenged by the MS community since such a change over a short time could likely be measurement error or random variation (Lavery et al., 2014). The Multiple Sclerosis Severity Score (MSSS) was also created to help judge disability progression and takes into account both the EDSS score and the disease duration (McMillan & Moore, 2006). Using the MSSS, an individual's disability can be compared to a distribution of scores amongst people who have had the disease for a similar duration (Roxburgh et al., 2005).

Smoking status may also relate to disability in MS patients. Manouchehrinia et al. (2014) utilized the MSSS with a large patient base from hospitals in the UK. Smoking status was obtained at the patient's first clinical visit and at each subsequent visit (Manouchehrinia, Weston, Tench, Britton, & Constantinescu, 2014). Patients were classified as current-, ex-, and non-smokers. Results indicated that current smokers with MS had a reduction of about 10 years of their life expectancy compared to non-smokers with MS using standardized mortality ratios. While that outcome could be attributed to other direct health consequences of tobacco exposure, the same group reported in a

subsequent study that ever smokers were 2.37 times more likely to be in upper quartile of severe disease than non-smokers as measured by the MSSS (Manouchehrinia et al., 2013, 2014). Smoking cessation seemed to improve disability prognosis suggesting the potential that those who quit smoking may be able to slow their MS disease progression compared to those who continue to smoke. They also showed that compared to non-smokers, current and ever smokers were 64% more likely to reach EDSS scores of 4 (relatively severe disability but fully ambulatory without aid) and 49% more likely to reach EDSS scores of 6 (severe disability using intermittent or unilateral constant assistance to walk 100 meters without resting). Weiland et al. (2014) also had comparable findings, further supporting evidence that cigarette smoking was associated with increased disability.

Another study has shown a trend between increasing number of pack-years smoked and MS disability progression ($p<0.001$ for trend) (Pittas et al., 2009). Pittas et al. (2009) noted that smoking was associated with increased EDSS scores and progression of clinical disability during the observation period even after adjustment for multiple confounders. They also found a strong trend in the number of pack-years smoked and Multiple Sclerosis Severity Scale (MSSS) progression of clinical disability and EDSS progression even after adjustment for multiple confounders ($p<0.001$ for trend).

These results impress the need to encourage those with MS to quit smoking. The proposed study is unique in that it aims to examine varying levels of secondhand TSE on disease burden in pediatric MS. If similar results are found with secondhand TSE and

pediatric MS, parents will need to be informed of the impact of this modifiable risk factor on their child's disease progression.

Secondhand Tobacco Smoke Exposure and Multiple Sclerosis

A report by the Surgeon General in 2007 stated that those who inhale tobacco smoke through secondhand sources are exposed to more toxic chemicals than those who are actively smoking the cigarette (DHHS, 2007). The report also suggests that there is no safe amount of exposure to tobacco smoke and even short amounts of exposure can interfere with the functioning of the heart and vascular system. It can also damage the lining of the airways, which can lead to more frequent asthma attacks and other lung problems (DHHS, 2007).

Secondhand tobacco smoke exposure (TSE) has been linked to other health problems associated with inflammatory response and immune system impairment in children including asthma, respiratory infections and ear infections (Bandiera, Richardson, Lee, & Al., 2011; Jin, Seiber, & Ferketich, 2013). Cardiovascular research has shown that those who are exposed to tobacco smoke have higher levels of oxidant gas exposure, leading to higher levels of reactive oxidant species (ROS) (De Prins et al., 2014). The increased free radicals then bind to cells in the body resulting to damage to DNA and mitochondrial DNA. It has also been suggested that through oxidative stress, TSE leads to endothelial stress creating a cascade of inflammatory response (Groner et al., 2015). Jin et al. (2013) found that exposure to TSE more than doubled the odds of child hospitalization due to asthma and increased the odds for emergency room visits by

37% (Jin et al., 2013). In 2011, Kabir et al. found that children exposed to secondhand tobacco smoke in the home were 50% more likely to have 2 or more neurobehavioral disorders compared to those who were not exposed to secondhand tobacco smoke in the home (Kabir, Connolly, & Alpert, 2011). Children aged 9-11 showed the greatest risk for neurobehavioral issues due to their length of time in a smoking home.

Only a few studies have examined the associations of MS and secondhand TSE. Hedstrom et al. (2011) discovered that the risk for MS in non-smoking adults exposed to tobacco smoke through secondhand sources increased by 30% (OR= 1.3; 95%CI 1.1-1.6). They also found an increased risk for developing MS (p=0.003 for trend) with a longer duration of exposure. This evidence points to the utility and public health significance of examining the association between TSE and MS risk in children.

Only one study has looked at TSE and risk of MS in children (Mikaeloff et al., 2007). This study in France found that parental smoking at home was significantly associated with risk for MS in children (RR=2.2; 95%CI 1.43-5.15). Importantly, these results were robust, even after adjusting for family history of MS, autoimmune disease, and socioeconomic status. The results from these two TSE and MS outcome studies suggest that beyond active smoking, exposure to tobacco smoke through secondhand sources may also increase the risk of MS development (Hedstrom et al., 2014; Mikaeloff et al., 2007). Further studies need to be conducted to extend the findings from adult studies to include a detailed assessment in a pediatric sample. The proposed study will address this gap as well as provide a possible mechanistic link between TSE, genetics and MS development.

Potential Biological Mechanisms Underlying Tobacco Smoke Exposure and Multiple Sclerosis Risk

Several studies have proposed a biological link between smoking and MS. It is hypothesized that tobacco smoke causes a disruption in the blood-brain barrier (BBB) and has a direct influence on the immune system. Contributing to the complexity of this interaction, cigarettes contain over 500 chemicals that are absorbed into the blood stream, each potentially with a set of biologic consequences or responses within the body (CDC, 2014). The following sections outline potential ways that TSE could be linked to MS.

Inflammatory Response

The characterization of the effects of smoking on the immune system is an active area of discovery. It is hypothesized that cigarette smoke results in a pathologic imbalance in immune function. In mouse studies, nicotine suppresses the pro-inflammatory cytokine IL-6 and inhibits other immune cell production (Naik et al., 2014). Rat studies have supported the immunosuppressive properties of cigarettes, and attribute this effect to the particulate phase of cigarette smoke (secondhand smoke inhalation). Another toxin, perhaps carbon monoxide, increases endotoxin exposure which is one of the most potent inflammatory agents known (Arnson, Shoenfeld, & Amital, 2010). Arnson et al. (2010) also reports that smokers have on average 30% more circulating polymorphonuclear neutrophils (systemic inflammatory response) than nonsmokers, and the number of circulating T-cells is also increased in cigarette smokers. The authors also suggests that smoke can release intracellular antigens, overwhelming the scavenging capacity of the immune system, and creating an abnormal immune response

in a subset of individuals. Bi-products of smoke production have also been shown to stimulate the production of peripheral T-lymphocytes (Arnson et al., 2010). Although increased immune responses may be caused by tobacco smoke exposure, it is unclear how these immune responses pass through the blood brain barrier (BBB), the tight junction between the CNS and the blood stream.

The Blood Brain Barrier

The BBB is a tight junction that regulates what can enter into the nervous system via the blood (Ballabh, Braun, & Nedergaard, 2004). A disruption in this barrier could potentially compromise the tightly regulated, limited immune surveillance within the brain and result in a damaging and unintended immune response within the brain itself (Alvarez, Cayrol, & Prat, 2011; Minagar & Alexander, 2003). In MS, immune cells inappropriately gain access to the central nervous system and result in damage to the myelin sheath (Minagar & Alexander, 2003; Rosenberg, 2012). One challenge with identifying the mechanisms behind MS causation is addressing why immune cells are able to pass through the BBB and attack myelin (the protective sheath surrounding nerves). Exactly how or why this happens is unclear. A review by Chang et al. (2014) noted that cigarette smoking may modulate the tight junction proteins in the BBB resulting in increased permeability. Nicotine may also modulate co-transporters in the BBB which results in increased extracellular potassium levels and cerebral edema (Chang, Ho, Wong, Gentleman, & Ng, 2014). Inflammatory mediators, including cytokines, are abnormally expressed in smokers, and cytokines can pass through regions

where there is absence of the BBB, or they can act on other cells to signal influx to the brain (Chang et al., 2014). Another theory is that tobacco smoke leads to alterations in the BBB through overproduction of reactive oxygen species (ROS), and thus allowing the entry of leukocytes into the CNS (Alvarez et al., 2011; Gilgun-Sherki, Melamed, & Offen, 2004). This mechanism is termed oxidative stress and is further described below.

Oxidative Stress

Oxidative stress occurs when there is an imbalance in concentrations of oxidizing agents and anti-oxidant properties in the cells. Smoking tobacco has been linked to cellular damage through oxidative stress and free radical production (Hecht, 1999; Naik et al., 2014; Phaniendra, Jestadi, & Periyasamy, 2015). Maternal smoking has shown to alter the adaptive and innate immune system of newborns, both releasing and inhibiting pro-inflammatory and anti-inflammatory mediators. The disruption in the immune system is theorized to be caused by high concentrations of free radicals in cigarette smoke (Jana & Pahan, 2007; Lu et al., 2000; Naik et al., 2014; van Horssen et al., 2008). This in turn leads to increased oxidative processes. When the cellular mechanisms to counteract oxidative stress are overwhelmed, cells can be irrevocably damaged. Through the oxidative process, excess amounts of nitric oxide (NO) can be produced along with inducible NO synthase mRNA (iNOS). The increased amount of reactive species could lead to cell damage and death through oxidizing lipids, proteins and DNA.

Excess amounts of both NO and iNOS have been found in the brain tissue of MS patients, and not in healthy control brain tissue (Bagasra et al., 1995; Calabrese et al.,

2003). Other markers of oxidative stress have also been elevated in MS patients compared to healthy controls. Koch et al. (2006) determined that serum diene was significantly increased in MS patients, though the amount of anti-oxidant species were relatively the same (Koch et al., 2006). Oliveira et al. (2012) also found markers of oxidative stress (CL-LOOH and carbonyl protein) to be significantly higher in patients with MS. Additionally they observed the same markers to be correlated with EDSS scores ($r= 0.32$; $p=0.003$) (Oliveira et al., 2012).

Importantly, researchers have hypothesized that these oxidizing agents may target endothelial cells, which has implications in the blood brain barrier (BBB) and the accessibility of immune cells to the CNS (Groner et al., 2015). Leukocytes entering the CNS lead to a cascade of demyelination, axonal damage, and oligodendrocyte loss.

Potential Interactions between Tobacco Exposure and Genetics

While the response to tobacco smoke noted above may lead to inflammatory attacks (such as those seen in ADS patients), it does not necessarily describe the chronic inflammatory problems expressed in MS. One potential mechanism is the influence of tobacco smoke particulates on DNA interactions altered through repeated inflammatory responses. The free radicals described above may also interact with DNA and cause genetic mutation or activate genes that lead to autoimmune disease. Cancer studies have shown that active tobacco smoking can influence gene expression (Gelboin, 1980; Hecht, 1999; Liu et al., 2010; McLemore et al., 1990). Cigarette smoking has been linked to CYP/A1 gene expression in lung cancer patients and altered regulation of this gene has

further been associated with lung tumors (McLemore et al., 1990). Additionally, this gene expression has shown to persist for years even after the person has quit smoking (Landi et al., 2008). Hecht et al. (1999) described a process by which smoking causes metabolic activation creating DNA adducts and carcinogen metabolites bind to DNA. If this process persists, it can lead to miscoding and permanent mutations (Hecht, 1999). Beane and colleagues (2007) further expanded upon this research by describing 28 genes which have irreversible damage in smokers. Furthermore, as the expression of genes is altered, smoking bi-products can lead to permanent mutations (Hecht, 1999) and irreversible damage (Beane et al., 2007; Landi et al., 2008).

Few studies have examined the impact of both smoking and genetic factors. Hedstrom et al. (2011) analyzed data from the Epidemiological Investigation of Multiple Sclerosis in a group of 16-70 year olds in Sweden (867 cases, 1209 controls). Smoking status was obtained as well as blood samples. The HLA-DRB1*15 haplotype, whose overexpression has previously been linked to MS (Caillier et al., 2008; Disanto et al., 2011), was assessed as well as several other HLA genes. Those with DRB1*15 and another HLA allele (A*03) were shown to have an increased odds for MS (OR=3.5 and 1.7 respectively) when adjusting for age, gender, residential area, and ancestry. The odds of MS was 60% higher in smokers compared to non-smokers (95%CI 1.3-2.0). An interaction was observed between the presence of DRB1*15, absence of HLA-A*02, and smoking on the risk for MS (OR=13.5, 95%CI 8.1-22.6). Without the genetic factors, the odds ratio was not significantly increased even among smokers (OR=1.4, 95%CI 0.9-2.1 for smokers without the genetic traits). MS risk appears to increase by HLA genotypes

and is influenced by smoking status. The interactions between HLA haplotypes and smoking are displayed in Figure 1 below (Hedström et al., 2011).

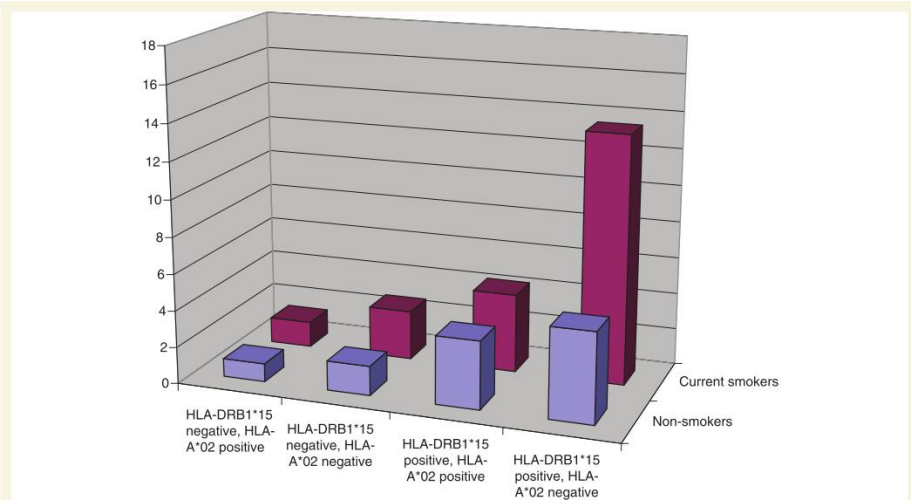

Figure 1.Interactions between smoking in adults and HLA genes. Source- Hedstrom AK, et al. Brain, 2011.

More recently, Briggs et al. (2014) also noted that a particular variant of N-acetyltransferases (NAT-1) was overexpressed in patients with MS compared to healthy controls. This relationship has been noted in several cancer studies as well since the NAT1 enzyme assists with the detoxification process and acetylation of molecules within the body (Bouchardy et al., 1998; Walker et al., 2009). The authors found that those who expressed a variant of the NAT1 gene and were also smokers were 5 times more likely to develop MS than never smokers (Briggs et al., 2014a).

In addition to active smoking, Hedstrom et al. (2014) also analyzed whether exposure to secondhand smoke, along with the same genetic markers referenced above, would show similar interactions in adult MS. They found that an interaction was still

present with passive smoke exposure and DRB1*15 and the absence of HLA-A*02. With the two genetic risk factors and exposure to passive smoking, the odds of MS was 7.7 times higher than those who did not have the genetic factors and were not exposed to smoke (OR 7.7; 95%CI 5.5-10.8). Figure 2 shown below depicts the interactions between the MS risk genes and secondhand TSE in adults (Hedstrom et al., 2014). Although not as pronounced as the interaction with active smoking, it appears that smoke exposure through passive sources still interacts with genes to produce risk for MS. It is unclear whether people with specific genes are already susceptible to MS or if differential expression (i.e., epigenetic change) due to exposure to cigarettes may occur.

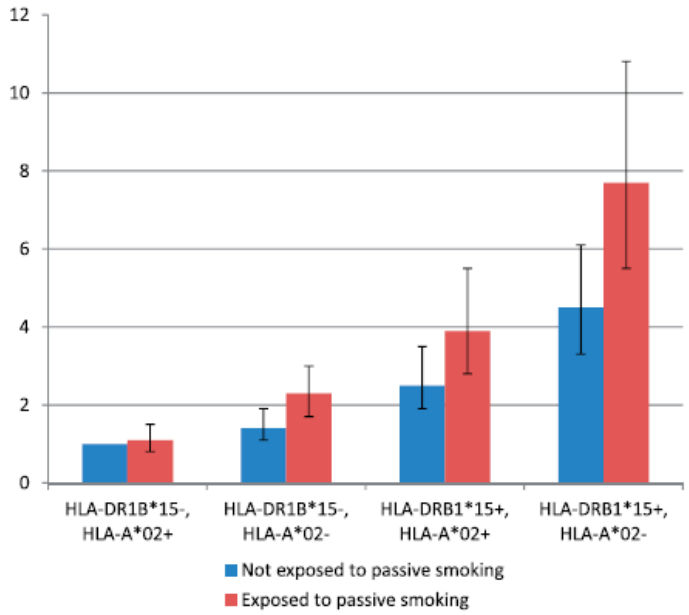

Figure 2. Interactions between passive smoke exposure and HLA genes. Source- Hedstrom AK et al. International Journal of Epidemiology, 2014.

Potential Interactions between Tobacco Exposure and Other Environmental Factors

In addition to the potential interactions between smoke exposure and genetics, other suspected environmental risk factors for MS have also been assessed for interaction with smoking in adult populations. The first study to review smoking status, genetics and prior EBV exposure interaction was published in 2010 (Simon et al., 2010). Simon et al. (2010) pooled risk ratios from the Nurse's Health Study, Tasmanian MS Study, and the Swedish MS Study. Genetic factors assessed included the HLA-DRB1*1501 haplotype. Participants were also asked whether they were current, past, or never smokers. Researchers noted an increased risk for MS for ever-smokers in all three studies, with a pooled risk ratio of 1.5 (95%CI 1.1-1.9). Increased risk was also observed for those who presented with HLA-DRB1*15 alleles (RR= 3.2; 95%CI 2.5-4.2) and for patients with higher anti-EBNA titers (Epstein Barr virus nuclear antigens, OR=2.6; 95%CI 1.8-3.8). Increasing EBV antibody titers with smoking exposure increased risk of MS significantly (OR=3.9;95% CI 2.7-5.7), and the researchers suggested a multiplicative interaction was present between the two exposures. Ever smokers with the highest EBV antibody titers had a seven-fold increase in MS (RR=7.4, p-value<0.001) (Simon et al., 2010).

Sundquivst et al. (2012) also had pronounced interaction findings between smoking, HLA genes, and antibodies for EBV. They found that the risk factors for MS are as follows: antibodies for EBV had an OR of 1.79 (95%CI 1.42-2.27), HLA-DRB1*15 had an OR of 3.24 (95%CI 2.54, 4.13), and ever smoking had an OR of 1.30 (95%CI 1.03-1.64). The highest risk observed came from those who smoke, who had higher antibodies for EBV, and had specific genetic markers [OR= 26.3 (95%CI 10.2-

67.9)]. The authors concluded that it is possible that many mechanisms may exist to increase the odds of MS. Furthermore, each genetic marker along with smoking could create the chronic immune response by which HLA mediates disease development; however, they did not observe a multiplicative interaction between antibodies for EBV and smoking, which Simon et al. had previously reported (Simon et al., 2010; Sundqvist et al., 2012).

The uniqueness of the proposed study is that exposure to tobacco smoke and potential interactions with genetic and other environmental factors on the odds for MS have yet to be assessed in the pediatric population. These interactions also have yet to be compared between MS patients and ADS patients. Importantly, it will be the first to examine whether TSE interacts with other known risk factors to exacerbate or increase the likelihood of MS disability. Results may help elucidate important environment and genetic interactions in children who are closer to biological onset of disease with a relatively shorter duration of environmental exposures.

Measures of Tobacco Smoke Exposure

Self-Report Measures

Most of the current research on TSE and MS has relied on questionnaires or interviews to assess smoking status and smoke exposure. Questionnaires generally ask if the subject (adult patient or pediatric patient caregiver) has ever smoked, the number of cigarettes smoked per day, and the duration of smoking. From these answers, subjects are classified into different categories depending on the study. Some used gross means of

"ever" versus "never" smoking status for smoking classification, while others created multiple categories based on the number of cigarettes per day reported. Length of time smoking was also considered, in addition to the number of cigarettes smoked. When assessing chronic smoke exposure, studies generally used the number of pack-years a subject reported, i.e. the number of years they spent smoking a pack of cigarettes per day.

Ideally, a self-report exposure assessment would utilize validated methods such as timeline follow-back methods which assists participants with recalling behaviors from up to two years prior (Sobell & Sobell, 1992). These guided interviews utilize calendars and other tools to help remind people of their behaviors. For example, a person can be asked if there are particular events that caused them to smoke inside instead of outside over the last week such as weather patterns or other incidents. Using special occasions can also help a person remember prior days for which they can relate their behaviors to. Additionally the timeline follow-back method (Brown et al., 1998) provides a way to assess changes over time in smoking behavior, particularly over a longer period of time. The downside of these coaching/interview sessions is the timing. Interviews generally take 1-2 hours to conduct so it can be time-consuming for both the patient and provider.

Brief self-report assessments are not always able to capture variations in behavior over time and can have biases such as inaccuracy of reporting. Despite these drawbacks, however, many self-report measures have shown to be reliable with regard to exposure assessment. Marrie et al. (2009) measured the level of agreement between two surveys conducted over two years asking about patients' smoking habits. They found that 96% of those who reported ever smoking on the first questionnaire reported ever smoking on the

second questionnaire (Marrie, Cutter, Tyry, Campagnolo, & Vollmer, 2009). The intra-class correlation coefficient was 0.73 for those entering age of smoking initiation and 0.90 for smoking cessation. Demographic information was assessed in relation to response reliability, and young participants, African-Americans, depressed participants, and those in lower socio-economic positions were less reliable with their responses.

Biological Measures- Cotinine

Biological methods can help contribute to the validity of self-report measures (Hovell, Zakarian, Wahlgren, Matt, & Emmons, 2000). Tobacco smoke is comprised of many chemicals, several of which may occur in the natural environment and are components of the body's natural metabolism (e.g. carbon monoxide). These constituents may also fluctuate in levels throughout the day, making the exact exposure amount difficult to assess and quantify. Nicotine concentrations in the body reflect degree of exposure to tobacco smoke (Benowitz, 1996; CDC, 2015). It is highly soluble in water and is metabolized through the liver. The proximate metabolite of nicotine is cotinine, which has been the gold-standard for measuring biological TSE (Benowitz, 1996; CDC, 2015). Approximately 70-80% of nicotine is converted to cotinine, so it offers a comparable estimate of the levels of nicotine within the body (see Figure 3 from Benowitz, 1996).

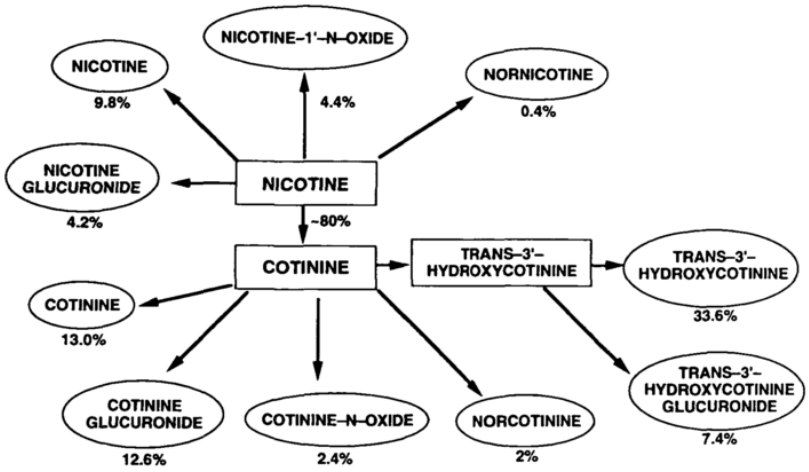

Figure 3. Metabolites of nicotine. Source- Benowitz, Epidemiol Rev, 1996.

Cotinine can be measured in the blood, urine, hair, and fingernails and offers good sensitivity and specificity (Benowitz, Bernert, Caraballo, Holiday, & Wang, 2008). It has a 16-18 hour half-life in the body, which is approximately the same in serum, urine, and saliva (Benowitz, 1996). The preferred matrix seems to be serum, which can be stable for years once it is frozen (Benowitz et al., 2008). Cotinine is a preferred measure to nicotine because unlike nicotine, the measurable amounts of cotinine remain relatively stable throughout the day, and the half-life of cotinine is much longer than nicotine (Benowitz, 1996). It has been used in several studies of TSE and health risks, and was found to be useful in quantifying the effects of smoke exposure on a variety of illnesses. In a review of TSE biomarkers, Benowitz (1996) comments that, "cotinine appears to be the most specific and most sensitive biomarker for exposure to nicotine from ETS [Environmental Tobacco Smoke]."

Cotinine measures also help classify even minor exposures to smoke. If parents indicate that they only smoke outside for example, a biological measure could show that the child is still exposed due to air currents, seeping of smoke through windows and doors, and other "third-hand" exposure sources. Third-hand smoke exposure refers to exposure to nicotine and tobacco smoke constituents through residues found in walls and carpets that are remnants from times where active smoking was present. Tobacco smoke remnants can also off-gas from smokers clothes even when they have smoked in another room or outside (Benowitz, 1996). The biological markers will also allow for the assessment of a dose-response relationship, which is difficult to determine with self-report measures. Results could help show parents that despite modifying habits (smoking only outdoors or not near their children), their children are still being exposed. They also may not be aware of all the sources of exposure mentioned above, and biological measures could help make them aware of these sources. Although cotinine measures are considered the gold-standard for measurement by some (Benowitz, 1996), it is not a perfect measure since it is subject to variations by individual metabolisms. The half-life is also longer in infants and young children compared to older children and adults (Hovell et al., 2000; Matt et al., 1999).

Only a few studies have examined the comparability between self-report measures and biological measures of TSE. In a study of 141 maternal smokers, Matt et al. (2000) found that survey methods significantly correlated with their child's urine cotinine samples (range- 0.40 to 0.59). They determined that mother's self-report of their child's exposure correlated with the biological measures and that the biological measures

correlated with environmental measures taken in the home. The authors also expressed that comprehensive measures of smoke assessment can help to provide more thorough estimates of TSE, so using multiple methods of sampling (self-report and biological) when possible was ideal. Similarly, McDonald et al. (2005) reviewed the sensitivity and specificity of self-reported smoking and serum cotinine levels in pregnant women. They hypothesized that pregnant women may not want to admit to smoking due to the negative connotations surrounding smoking during pregnancy; however, they found that the specificity was 100% and sensitivity 97.6% between self-report and biological confirmations of exposure. Several other studies have found similar correlations between parent's self-reported smoking habits and biological measures from the child (Bahçeciler, Barlan, Nuhoğlu, & Başaran, 1999; Crawford et al., 1994; Emmons et al., 1994; Greenberg, Haley, Etzel, & Loda, 1984; Hovell et al., 2000; Marbury, Hammond, & Haley, 1993). Hovell et al. (2000) reports that cotinine measures can help contribute to the validity of the self-report measures and both can be satisfactory measures of exposure (Hovell et al., 2000).

Only one study has previously utilized biological measures to assess smoking and MS risk. Salzer et al. (2013) found that elevated cotinine levels were associated with risk for MS in those younger than the median age of 26 years (OR=2.2, 95%CI 1.3-3.8) (Salzer et al., 2013). Risk estimates determined from the self-report questionnaire were found to be similar to the risk estimates for cotinine levels, although no dose response relationship was found with cotinine levels and risk for MS. No other studies have

assessed the association between MS and TSE using measures of cotinine, including within the pediatric population.

Significance

To date, no research has been conducted in the pediatric MS population that has been able to verify exposure to tobacco smoke using biological measures of TSE. This study is also one of the first to examine TSE differences between pediatric MS patients, ADS patients, and healthy controls. Additionally, this is one of the first studies to determine if relationships exist between TSE and other risk factors which may distinguish MS patients from ADS patients. If TSE is associated with MS risk, then intervention methods will need to be tailored to persuade parents and caregivers to protect their child from all sources of TSE, especially if increased exposure leads to worsening of the disease for their children. Tobacco smoke exposure is a modifiable risk factor for this vulnerable group and multi-level strategies can be employed utilizing the pediatrician-family relationship to both help motivate parents to quit smoking and lessen their child's exposure to tobacco smoke (Lepore et al., 2013). Findings from the proposed study will aid in recognition of risks for this specific population. This study may also provide potential insight into the interplay between TSE and its impact on the pro-inflammatory cascade, which may be informative for other inflammatory and autoimmune disorders as well.

Hypotheses and Underlying Assumptions

Aim 1: To examine the relationship between secondhand tobacco smoke exposure and pediatric demyelinating disease.

Hypothesis 1a: Reports of TSE in the home will be higher among parents of children with MS compared to healthy controls.

Hypothesis 1b: Reports of TSE in the home will be higher in children with ADS compared to healthy controls.

Hypothesis 1c: Reports of TSE in the home will be higher in children with MS compared to patients with ADS.

This will be one of the first studies exploring the association between TSE and pediatric demyelinating disease, and differentiating exposure effects between MS and ADS patients. TSE will also be validated using a biomarker of exposure (serum cotinine, a metabolite of nicotine). Based on several findings from smoking studies in adults, it is assumed that children exposed to tobacco smoke in the home will be at increased odds for MS compared to those not exposed. Additional analyses within this aim clarify exposure effects in three groups: 1) MS patients, 2) ADS patients, and 3) healthy controls. Logistic regression models will be built assessing the effects of TSE on the odds for MS compared to healthy controls and the odds of ADS compared to healthy controls. A model will also be created to examine the effects of TSE on the odds for MS compared to ADS patients. This relationship is important to investigate because the mechanism behind why some patients only have one demyelinating attack (ADS) and others have

multiple over several years (MS) is unknown. Variance in exposures may provide insight into why some patients only have an isolated attack while others develop MS.

Aim 2: To assess the moderating effects (interaction) of secondhand tobacco smoke exposure on other known risk factors for pediatric MS patients compared to pediatric ADS patients.

Hypothesis 2a: Patients exposed to tobacco smoke who also have HLA-DRB1*15 alleles will be at increased odds for MS compared to ADS patients.

Hypothesis 2b: Patients exposed to tobacco smoke with low serum vitamin D will be at increased odds for MS compared to ADS patients.

Hypothesis 2c: Patients exposed to tobacco smoke with prior EBV exposure will be at increased odds for MS compared to ADS patients.

Previous research has shown that specific HLA alleles may predispose patients to MS, when compared to both healthy controls and ADS patients (Caillier et al., 2008; Disanto et al., 2011). Additionally, the HLA-DRB1*15 allele has exhibited the strongest genetic effect on risk for MS (De Jager et al., 2008). More recent evidence has shown a possible interaction between secondhand tobacco smoke exposure and the presence of HLA-DRB1*15 alleles in adults (Hedstrom et al., 2014). The current study is one of the first studies to assess this relationship in the pediatric population. Further analyses will also investigate associations between TSE in combination with other environmental exposures (serum vitamin D levels and prior EBV exposure), and the odds for developing MS compared to ADS patients. Low vitamin D has been associated with increased risk for

MS in both the pediatric and adult populations (Hanwell & Banwell, 2011; Munger et al., 2004). Therefore, it is hypothesized that the presence of both TSE and low vitamin D levels will increase the odds of MS compared to ADS patients. Patients with MS are also 3 times more likely to have been exposed to EBV prior to MS diagnosis (Alotaibi et al., 2004; B Banwell, Krupp, et al., 2007). Additionally, prior research has noted a multiplicative interaction between active smoking and prior EBV infection in adults (Simon et al., 2010; Sundqvist et al., 2012). It is assumed that exposure to prior EBV infection in conjunction with secondhand TSE will increase the odds for developing MS in the pediatric population as well.

Aim 3: To determine whether exposure to tobacco smoke relates to increased disease burden in pediatric MS patients as measured through neurological disability functional scores.

> *Hypothesis 3a*: Children who are reportedly exposed to tobacco smoke in the home will have higher neurological disability scores indicating a greater burden of disease.
>
> *Hypothesis 3b*: Children who have greater levels of cotinine (indicating higher exposure to tobacco smoke) will have increased neurological disability scores indicating a greater burden of disease.

Prior studies assessing the impact of active smoking on disease burden have shown that active smokers have a higher disease burden than non-smokers (Manouchehrinia et al., 2013; Weiland et al., 2014). Additionally, a trend has been reported between increased

amounts of smoking and MS progression and disease burden (Pittas et al., 2009). Specifically, this study will examine at the impact of TSE on a child's disease burden measured through neurological functional scores assessed by study neurologists. The hypotheses will be assessed in two ways. First, self-report measures of exposed/not exposed will be examined in conjunction with functional scores to determine if MS patients who are exposed to tobacco smoke have more disease burden than those who are not exposed to tobacco smoke. Functional measures will be assessed at baseline, 2 years, and 3 years following symptom onset. Secondly, cotinine values will be categorized as no exposure, low exposure, and high exposure to determine if increased exposure leads to increased burden of disease in MS patients over time.

CHAPTER 3

STUDY METHODS

Study Overview

This study utilized data from the Canadian National Demyelinating Disease Study which was introduced in Canada in September of 2004. Data were collected on participants through 2013. The parent study aimed to collect information from all incident cases of demyelination over several sites in Canada, and more recently, the Children's Hospital of Philadelphia (CHOP) was added as a collecting site (N=24 collecting sites).

Patients were diagnosed within the hospital or clinic setting at the participating hospital and were followed for at least 3 years to confirm diagnosis of multiple sclerosis versus acquired demyelinating syndrome. Information was collected on each patient using neuroimaging techniques, blood sampling, genetic testing, and environmental questionnaires. Thorough neurological and physical exams were performed at baseline (first symptom onset), 3 months, 6 months, 12 months, and then annually after the initial visit.

The analysis of the current study was a case-control study design. Information on exposures to tobacco smoke and other environmental factors was assessed at baseline. This study was approved by the Institutional Review Boards at each of the participating sites in Canada and at CHOP. The current analysis was reviewed by the Temple University IRB and was found to be exempt from full review (exempt letter in the Appendix B).

Description of Participants

Children under the age of 16 who presented to participating hospitals within 30 days of symptom onset of the initial demyelinating event were approached for recruitment. Participants gave consent at the enrolling site. Demyelinating diagnoses included for recruitment were clinically isolated syndromes, transverse myelitis, optic neuritis, and acute disseminating encephalomyelitis. Only participants who have tobacco smoke exposure information collected from questionnaires within 1 year of disease onset were used in this analysis. Participants were classified into disease and non-disease categories using the methods described below.

Multiple Sclerosis Patients (N=77)

Multiple sclerosis (MS) patients were diagnosed using the McDonald 2005 criteria which was described in the introduction (Polman et al., 2005). Generally, differential diagnosis is made by a second demyelinating attack, which distinguishes an MS patient from a monophasic ADS patient, but further classification can be made using magnetic resonance imaging (MRI). For this study, confirmation of MS was decided by a panel of pediatric neurologists using guidelines set by the 2005 McDonald Criteria:

1) A second attack of demyelination occurring more than 30 days from the initial event (confirmed new neurologic deficits persisting for 24 hours or longer in the absence of fever or infection); or,
2) A second MRI scan showed new lesions meeting criteria for dissemination in time.

Monophasic Acquired Demyelinating Syndrome Patients (N=248)

Monophasic Acquired Demyelinating Syndrome (ADS) patients were defined as patients who presented with a single demyelinating attack and at the study end point did not develop further evidence (clinical or radiographic) of disease. Patients were followed over several years (at least 3) to confirm the diagnosis of monophasic ADS which is consistent with clinical practice and expert recommendations. Diagnosis was verified by a panel of experts in pediatric MS.

Healthy Controls (N=69)

Healthy controls were selected through voluntary participation at the primary institution and one other participating site (the Hospital of Sick Children, Toronto, ON; Alberta Children's Hospital, Calgary, Canada). Controls were required to have no existing neurological conditions, inflammatory diseases, or a history of MS in their family. Healthy controls were recruited towards the end of the study enrollment between 2009-2012. Since genetics and blood sample analysis were conducted for MS and ADS patients prior to healthy control enrollment, a majority of the healthy control group has not yet had blood samples analyzed, and therefore, information is not available for many of the covariates assessed for this study. Therefore, data from healthy controls will only be utilized for Aim 1.

Outcomes of Interest

Aim 1- Primary Outcomes

Aim 1 is the diagnosis of MS as described above. TSE was compared between MS patients and healthy controls and between MS patients and ADS patients. The secondary outcome was the diagnosis of ADS and the effects of tobacco smoke exposure was compared to healthy controls.

Aim 2- Outcomes

The primary outcome of interest for aim 2 is the diagnosis of MS. Effects of exposures will be compared between MS and ADS patients only.

Aim 3- Outcomes

For the third aim of this analysis, the outcome is disease burden as measured by neurological disability functional scores, along with the associated symptoms:

- Pyramidal function- trouble moving limbs or weakness in limbs;
- Cerebellar function- tremor, loss of coordination, loss of control of bodily movements;
- Brainstem function- problems swallowing, problems talking, or experiencing rapid eye movement;
- Sensory- loss of sensation, numbness;
- Bowel and bladder function- loss of control of bowel and/or bladder;
- Vision- difficultly with vision or loss of vision.

Each function is rated on a scale from 0 (no disability) to 6 (severe disability). The scoring system is based on the Expanded Disability Status Scale (Kurtzke & Al., 1983), a standard scoring method used to quantify disease outcome/burden in patients with MS. The scores were dichotomized for analysis (0= minimal functional symptoms, 1=more than minimal symptoms) to compare the impact of tobacco smoke exposure on low vs high impairment. Only cases of MS were included in this analysis.

Primary Independent Variable- Tobacco Smoke Exposure

Exposure to tobacco smoke through secondhand sources was the main exposure of interest in this study. Tobacco smoke exposure (TSE) was determined in two ways: parent self-report of patient's exposure through questionnaires and confirmation of exposure through serum cotinine analysis. Parent self-report was the primary method used to identify pediatric patients exposed to tobacco smoke within the first year of their symptom onset. Because some of these patients did not have exposure information at baseline from questionnaires (N=13), serum cotinine at baseline was assessed to confirm that TSE was present at symptom onset. A detailed description of the collection methods follow.

Parent Self-Report

To assess the child's smoke exposure, parents were asked:

"Does anyone in the patient's household smoke while living with the patient?"

If the parent answered "yes", they were further asked:

2) How many people smoke;

3) How old was the patient when exposed to TSE smoke in the home;
4) How many cigarettes per day are/were consumed (on average) in the patient's household/car;
5) If no-one in the home smokes now, age of the patient when smoking ceased.

Data from the questionnaires was analyzed as a dichotomous variable indicating 1) exposed to tobacco smoke in the home at disease onset, and 2) not exposed to tobacco smoke in the home at disease onset. Although all of the above questions were asked of the parents, only 28% answered questions 2-5 which provided more details about exposure (i.e. number of cigarettes, age when exposure started). Therefore, serum cotinine values (biological measure of tobacco exposure) were used to help inform on quantity of tobacco smoke exposure.

Child's Serum Cotinine

For parents who indicated that a smoker was present in the home of the child, the child's serum cotinine was analyzed. When possible, samples from the baseline visit were analyzed and if baseline samples were not collected, a serum sample was analyzed within 3 months of symptom onset. Analysis of serum cotinine was performed at The Children's Hospital of Philadelphia in the Clinical Research Core laboratory. Samples were sent securely from the Montreal Experimental Therapeutics Laboratory, which stored frozen serum samples from participants in the study. Cotinine was assayed using Enzyme-Linked Immunosorbent Assay (ELISA) which has been a validated test for determining serum cotinine levels (Abnova Corporation). Procedures were followed per the assay

protocol. Control runs were used on each tray to determine inter-tray variability. Values were stored in a master data file as ng/mL. For all patients who did not have TSE information available from environmental questionnaires at baseline, cotinine values were used to verify exposure status at baseline/symptom onset. Cotinine values of 0.8 ng/mL or greater classified participants as being exposed (Benowitz et al., 2008; Benowitz, 1996). Additionally, thirteen MS patients did not have self-report information available. To help retain sample size, cotinine values were used to classify these patients within the dichotomous variable as exposed or not exposed to tobacco smoke at baseline.

Although cotinine values aided in verification of exposure status, analysis of serum cotinine was only possible for patients whose parents indicated their exposure to tobacco smoke in the home. In order to investigate the effects of smoke exposure as a continuous variable in Aim 3, cotinine values were imputed for the missing data among children reported as not exposed to tobacco smoke. Since 100% of those unexposed were missing cotinine data, a random number generator was used to impute values between 0 and 0.8, which would be indicative of no exposure in this population since 0.8 was the cutoff used to confirm exposure (Benowitz et al., 2008). Because people can be exposed to small amounts of nicotine from other sources, non-zero values were selected for imputation. This measure is limited because it assumes that children whose parents said they were not exposed were truly not exposed.

Covariates

Based on prior research, several covariates were assessed as part of the analysis for the study and are described in more detail below.

Age and Gender

Pediatric MS is known to differ by age and gender. The ratio of girls to boys is similar in children under 11 but changes to a 2.6:1 ratio of girls to boys in older children (Yeh et al., 2009, Lavery et al., submitted manuscript). Additionally, ADS patients generally present at a younger age than MS patients, so age difference between the two groups is expected. It was also assumed that older children are more likely to have longer exposure to tobacco smoke. Information from the environmental questionnaires did not accurately assess the length of time exposed to smoke since too much data was missing (~50%), therefore, it was assumed that older patients likely had longer exposures than the younger patients and thus may impact findings. Age may also be a factor in the analysis utilizing cotinine, since cotinine metabolizes differently in older children compared to younger children. Because of these factors, both age and gender will be adjusted for within the analyses in aims 1 and 2.

Genetic Assessment

Whole blood samples were obtained at each visit for most patients within the study. Of the participants, only a subset of MS and ADS patients (N=266 children) have previously been typed for HLA alleles using PCR amplification. Specifically, each

sample was assessed for 13 of the HLA-DRB1 alleles which have previously been linked to MS. Based on prior research, this study specifically looked at the presence of DRB1*15 because of its suspected association with MS (Barcellos et al., 2003; Disanto et al., 2011; Lincoln et al., 2005; Masterman et al., 2000). A dichotomous variable was created indicating 1) the presence of DRB1*15 alleles, or 2) no DRB1*15 alleles present. Genetic information was only available for 13 of the 69 healthy controls, so effects of these alleles were only considered between MS and ADS patients.

Vitamin D

Serum vitamin D levels at baseline was also considered as a covariate within analyses. Vitamin D plays a significant role within immune system function and low levels have been associated with MS risk (Hanwell & Banwell, 2011; Maruotti & Cantatore, 2010). The effects of serum vitamin D levels will be assessed as a continuous variable in models as well as a dichotomous variable. The dichotomous variable will be created based on previously defined levels which have indicated the most significant risk, 1) vitamin D levels below 50 nmol/L, and 2) vitamin D levels greater than or equal to 50 nmol/L (Banwell et al., 2009). Serum vitamin D was only analyzed in a sample of MS and ADS patients so will not be compared in the models with healthy controls.

Epstein Barr Virus

Epstein Barr Virus (EBV) serology was also considered for analysis as prior studies have shown that prior EBV exposure is related to MS risk (Alotaibi et al., 2004;

Banwell, Krupp, et al., 2007; De Jager et al., 2008). Remote EBV infection was assessed in a prior analysis which determined whether a patient has been exposed to the virus at some point during their life. A dichotomous variable will be created to indicate 1) prior exposure to EBV, and 2) no prior exposure to EBV. Prior exposure to EBV was only analyzed in a sample of MS and ADS patients so will not be compared in models with healthy controls.

Power analysis

Data for this study includes patients who presented with a demyelinating disease in Canada. All patients who presented to one of the participating sites with demyelination within 30 days of symptom onset were approached to participate in the study. A post-hoc power analysis was calculated for the primary outcome analysis in Aim 1 and Aim 2, using information about exposure prevalence in MS patients compared to the healthy control group and MS patients compared to ADS patients (Table 1). Using an alpha of 0.05 and anticipating an odds ratio of 2 (Mikaeloff et al., 2007), the power for analyzing the effects of TSE on odds for MS compared to healthy controls was approximately 50%. With similar parameters, the power for analyzing the effects between MS patients and ADS patients was approximately 70%. Since the power for this study is low, results will need to be interpreted with caution since the primary analysis of this study is under-powered.

Table 1

Post-hoc estimation of study power using primary comparisons from Aims 1 and 2

Comparison	Alpha	Sample Size A	Sample Size B	OR to detect	Calculated Power
MS Patients, Healthy Controls	0.05	77	69	2	49.13%
MS Patients, ADS Patients	0.05	77	248	2	72.02%

Data Analysis Plan

All analyses were conducted using Stata software version 12.0 (College Station, TX). All data was first examined for any outliers and continuous variables assessed for normality. Demographics were compared among disease groups and healthy controls using t-tests (mean comparison of continuous variables) and chi-square tests (comparison of categorical variables) where appropriate.

Aim 1 Analyses

Aim 1 tests three hypotheses examining the effect of TSE on the odds for demyelinating disease. The primary outcome for Aim 1 is the diagnosis of MS, which has previously been recorded and validated by pediatric neurologists. As described previously TSE will be analyzed as a dichotomous independent variable indicating exposed or not exposed.

For the primary endpoint (MS) logistic regression will be used to determine the odds for developing MS given exposure to secondhand tobacco smoke in the home. This relationship will be compared to both healthy controls and ADS patients. The effects of

TSE on a secondary outcome, ADS, were also assessed using logistic regression, comparing exposures in this group to healthy controls.

For models comparing disease groups to healthy controls, only age and gender could be controlled for within the models (*Aim 1a&b*). Information on prior EBV exposure and vitamin D was unavailable for healthy controls and only 19% had information on HLA; therefore, these covariates could not be controlled for within the models. For the model assessing TSE and the odds of MS compared to ADS patients, vitamin D, prior EBV exposure, and HLA genes were considered for the final model along with age and gender (*Aim 1c*). Univariate analyses were used to determine which variables were added to the final model. Any associations that had a p-value of 0.25 in univariate analysis were considered for the final models (Hosmer & Lemeshow, 2000). Model fit was estimated using Hosmer-Lemeshow goodness of fit techniques.

Aim 2 Analyses

Aim 2 tests the moderating effect of TSE on the relation between other known risk factors and MS compared to ADS patients. Each additional exposure (HLA-DRB1*15 [*Aim2a*], serum vitamin D [*Aim2b*], and prior EBV exposure [*Aim2c*]) was assessed for interaction effects (moderation) with the primary study exposure TSE. Interaction analysis was performed for each exposure relationship using guidelines offered by Szklo and Nieto, Rothman, and VanderWeele (Rothman, Greenland, & Walker, 1980; Szklo & Nieto, 2007; VanderWeele, 2013):

1. Stratified analysis, which helps determine the impact of each variable on the odds for MS in the absence of the other factor, as well as the odds for MS when both

factors are present, was first performed for each covariate in relation to TSE. The stratified analysis uses those not exposed to either factor as the reference group. This analysis also allows for the assessment of additive interaction, which would imply that the combined effect of both exposures is greater than the sum of the individual factors (Gordis, 2014; Szklo & Nieto, 2007).

2. The presence of additive interaction was determined using the formula below recommend by VanderWeele and Rothman et al. (Rothman et al., 1980; VanderWeele, 2013):

   Interaction OR – Var1 OR – Var 2 OR + 1 = Excessive Risk

   If the excessive risk was greater than 0 then additive interaction was considered present.

3. An interaction term was created between each exposure and TSE (ex: Vitamin D*TSE). Regression models were built with and without the interaction term, and a likelihood ratio test was performed to determine if the interaction term improved the fit of the logistic regression model. The interaction term specifically helps to determine whether multiplicative interaction is present, and if so, does it add value to the model assessing the odds for MS compared to ADS. Models were also adjusted for age and sex.

For any significant interactions found through this analysis, attributable proportions (AP) were calculated using the formula supplied by VanderWeele (2013):

$$\mathbf{AP} = \frac{\mathbf{Interaction\ OR - OR1 - OR2 + 1}}{\mathbf{Interaction\ OR - 1}}$$

The AP is the estimated proportion of disease which can be attributed to the interaction between the exposures. Although since the relative risks were unknown, odds ratios were used to estimate the relative risk (Hedstrom et al., 2014; VanderWeele, 2013).

Aim 3 Analyses

Aim 3 explores the effects of TSE on disease burden in MS patients. This association was measured in 2 ways:

1) The dichotomous smoke exposure variable was used to determine if exposure to tobacco smoke was related to functional scores for MS patients at baseline, 2 years, and 3 years following symptom onset. The proportion of patients who presented with more than minimal symptoms in each functional measure was assessed and compared between the exposed and unexposed groups. Chi-square tests were used to detect difference in the proportion of patients within the exposed and unexposed groups (*Aim3a*).

2) Continuous cotinine values were categorized to represent high exposure (values >1.094 ng/mL), low exposure (0.8≤1.094 ng/mL), and no exposure (<0.8 ng/mL). A 50% median cut-point in cotinine was used to classify high or low exposure. Chi-square tests were used to determine the proportion of patients presenting with more than minimal symptoms at baseline (*Aim3b*).

Missing Data Analysis

Participant information was assessed at the beginning of the study to verify missing data. Information on the primary outcomes and primary exposure was not

missing in this study population. Only a few ADS subjects were missing demographic information (age and sex) and were excluded from the logistic regression analyses. Because of the timing of blood sample analysis, some MS and ADS patients were missing data on vitamin D, or EBV status. Most healthy controls were missing information on HLA and no information was available for healthy controls on serum vitamin D levels or prior EBV exposure. Since the majority of the missing covariates are because of the timing of enrollment and not related to other factors, missing data for the covariates are likely to be missing completely at random (MCAR) (Greenland & Finkle, 1995; White & Carlin, 2010). With MCAR data, complete case analysis should be valid (Greenland & Finkle, 1995; White & Carlin, 2010); however, as a statistical exercise, missing imputation analysis was performed for the missing data to determine how improved sample size would affect the outcome of the analyses for Aims 1 and 2.

Since all healthy controls were missing information on vitamin D and EBV infection, values could not be imputed for healthy controls. In addition, although 13 of the 69 healthy controls had information on HLA alleles, none of the 13 presented with HLA-DRB1*15, which limited the ability to impute genetic information for healthy controls as well. Therefore, missing data analysis was restricted to analyses involving MS patients and ADS patients (Aims 1c&2). A full description of the results from the missing data exercise is presented in the results section.

Multiple imputations (MI) are arguably one of the most efficient ways to maximize a dataset with missing data (Donders, van der Heijden, Stijnen, & Moons, 2006; Graham, 2009; White & Carlin, 2010), although the methods assume that data are

missing at random (MAR) rather than MCAR. MI replaces missing values with multiple sets of simulated values, analyzes each created and complete dataset, and automatically adjusted the parameters for uncertainty of the missing data. Multiple imputations using chained equations (MICE) was performed using the *ice* program within Stata statistical software (Stata Corp, College Station, TX). The MICE method uses a sequence of univariate imputations to impute data, but also allows varying types of data (e.g. binary variables) to be entered in to the models.

A series of 40 imputational sets were used in order to maximize validity of the imputed set (Donders et al., 2006; Graham, 2009; White, Royston, & Wood, 2011). Imputations were created based on the participants' status as an MS or ADS patient, utilizing age as predictor for imputations. The analyses for Aim 1c and Aims 2a-c were repeated using the imputed datasets. The results of the imputed dataset are presented in a side-by-side comparison with the complete case analysis (only participants without missing data). Z-scores will be used to assess statistically relevant changes in estimates between the complete case analysis and MI analysis.

Ethical Considerations

This collection of data for this study was approved by the Institutional Review Boards of each collecting site. The Children's Hospital of Philadelphia was among these sites and received approval as well. For the proposed analysis, no patient identifiers will be used. The Institutional Review Board at Temple University approved the secondary analysis of this dataset and determined the study to be exempt from review (approval

letter in Appendix A). Data has been stored on a secured server at The Children's Hospital of Philadelphia.

CHAPTER 4

RESULTS

Baseline Characteristics

Comparison of baseline characteristics is presented in Table 2. Only information on gender and age was available for healthy controls. Age at onset was significantly different between MS (mean age 12.6 ± 3.4) and ADS (mean age 8.8 ± 4.5) patients, which was expected based on prior research. Healthy controls mean age was not significantly different from MS patients (mean age 14.4 ± 2.2 for healthy controls), but was different from ADS patients who were significantly younger on average. Distribution of gender also differed significantly between the three groups. Twice as many MS patients were female (67%) compared to a fairly even distribution of gender among ADS patients (51% female). Gender distribution of controls was in between the two disease groups. Race and ancestry were fairly similar between the ADS and MS patients, which were mostly made up of white people of European decent.

Few subjects had family history of MS from any family members, and this number did not differ significantly between the two groups. Presentation of initial phenotype varied between MS and ADS patients as ADS patients were more likely to present with ADEM, which was expected.

Table 2

Baseline characteristics of study participants

	Multiple Sclerosis N =77	**Monophasic ADS N =248**	**Healthy Controls N=69**	**p-value**
Age at Onset (years)	12.6 (3.4)	8.8 (4.5)	14.4 (2.2)	<0.001
Female (N;%)	52 (67%)	126 (51%)	41 (59%)	0.015
Ratio F:M	2:1	1.03:1	1.46:1	
Ancestry (N;%)				
European	44 (60%)	144 (62%)		0.59
Non-European	29 (40%)	89 (38%)		
Race (N;%)				
White	56 (79%)	184 (81%)		0.756
Non-White	15 (21%)	43 (19%)		
Phenotype at Onset (N;%)				
Monofocal	55 (71%)	197 (82%)		0.052
Polyfocal	22 (29%)	44 (18%)		
ADEM	3 (4%)	74 (30%)		
Family History of MS (N;%)				
First-degree Relative	1 (1.3%)	4 (1.6%)		0.567
Any	6 (7.8%)	12 (4.8%)		

Identification of Exposure

Information on the primary exposure of interest (secondhand tobacco smoke) is presented in Table 3. Approximately 34% of parents in the MS group and 30% of parents in the ADS group reported smoke exposure while only 26% of healthy control parents reported the presence of a smoker in the child's home. All groups reported higher levels of smoking than the general population of Canada which is approximately 19.9% for all of Canada and 19.4% for the province of Ontario (Schwartz, 2013).

Cotinine was analyzed for all subjects whose parents reported smoking and for 13 MS subjects whose smoking status was not ascertained from parent report. The average serum cotinine values were significantly higher in the MS group (mean= 7.09 ± 20.2) compared to the ADS patients (mean =1.84 ± 6.4) and healthy controls (mean=0.83 ± 0.03), with greater range of values in the MS group. Median cotinine values between the groups were more comparable, with both MS and ADS patients having slightly higher median values compared to the healthy controls. These results were reflective of two significantly higher values for cotinine in the MS group compared to the rest of the study participants.

Table 3

Exposure to tobacco smoke in the home among pediatric MS patients, ADS patients, and healthy controls

	Multiple Sclerosis N = 77	**Monophasic ADS** N =248	**Healthy Controls** N= 69	**p-value**
Parent -reported Child Exposure	26 (34%)	75 (30%)	18 (26%)	0.567
Cotinine	N=25	N=69	N= 14	
Mean Concentration (ng/mL)	7.09 (20.2)	1.82 (6.4)	0.83 (0.03)	<0.001
Median Concentration (ng/mL)	1.04	1.05	0.83	<0.001

Distribution of covariates

Prior genetic analysis was conducted for 196 patients with ADS (79%) and 74 patients with MS (94%). Additional information on prior EBV infection and baseline serum vitamin D levels was obtained for fewer patients (sample size for each data element recorded in Table 4). As mentioned in the methods, limited information was available for genetics and no information was available on other exposures for healthy controls; therefore, their information was not included in Table 4.

Compared to ADS patients, more MS patients had one or more HLA-DRB1*15 alleles (42% compared to 28%). Among the MS patients (N=49) and ADS patients (N=134) with available EBV exposure data, a majority of MS patients had prior exposure to the virus (78%) whereas only 39% of ADS patients has a prior exposure to the virus. MS patients also had significantly lower vitamin D levels (p=0.005) and a higher

proportion were in the lower tertiles of vitamin D exposure when compared to ADS patients (47% of MS patients compared to 30% in ADS patients).

Table 4

Distribution of covariates between pediatric MS and ADS patients

	Multiple Sclerosis	**Monophasic ADS**	**p-value**
HLA-DRB1*15	N=74	N=196	0.037
One or more alleles	31 (42%)	55 (28%)	
EBV Remote Infection	N=49	N=134	<0.001
Positive	38 (78%)	52 (39%)	
Serum Vitamin D	N = 39	N=126	0.005
Mean Concentration (nmol/L)	51.9 (21.9)	64.6 (25.6)	
Vitamin D Tertiles (nmol/L)	N = 39	N=126	0.091
<=49.8	18 (47%)	38 (30%)	
49-9-74.0	14 (36%)	44 (35%)	
>=74.1	7 (18%)	43 (34%)	

Aim 1- TSE and MS

Logistic Regression Analysis

Univariate analyses were first conducted to determine the odds of MS based on exposure to secondhand tobacco smoke in the home, which was coded as a dichotomous variable of exposure (0=not exposed, 1=exposed). Models were created to compare MS patients to healthy controls, ADS patients to healthy controls, and MS patients to ADS patients. Models using healthy controls as the comparison group were adjusted for age and sex. Table 5 displays the results from these analysis comparing disease groups to healthy controls. The Hosmer-Lemeshow Goodness of Fit test for both models had a p-value over 0.05, and we can infer that the models fit the data well.

Table 5 - Aim 1a,b

Logistic regression models associating the effects of TSE between MS patients and healthy controls and monophasic ADS patients and healthy controls, adjusting for age and sex

Model	**MS vs HC (Aim 1a)**	**ADS vs HC (Aim 1b)**
	N=144	N=317
Tobacco Smoke Exposure	1.84 (0.86, 3.95)	2.24 (1.08, 4.63)*
Age	0.78 (0.67, 0.90)	0.60 (0.52, 0.69)
Sex	1.47 (0.72, 3.02)	0.81 (0.42, 1.57)

Pseudo R^2	0.08	0.33
Goodness of Fit p-value[±]	0.467	0.996

[±] Hosmer-Lemeshow Goodness of Fit test
*Significant at the 0.05 level

The odds for MS compared to healthy controls was 1.84 times higher for those exposed to tobacco smoke in the home, although the increase was not significant at the 0.05 level (*Aim1a*). However, children who were exposed to tobacco smoke in the home were 2.24 times more likely to have ADS compared to healthy controls (OR=2.24; 95%CI 1.08, 4.63), which was significant at the 0.05 level (*Aim1b*).

The final model comparing MS patients to ADS patients (*Aim1c*) contained age, sex, EBV remote infection status, HLA-DRB1*15 alleles, and vitamin D levels since all variables were independently identified as significant risk factors for MS (Table 6). TSE did not significantly impact the odds of having MS compared to ADS (OR=0.98; 95% CI 0.41, 2.35). Each individual factor was independently associated with MS risk; however when put into a model together, the significance of some of the risk factors was reduced.

Table 6- Aim 1c

Logistic regression models associating the effects of TSE between MS patients and monophasic ADS patients, adjusting for age, sex, EBV remote infection, HLA-DRB1*15 alleles, and vitamin D .

Model	**MS vs ADS**
	N=157
Tobacco Smoke Exposure	0.98 (0.41, 2.35)
Age	1.19 (1.06, 1.35)*
Sex	1.36 (0.59, 3.15)

EBV Remote Infection	2.61 (1.09, 6.28)*
HLA-DRB1*15 Alleles	1.89 (0.83, 4.34)
Vitamin D Levels	0.99 (0.97, 1.00)*
Pseudo R^2	*0.174*
Goodness of Fit p-value[±]	*0.04*

[±]Hosmer-Lemeshow goodness of fit test
*Significant at the 0.05 level

The effects of HLA-DRB1*15, which were significantly associated with odds for MS in univariate analysis, were also no longer significant in the full model although the sample size was reduced in the full model due to missing data for other risk factors. Vitamin D levels were also only moderately significant when added to the full model as a continuous variable. The Hosmer-Lemeshow Goodness of Fit test indicates that the model does not completely fit the data, therefore, other potential variables may be impacting this relationship or the sample size may be limiting the data.

Aim 2- Relationship of MS Risk Factors

The considered covariate risk factors (HLA-DRB1*15, vitamin D, and prior EBV infection) were related to increased odds for MS independently (compared to monophasic ADS patients); however, when all of the factors were entered into a logistic regression model, significance of some of these factors decreased. Stratified analyses were then conducted to determine if interaction was present between each exposure variable and TSE and to examine the differing effects of these multiple exposures on the odds for MS. Results from each sub analysis comparing these multiple exposures are described below.

*Aim 2a- Tobacco Smoke Exposure and HLA-DRB1*15 Alleles*

Table 7 shows the effects of TSE on MS risk when stratified by the presence and absence of HLA-DRB1*15 alleles. Compared to patients with ADS, the presence of HLA genes in the absence of TSE did not significantly increase the odds for MS. Similar results were found for odds of MS in the presence of TSE without the presence of HLA genes. Importantly, when both HLA genes and TSE were present, the odds for MS significantly increased to 3.23 (95%CI 1.04, 9.79). This suggests that patients who present with HLA-DRB1*15 alleles may be more susceptible to MS when exposed to tobacco smoke.

Table 7 - Aim 2a

Stratified analysis assessing the effects between TSE and HLA-DRB1*15 alleles at disease onset and odds for MS

TSE	HLA-DRB15	Number of Patients MS/ADS	OR (95%CI)	p-Value
-	-	27/97	1	
-	+	21/45	1.68 (0.81, 3.45)	0.131
+	-	15/44	1.22 (0.55, 2.66)	0.584
+	+	9/10	3.23 (1.04, 9.79)	*0.021

* Significant at the 0.05 level

The relative excess risk due to potential interaction between TSE and HLA genes was also calculated using the formula below recommend by VanderWeele and Rothman et al. (Rothman et al., 1980; VanderWeele, 2013):

Interaction OR – Var1 OR – Var 2 OR + 1 = Excessive Risk

3.23 -1.68- 1.22 +1 = 1.33

Since the excess risk is more than 0, then additive interaction is considered to be present (Szklo & Nieto, 2007; VanderWeele, 2013). This result suggests a slight positive, additive interaction between HLA genes and tobacco smoke exposure to increase the odds for MS compared to ADS.

Additionally, multiplicative interaction of HLA genes with TSE was assessed using an interaction term in logistic regression models. Table 8 shows the logistic regression models with and without the interaction term.

Table 8 – Aim 2a

Results of logistic regression models with and without the presences of the TSE*HLA interaction term

	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	1.12 (0.59, 2.14)	1.17 (0.54, 2.57)
HLA	2.20 (1.18, 4.10)*	2.30 (1.09, 4.86)*
Age	1.25 (1.15, 1.36)*	1.25 (1.15, 1.36)*
Sex	1.81 (0.99, 3.32)	1.82 (0.99, 3.34)
TSE*HLA	--	0.86 (0.22, 3.37)
Pseudo R^2	0.144	0.143
Likelihood-ratio test p-value = 0.83		

* Significant at the 0.05 level

The interaction term was not significant in the model (p-value= 0.829). The results of a likelihood ratio test returned a p-value of 0.823, which indicates that the null model (without the interaction term) should not be rejected, implying that the interaction term does not add to the fit of the model. Therefore, any interaction present between the

exposures is unlikely to be multiplicative.

The attributable proportion was also calculated, which is the proportion of the disease that is estimated to be due to the potential interaction between the two exposures (Hedstrom et al., 2014; Rothman et al., 1980; VanderWeele, 2013):

$$\mathbf{AP} = \frac{3.23 - 1.68 - 1.22 + 1}{3.23 - 1} = \mathbf{0.60}$$

The calculated attributable proportion accounted for 60% of MS risk that could be attributed to the presence of both TSE and HLA alleles. However, since we are using the odds ratio to approximate the relative risk, the attributable proportion is just an estimate of the values we might see if the odds ratios are similar to the relative risk in this population.

Aim 2b- Tobacco Smoke Exposure and Vitamin D

Similar analyses were performed examining the combined effects of TSE and vitamin D levels to determine if interaction was present (Table 9). Serum vitamin D levels under 50 nmol/L were considered as a risk factor for MS based on prior research (Banwell et al., 2011).

Table 9 - Aim 2b

Stratified analysis assessing the effects between TSE and low serum vitamin D at disease onset and odds for MS

TSE	Vitamin D (<50)	Number of Patients MS/ADS	OR (95%CI)	p-Value
-	-	41/149	1	
-	+	9/25	2.03 (0.90, 4.54)	0.087
+	-	17/62	0.98 (0.37, 2.55)	0.963
+	+	9/13	2.89 (1.12, 7.46)	*0.028

* Significant at the 0.05 level

Neither decreased vitamin D or TSE were significantly related to risk of MS without the presence of the other factor. However, having low vitamin D and being exposed to secondhand tobacco smoke in the home significantly increased the odds for MS by 2.89 (95%CI 1.12, 7.46) when compared to ADS patients.

Presence of additive interaction was again assessed (Rothman et al., 1980; VanderWeele, 2013):

IR = 2.52 – 1.00 – 1.31 +1 = 1.21

Since this value was over 0, we can again assume that interaction is present (Szklo & Nieto, 2007; VanderWeele, 2013). This relationship suggests a slight positive additive interaction, although the sample size in the stratified groups limits interpretation.

Logistic regression models were created to include an interaction term between TSE and vitamin D. Table 10 shows the results of the models with and without the interaction term.

Table 10- Aim 2b

Results of logistic regression models with and without the presences of the TSE*Vitamin D interaction term

	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	0.93 (0.51, 1.70)	0.70 (0.25, 1.95)
Vitamin D	1.29 (0.65, 2.53)	1.37 (0.57, 3.30)
Age	1.28 (1.17, 1.39)*	1.29 (1.16, 1.43)*
Sex	1.80 (1.01, 3.22)*	1.87 (0.91, 3.86)
TSE*Vitamin D	--	1.70 (0.38, 7.74)
Pseudo R^2	0.148	0.151
Likelihood-ratio test p-value = 0.335		

*Significant at the 0.05 level

The interaction term was not significant (p=0.335), and a likelihood ratio test confirmed that the model was stronger without the interaction term (rejected the hypothesis that the model with the interaction term was better, p=0.336). This suggests that the increase in OR is additive rather than multiplicative (Gordis, 2014; Szklo & Nieto, 2007; VanderWeele, 2013).

The attributable proportion of the exposures was calculated:

$$\mathbf{AP} = \frac{\mathbf{2.52 - 1.00 - 1.31 + 1}}{\mathbf{2.52 - 1}} = \mathbf{0.80}$$

The attributable proportion shows that a large proportion of disease (80%) can again be attributed to the presence of the two exposures. However, since we are using the odds ratio to approximate the risk ratio in this calculation, interpretation of this finding is limited.

Aim 2c- Tobacco Smoke Exposure and Prior Epstein Barr Virus Exposure

Lastly, a stratified analysis assessed the relationship between TSE and prior Epstein Barr Virus (EBV) exposure (Table 11). While both exposures together increased the odds of MS, the main driver of the increase was prior exposure to EBV. Although TSE increased the odds of MS when combined with EBV, it also expands the variance of the measure. The increased odds may have had a significant additive effect, but this was unable to be determined because of small sample size in the MS group specifically.

Table 11 – Aim 2c

Stratified analysis assessing the effects of TSE and prior EBV exposure and the odds for MS

Passive Smoking	Prior EBV Exposure	Number of Patients MS/ADS	OR (95%CI)	p-Value
-	-	10/65	1	
-	+	21/33	4.13 (1.62, 10.9)	*0.001
+	-	1/17	0.38 (0.01, 3.07)	0.360
+	+	15/19	5.13 (1.79, 14.9)	*0.043

* Considered significant at the 0.05 level.

Adding an interaction term to logistic regression models showed that these two variables did not interact on a multiplicative level (p=0.297). Table 12 shows the results of the likelihood ratio test, which also indicated that the model was stronger without the

interaction term (p-value= 0.255).

Table 12 – Aim 2c **Results of logistic regression models with and without the presences of the TSE*EBV interaction term**		
	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	0.78 (0.34, 1.76)	0.28 (0.03, 2.50)
EBV Remote Infection	3.85 (1.70, 8.72)*	2.99 (1.19, 7.52)*
Age	1.25 (1.12, 1.40)*	1.25 (1.12, 1.40)*
Sex	1.74 (0.80, 3.78)	1.71 (0.79, 3.72)
TSE*EBV	--	3.51 (0.33, 37.3)
Pseudo R^2	0.192	0.198
Likelihood-ratio test p-value = 0.255		

*Significant at the 0.05 level

The low sample size in the category of MS patients exposed to tobacco smoke but with no prior EBV exposure data may be limiting the results shown; however, it appears that TSE and EBV could work in conjunction with each other, although the full effect of this relationship needs to be studied in a larger sample size.

Aim 3- Effects of TSE on Disease Outcome using Serum Cotinine

The effect of TSE on disease burden in MS patients (N=77) was measured through functional scores presented at baseline as previously described. Table 13 shows the proportion of MS patients who presented with more than minimal problems as determined by neurological disability functional measures.

Table 13 – Aim 3a

Impact of exposure to tobacco smoke on functional scores in MS patients at baseline, 2 years, and 3 years following incident attack, proportion with more than minimal disability

	Baseline (N=77)			**2 Years Post Onset (N=76)**			**3 Years Post Onset (N=54)**		
	TSE	*no TSE*	*p-value*	*TSE*	*no TSE*	*p-value*	*TSE*	*no TSE*	*p-value*
Pyramidal	56%	48%	0.515	26%	25%	0.924	35%	32%	0.842
Cerebellar	43%	28%	0.186	30%	18%	0.234	40%	12%	0.016
Brainstem	26%	22%	0.770	9%	0%	0.058	0%	6%	0.269
Sensory	39%	38%	0.926	13%	13%	0.980	20%	26%	0.591
Vision	32%	29%	0.760	18%	11%	0.424	21%	13%	0.445
Bowel/bladder	19%	8%	0.160	14%	3%	0.088	11%	3%	0.252

* Significance of results adjusted for Bonferroni correction of multiple comparisons (p=0.05/6 = 0.008).

Across all functional scores, a higher percentage of MS patients presented with more than minor functional problems at baseline in the exposed group compared to the unexposed group, although results were not statistically significant after adjusting for the Bonferroni correction for multiple comparisons in the same dataset. More than minimal cerebellar functions were presented in 43% of those exposed to tobacco smoke compared to 28% of those not exposed to tobacco smoke indicating more problems with coordination and body control in the exposed group at symptom onset, although the difference was not significant. Functional measures were explored further in the MS patient group 2 years and 3 years following disease onset, although some patients were lost to follow up at year 3 (N=23 lost to follow up). Those who were exposed to tobacco smoke (as indicated with baseline self-report measures) had more cerebellar issues 3

years after disease onset (40% compared to 12% in unexposed group), although this result may be impacted by those who were lost to follow up. The decrease in cerebellar function at 3 years is indicative of loss of coordination or loss of control of bodily movements, which only worsens as the disease progresses.

Additionally, three levels of cotinine exposure were created to compare levels of exposure to baseline symptom onset in MS patients: no exposure (<0.08), low cotinine (0.08≤1.094), and high cotinine (>1.094). The results from this "dose response" assessment were inconclusive (*Aim3b*). If the hypothesis about greater TSE relating to decreased function was supported, we would have observed a higher proportion of patients presenting with more than minimal symptoms at baseline in the high exposure category. A higher proportion of patients in the high cotinine category presented with more than minimal cerebellar and visual function symptoms compared to the low and no exposure categories, however the sample size in all categories was low. Other functional scores showed higher proportions of patients in exposure overall (either low or high exposure) which is supported by the results presented in Table 13, although these differences are not statistically significant. Limitations of these metrics are presented in the discussion.

Missing Data Analysis

As described in the methods, a large amount of data was missing for the primary covariates in this study. Table 14 below displays the number and proportion of missing data for MS patients and ADS patients. Although a large proportion of information on

vitamin D and EBV infection are missing for the study population, this is largely because the majority of those missing data were enrolled in the study after the blood sample analysis.

Table 14 – Missing Data Analysis

Summary of missing covariate data for MS and ADS patients

	MS Patients (N=77)	ADS Patients (N-=248)
HLA-DRB1*15	3 (4%)	52 (21%)
Vitamin D	38 (49%)	122 (49%)
EBV Exposure	28 (36%)	115 (46%)

A comparison was also made of demographic factors between patients missing data and patients not missing data. The mean age did not differ between those missing and not missing data in both MS patients (p=0.74) and ADS patients (0.35). The proportion of males and females and the proportion of patients exposed/not exposed to tobacco smoke in the home was also not significantly different for patients (MS and ADS) missing and not missing data. Aim 1c and Aims 2a-c analyses were repeated and compared to the complete case analysis as described below.

Multiple Imputations

Table 15 below provides the number of complete cases, the number of imputed cases, and the total sample size for MS and ADS patients after imputation. Complete

covariate exposure information was only available for 61% of MS patients and 54% of ADS patients. After imputations, a total of 77 MS patients and 248 ADS patients were considered for analysis.

Table 15 – Multiple imputation analysis

Number of complete and imputed observations in each group

	MS Patients			**ADS Patients**		
	Complete N	*Imputed N*	*Total N*	*Complete N*	*Imputed N*	*Total N*
HLA-DRB1*15	71	6	77	195	53	248
Vitamin D	51	26	77	152	96	248
EBV Exposure	47	30	77	134	114	248

Aim 1c Multiple Imputation Analysis Comparisons

Aim 1c assessed the effects of TSE on the odds for MS compared to ADS, while adjusting for age, sex, prior EBV infection, presence of HLA-DRB1*15 alleles, and serum vitamin D levels. Table 16 shows results for the logistic regression model before imputation (complete case analysis) and after imputation. The results of the multiple imputation (MI) analysis were similar to the complete case analysis (CCA), although the standard errors were smaller and confidence intervals slightly narrower. A Z-test comparing the estimates showed they were not significantly different. TSE was still not significantly related to the odds of MS compared to ADS, even when controlling for other suspected risk factors and confounders. Therefore, missing covariate data is unlikely to change the relationship in this study.

Table 16- Aim 1c Comparisons

Multiple imputation analysis for Aim 1c- Logistic regression models associating the effects of TSE between MS patients and monophasic ADS patients, adjusting for age, sex and using imputed values for EBV remote infection, HLA-DRB1*15 alleles, and vitamin D

Model	**MS vs ADS**	**MS vs ADS**	**Model Comparison**
	Complete Case Analysis	*Multiple Imputation Analysis*	*p-value*
Tobacco Smoke Exposure	0.81 (0.35, 1.84)	0.84 (0.44, 1.61)	0.530
Age	1.25 (1.11, 1.41)*	1.26 (1.15, 1.38)*	0.572
Sex	1.81 (0.83, 3.98)	1.71 (0.92, 3.20)	0.504
EBV Remote Infection	3.68 (1.61, 8.43)*	3.43 (1.46, 8.08)*	0.521
HLA-DRB1*15 Alleles	1.90 (0.88, 4.09)	2.11 (1.10, 4.03)*	0.576
Vitamin D Levels	1.07 (0.48, 2.40)	1.45 (0.66, 3.15)	0.518

*Significant at the 0.05 level

The only difference in measures was the change of significance of HLA-DRB1*15 in the full model; however, this difference is likely due to the narrower standard errors that multiple imputations estimates.

Aim 2- Multiple Imputation Analysis Comparisons

Aim 2 assessed for any potential interacting effects between TSE and the other suspected risk factors for MS. Both stratified analyses and logistic regression models were created to match the analyses for Aim 2. CCA results were again compared to MI results through side-by-side presentation. The results of Aim 2a are displayed below and the tables for Aims 2b and 2c are shown in Appendix A.

The observed effects with the CCA did not change after MI for each of the

analyses for Aim 2a-c, although standard errors were slightly smaller and the confidence intervals slightly narrower in the multiple imputation analysis compared to the CCA. For Aim 2a, a slight additive interaction (IRR=0.7) still appeared to be present between TSE and HLA-DRB1*15 alleles after MI, although slightly less than the CCA (IRR=1.33) (Table 17). The odds for MS were the highest for patients who had both exposure to tobacco smoke in the home and who also presented with HLA-DRB1*15 alleles (OR=2.77; 95%CI 1.06, 7.18).

Table 17 – Aim 2a Comparisons

Multiple imputation analysis for Aim 2a- Stratified analysis assessing the effects between TSE and HLA-DRB1*15 alleles at disease onset and odds for MS

		Complete Case Analysis		*Multiple Imputation Analysis*	
TSE	**HLA-DRB15**	**OR (95%CI)**	**p-Value**	**OR (95%CI)**	**p-Value**
-	-	1		1	--
-	+	1.68 (0.81, 3.45)	0.131	1.85 (0.94, 3.66)	0.074
+	-	1.22 (0.55, 2.66)	0.584	1.22 (0.60, 2.45)	0.586
+	+	3.23 (1.04, 9.79)	*0.021	2.77 (1.06, 7.18)	*0.036

*Significant at the 0.05 level

Adding an interaction term also did not significantly change the logistic regression model, and estimates were fairly similar between CCA and MI (Table 18). A Z-test was performed for each of the estimates between CCA and MI and results were not significantly different (values not shown).

Table 18 – Aim 2a Comparisons

Multiple imputation analysis for Aim 2a- Results of logistic regression models with and without the presences of the TSE*HLA interaction term

	Complete Case Analysis		*Multiple Imputation Analysis*	
	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	1.12 (0.59, 2.14)	1.17 (0.54, 2.57)	0.98 (0.53, 1.80)	1.05 (0.49, 2.24)
HLA	2.20 (1.18, 4.10)*	2.30 (1.09, 4.86)*	2.20 (1.18, 4.10)*	2.36 (1.12, 4.99)*
Age	1.25 (1.15, 1.36)*	1.25 (1.15, 1.36)*	1.29 (1.18, 1.40)*	1.29 (1.18, 1.40)*
Sex	1.81 (0.99, 3.32)	1.82 (0.99, 3.34)	1.81 (1.01, 3.26)*	1.83 (1.02, 3.28)*
TSE*HLA	--	0.86 (0.22, 3.37)	--	0.80 (0.21, 3.05)

*Significant at the 0.05 level

Similar analyses were repeated for Aim 2b&c. Tables of results are shown in Appendix A. The results indicate that MCAR can be assumed since the estimates after imputation did not significantly change. Further discussion of this point is provided in the next chapter.

CHAPTER 5

DISCUSSION

Summary of Results

Risks for the development of pediatric MS are still relatively unknown. This study aimed to address several of these gaps by analyzing a potential risk factor for MS, secondhand tobacco smoke exposure. This study was also able to examine the potential effects of TSE on monophasic ADS compared to healthy controls, which has not previously been assessed in the pediatric population. Furthermore, this was one of the first studies to examine the relationship of TSE in conjunction with genetic and other environmental factors to help describe the potential biological mechanism behind which TSE may be impacting MS in children, specifically compared to patients with ADS.

Results from these analyses suggest that although TSE may not be a risk factor for pediatric MS, it may be a primary risk factor for ADS (OR=2.2; 95%CI 1.08, 4.63). This finding indicates that other factors are needed in order for MS to occur in children, whether genetic or environmental, and are likely needed in some combination to increase the odds of MS over a monophasic ADS attack. The biological mechanism behind the exposures seen in this study are not fully understood and are beyond the scope of this study to explore; however, slight additive interactions between HLA and TSE and vitamin D and TSE both indicate ways in which TSE may influence the occurrence of disease. These exposure relationships may also highlight that multiple risk factors which significantly increase the likelihood of chronic disease (MS versus ADS). A detailed discussion of findings from each study aim follows.

Discussion of Aim 1

TSE was not significantly associated with increased odds for MS compared to healthy controls (*Aim 1a*, OR= 1.84; 95%CI 0.86, 3.95) but was significantly associated with higher odds of monophasic ADS compared to healthy controls (*Aim 1b*, OR=2.24; 95%CI 1.08, 4.63). Although slightly different from our hypothesis, this result may highlight the difference in occurrence of ADS versus MS. TSE was sufficient to increase the odds for ADS, but perhaps in order for MS to occur, other genetic and/or environmental factors must be present. The power of the study and the sample size may also have had an impact on these findings.

The results of this study varied from the other two studies which examined passive exposure to tobacco smoke and risk for MS. Only one prior study has looked at the effects of TSE on pediatric MS (Mikaeloff et al., 2007). They found that passive smoke exposure increased the risk of pediatric MS by 2 fold, which is similar to the effect noted in this study when controlling for age and sex, although our results were not significant (OR= 1.84; 95%CI 0.86, 3.95). The prior study was collected in a much larger patient population and compared to a large control population in France. Mikealoff et al. (2007) were also able to ascertain length of time exposed to TSE, which the current study was not able to assess. Interestingly, the population in France has a much higher percentage of children who were exposed to tobacco smoke at home compared to this study (62% of cases and 41% of controls). Although their findings were significant, they were not able to confirm smoke exposure through biological mechanisms as this study was able to do, nor did they assess differences between MS patients and ADS patients.

Hedstrom et al. (2014) also found that non-smoking adults exposed to tobacco smoke through secondhand sources were at an increased risk for MS (OR=1.3; 95% 1.1, 1.6). Length of time exposed also increased the risk for MS (p=0.003).

Other studies assessing the relationship between active smoking and MS in adults also differed from Aim 1 study results. Similar to other passive smoke exposure studies, significantly increased risks for MS have been associated with active smoking in adults (Ramagopalan et al., 2013; Salzer et al., 2013). The effect may be less pronounced in this study because lung irritation from smoke might be less in passive exposures (or cause less inflammatory response) compared to active smoking. Our cotinine analysis showed that while parents indicate that they do smoke, participants' exposure was still fairly low compared to cotinine values observed in active smokers (Benowitz et al., 2008). Information on the primary location of smoking and average cigarettes smoked per day was not available for most patients, so cotinine values could not be correlated with these factors. Studies in adults also may have more pronounced findings than the current study because they most likely have longer periods of exposure than a child would, and the current study is underpowered. Most of the adult studies were also able to assess quantity of exposure. These studies were also not able to assess the impact of smoking or smoke exposure on the odds for ADS. This is a distinguishing factor for this study and interestingly TSE was related to increased odds for ADS when compared to healthy controls.

Discussion of Aim 2

*Aim 1a- Tobacco Smoke Exposure and HLA –DRB1*15 alleles*

Although Aim 1 did not find significant differences in exposure between MS and ADS patients, other factors may be at play to cause MS over ADS. Therefore, Aim 2 explored the effects of multiple exposures on the odds for developing MS compared to ADS. Patients who were exposed to tobacco smoke in the home who also were positive for HLA-DRB1*15 alleles were 3.2 (95% CI 1.04, 9.79) times more likely to have MS compared to patients with ADS. Perhaps people with HLA-DRB1*15 alleles are more susceptible to immune system malfunction and the chemicals inhaled through passive smoke exposure influence this susceptibility and lead to an inflammatory cascade (Arnson et al., 2010; Breton et al., 2012; Gill, Krishnan, & Dozor, 2014). TSE may also trigger an immune response in ADS patients, but without the presence of genetic susceptibility, the exposure is not sufficient enough to cause MS. Masterman et al. (2000) also remarked that patients who have these alleles may have a "head start" to developing disease, and those without the allele would require longer exposure or more exposures to cause disease (Masterman et al., 2000).

Prior studies in Sweden noted the same interacting effects between DRB1*15 alleles and exposure to tobacco smoke, though only comparing healthy controls and MS patients (Hedstrom et al., 2014). Although the study was conducted in adults who may have had longer exposure to tobacco smoke, they reported a combined OR of 4.7 (95%CI 3.7, 6.0) when both smoke exposure and HLA-DRB1*15 alleles were present. The results were similar to the findings of the present study, which analysis showed that exposure to

both HLA-DRB1*15 alleles and tobacco smoke lead to an increased OR of 3.23 (95%CI 1.04, 9.79). The current study also could only examine added risk in the MS population compared to ADS patients, but since ADS patients are more genetically similar to healthy controls, the results would be expected to be similar when compared to healthy controls (Banwell et al., 2011; van Pelt et al., 2013). This was a strength of the current study since it helped assess whether differences in multiple exposures was a distinguishing factor between MS and ADS patients.

Aim 2b- Tobacco Smoke Exposure and Vitamin D

Analyses in Aim 2 also found slight additive interaction between low levels of vitamin D and TSE. Patients with both exposures were 2.5 (95%CI 1.08, 6.30) times more likely to have MS compared to ADS, although sample size and low power may have affected results. Prior to the analysis of this study, it was hypothesized that increased TSE may lead to decreased uptake of vitamin D in the blood, which could contribute to immune system dysregulation (Banihosseini, Baheiraei, Shirzad, Heshmat, & Mohsenifar, 2013; Lee, Kim, Lim, & Hong, 2015).

While originally thought to be only responsible for calcium uptake in the bones, vitamin D has received more notice recently as an important regulator within the immune system. Lower vitamin D has been associated with increased infections in patients had linked with other autoimmune diseases including rheumatoid arthritis, diabetes mellitus, and inflammatory bowel disease (Aranow, 2011). Prior research has shown that active

smokers have significantly decreased levels of vitamin D (Lee et al., 2015; Manavi, Alston-Mills, Thompson, & Allen, 2015; Ren et al., 2016; Shinkov et al., 2015).

Results from this analysis showed that combined TSE and low vitamin D exposure have an additive effect on the odds for MS compared to ADS patients, however because of the timing of data collection, it is unclear whether vitamin D is influenced by exposure to tobacco smoke or whether the two risk factors work as separate mechanisms. It is also unclear whether the decrease in vitamin D is because of biological mechanisms or due to other psychosocial factors that were not assessed in this study. This was one of the first studies to examine interactions between these two exposures in the MS population. More studies would benefit from looking at the potential interactions between these factors in a larger sample.

Aim 2c- Tobacco Smoke Exposure and Epstein Barr Virus Exposure

The most notable increase in odds for MS in this study was seen with the inclusion of prior EBV infection, although the relationship with TSE could not be accurately assessed due to very low sample size. Through logistic regression analysis and stratified analysis, EBV remained consistently a risk factor for MS compared to ADS patients. Simon et al, (2010) hypothesized that some component of TSE alters the immune system's response to EBV infection, thus working in conjunction (and independently) with each other to increase MS risk (Simon et al., 2010). The authors also mention that both EBV and nicotine metabolism have similar molecular pathways, which possibly helps explain the interacting effect with immune function.

Results from previous studies assessing interaction between TSE, EBV, and genetics have varied. Simon et al. (2010) found that an interaction was present between EBV titers and smoking, with the combination of the two factors increasing the odds for MS by 3.9 (95%CI 2.7, 5.7). Although the current study was lacking the sample size to determine if a true interaction was present, the increase in odds ratio seen when both exposures were present suggests evidence for this hypothesis. In a study of adult women, de Jager et al. (2008) found a multiplicative interaction between higher titers for EBV and HLA-DRB1*15 alleles. They did not find an interaction with ever smoking, but the risk ratio was elevated further when adding this risk factor. Other studies have not shown the same multiplicative effect, but have shown that the combination of multiple exposures can lead to increased risk. Sundqvist et al. (2012) found that if a person was exposed to smoking, high EBV titers, HLA-DRB1*15 alleles and were lacking HLA-A*02 alleles, the odds for MS was 26.3, although the confidence interval was very wide (95% 10.20, 67.9) (Sundqvist et al., 2012).

Summary of Aim 2 Findings

The relationships between these exposures (TSE, HLA genes, vitamin D, and EBV) help to illustrate that there may be a mechanistic and possibly additive effect of being exposed to multiple environmental factors at the same time. Additionally, smoke exposure could modify proteins within the lung, and once these autoimmune memory cells are created within the lungs, more smoke exposure triggers more inflammatory responses (Arnson et al., 2010; Johannsen et al., 2014). These inflammatory responses

could potentially take on migratory patterns which can pass through to the CNS through cross reactive peptides (Chang et al., 2014; Sundqvist et al., 2012). The impact of this relationship should be explored further in a larger population of patients and healthy controls in a longitudinal cohort. These mechanisms could also be explored in other autoimmune diseases for which the biological mechanism is unknown. Perhaps the impact of tobacco smoke exposure or the interactions of other environmental exposures leads to genetic changes in these patients causing immune system dysregulation in other organ systems.

Discussion of Aim 3

From this study, it is unclear how TSE affects disease burden in pediatric patients. Children exposed to tobacco smoke in the home were more likely to present with more than minimal neurological function symptoms, but no significant differences were seen. Additionally, a trend was not observed between increasing TSE (higher cotinine levels) and worsening of symptoms. These results differed from what was hypothesized based on prior adult studies. Manouchehrinia et al. (2013) found that current and ever smokers were 64% more likely to have higher disability scores than nonsmokers. Pittas et al. (2009) also showed a trend with increasing smoke exposure and increasing disability. Similarly, Weiland et al. (2014) found evidence of an association between cigarette smoking and poorer health outcomes in terms of disability and health-related quality of life.

Although the current study results did not indicate an association with the level of exposure, there are limitations of using functional scores as a measure of disease burden.

Of note, pediatric patients are less likely to show signs of irreversible disability until 10-20 years following disease onset (Renoux et al., 2007), therefore the effects of TSE should ideally be assessed several years after disease onset. These limitations are discussed further in the following section.

Missing Data Analysis

The parent study for this analysis enrolled subjects over several years, therefore, some missing data was expected. Also, the analysis of blood samples for this study was conducted after a few years of enrollment on the available participants at the time. This created a decreased sample size for the main covariates assessed in the current study, since all subjects were not enrolled at the time the analysis was carried out. This also limited the ability to adjust for other factors in the analyses using healthy controls, since healthy children were not enrolled until after the blood samples were analyzed.

Because other covariates and outside factors were an unlikely cause of the missing data, it was assumed that the data for the covariates was missing completely at random (MCAR). However, to confirm this assumption, multiple imputations (MI) were performed to estimate effects of TSE on MS patients compared to ADS patients with a more complete dataset. MI analysis infers that data are missing at random (MAR), so differences seen between the estimates would argue that the data was not MCAR. The results of the MI analysis, however, were fairly similar to the complete case analysis (CCA), and p-values showed that any slight differences between the measures were not

significant. These findings give little reason to assume data are not MCAR and even less reason to show that the data would be missing not at random (MNAR).

Reporting data based on CCA or by MI analysis have strengths and limitations. CCA is less efficient than MI, but is likely unbiased when data is MCAR since it represents an assumed random selection of the study population (Donders et al., 2006; van der Heijden, T. Donders, Stijnen, & Moons, 2006; White & Carlin, 2010). Missing data for covariates can limit reportable information, particularly when seen in large percentages as this study did. MI analysis gives sound results with respect to bias and precision, although it requires the use of predictors to model imputations, which may not be valid for MCAR data (White & Carlin, 2010). White and Carlin (2010) argue that with values only measured once, MI & CCA are practically the same. They also note that just despite MI having smaller standard errors, it does not mean it is a more precise estimate.

A sensitivity analysis was not performed for this study, because missing data was assumed to be missing completely at random and very unlikely to be missing not at random. Results from preliminary analyses around missing data as well as the results from the MI analysis have substantiated this assumption. The one difference in measures between the two analyses was the change in significance of HLA genes within the full model comparing MS to ADS patients (Aim 1c). This difference was likely seen because more subjects who had HLA information could be used in the analysis who were missing data on vitamin D and prior EBV exposure. MI allowed more subjects with HLA to be included within this assessment. This may also be due to the estimate corrections within MI (White & Carlin, 2010).

Study Strengths

This study had several key strengths. First was the ability to examine a large group of ADS patients whose diagnoses was confirmed over several years of observation. The ability to confirm diagnosis after several years likely led to reduced misclassification of disease. The sample size was also large for a pediatric demyelinating disease study as the time the data was collected.

Another strength was the ability to quantify exposure using serum cotinine measures, specifically at baseline when patients were first experiencing symptoms of disease. Using this biomarker also helped to confirm the self-report exposure status obtained from parental report which other studies have not been able to do. Serum cotinine also helped categorize "dose" of exposure since self-report quantity of exposure could not be ascertained for most patients. For those parents who indicated that they only smoke outside, a comparison was made looking at the cotinine levels in their children (data not shown). While some had lower cotinine values than others, they still showed positive exposure. The knowledge gained from this sub-exploration will also help explain to parents that although they insist that they only smoke outside, their child is still exposed to small amounts of tobacco smoke either through smoke drift or proximity to the parent after they have smoked a cigarette. Results in Aim 3 were limited in assessing the effects of TSE on disease burden, therefore, this area needs to be explored further in the future.

Another strength of this study was the ability to compare MS patients to monophasic ADS patients. Although TSE comparisons have been shown between MS

patients and healthy controls, this is one of the first studies to examine this relationship using ADS patients as a comparison. Distinguishing the risk in these two groups was important for examining differences in potential causal mechanisms behind the two disease occurrences.

Study Limitations

Despite these strengths the study also had limitations, but these limitations can help to guide future research. The analysis for this study was completed utilizing data collected previously on a cohort of patients in Canada. While the sample size was large for a pediatric demyelinating disease study, the power was low and thus interpretation of the results must be done cautiously. Greater numbers are also needed to do more detailed interaction analyses to assess the potential effects of multiple exposures.

The findings from Aim 1 could also have been influenced by the selection of the healthy control population. In Canada, the smoking prevalence is ~19-20%, yet 26% of the healthy control families indicated that their children were exposed to secondhand tobacco smoke in the home. It is unclear why the study population is exposed to higher self-reported smoking rates than the general population. Since socioeconomic variables were not available to analyze, we could not assess whether this difference is due to social factors.

Additionally, no information on environmental exposures in healthy controls was available for analysis within this population. While analysis using healthy controls could be adjusted for age and sex, future analysis would benefit from exploration of other potential risk factors that could be adjusted for within the regression models. A future

study should aim to age and sex match controls with both MS and ADS patients in a much larger population to explore these risk factors further, particularly if multiple risk factors can be included for interaction. This study was able to emphasize the need for future research using a control population since significant interacting effects were present when comparing odds for MS to ADS patients.

Furthermore, although information was available from prior genetic analyses for MS and ADS patients, only information on HLA-DRB1*15 alleles was available for this analysis. Results in this study could potentially have been influenced by the presence or absence of other alleles as noted in previous studies (such as HLA-A*02) or on other alleles that have not yet been discovered (Hedstrom et al., 2014). At the time of the genetic analyses for the current study, the influences of these other factors had not yet been established.

Detailed information about exposure assessment in this study was also difficult to define. Although parents were asked a series of questions about their smoking habits including length of time smoking around their child, most frequent smoking location, and number of cigarettes smoked per day, these questions were not often answered by the parent (~50% of parents did not answer 1 or more of these questions). Length of time exposed to tobacco smoke could therefore not be ascertained for many patients. Future studies will also work to develop better questionnaires which will assess the timing of exposure, the frequency of exposure, and the amount of exposure over time for the patients. Of particular interest is the length of time that children have been exposed to tobacco smoke, particularly because studies of active smokers have shown that the length

of time smoking heavily influences disease progression and outcomes (Di Pauli et al., 2008; Manouchehrinia et al., 2013; Pittas et al., 2009). Additionally, in adult patients who have quit smoking, their disease progression is slowed in comparison to still active smokers (O'Gorman et al., 2014), indicating that parents who quit smoking around their children may be able to positively influence their child's prognosis.

Although the availability of serum samples for use in cotinine analysis was a strength of this study, the timing of the blood collection is also important. For this study, serum samples were selected that were close to baseline or within 3 months of disease onset when baseline samples were not available. However, MS patients and many ADS patients may have had blood draws during a hospital admission. Blood collection may not have occurred when the patient first arrived to the hospital and may have taken several days as an inpatient before research blood was available for collection. Longer stays in the hospital before blood samples were taken, would have lowered serum cotinine values since the half-life of cotinine is 15 hours. A pilot analysis was conducted for 10 people to determine the variation between multiple samples at different time points for the same patient (data not shown). Serum samples that were measured around a year after the baseline measurement increased by 0.8 ng/mL on average which was a 39% average increase in serum cotinine values. This helps illustrate that the cotinine samples may be underestimating the risk for MS seen in this population.

Seasonal timing of blood samples may also affect the levels of vitamin D. People tend to have lower serum D levels during the winter months when they are less exposed

to sunlight. Fortunately, in this study the serum concentrations by season were not significantly different and therefore should not have affected results (data not shown).

Future studies would benefit from drawing blood upon arrival to the hospital at the time of disease presentation, although due to the illness and nature of disease, this may be challenging to do. Better timing of collection would allow for a more accurate assessment of the amount of tobacco smoke the child has recently been exposed to (Benowitz, 1996). Passive monitoring devices in the home could also provide more information on exposure levels (Marbury et al., 1993). Additionally, future analyses should utilize gas chromatography for the analysis of cotinine since it is much more sensitive for low levels of exposure (<1ng/mL of exposure) (Matt et al., 2000; Matt, Bernert, & Hovell, 2008). The ELISA method, though very accurate with high levels of exposure, is less sensitive with low levels of exposure around 1ng/mL (Abnova Corp. kit reference). The assessment also has difficulty quantifying values less than 1ng/mL. Gas chromatography could not be used for the current study due to the expense and limited availability in the U.S.

The use of neurological functional scores for measuring disease burden for Aim 3 has limitations. The EDSS is commonly used to assess disability in MS subjects but is often criticized as a subjective measure. Therefore, neurological function scores were obtained as part of the Canadian Demyelinating Disease Registry; however, analysis of these measures has not been validated for use in MS or ADS. Furthermore, it is difficult to compare disability affecting different domains within one score (i.e. brainstem disease versus visual impairment); therefore, in the present study, data was presented by

neurologic system rather than as a single score. Other factors that capture disease burden in MS include annualized relapse rate and MRI lesion burden. This study was unable to examine the effects of TSE on these measures or quality of life, which could relate to the child's disease progression in the earlier phase of disease and are of interest for future studies. Since repeat measures of cotinine were unavailable, we had to estimate the effect of baseline exposure on longitudinal outcomes.

Lastly, this study did not have access to information about other risk factors or potential psychosocial variables that may play a role in both the exposure to tobacco smoke and MS risk. For example, stressful life events have been investigated as a risk factor for MS and demyelinating events. A meta-analysis by Mohr et al. (2004) found a significantly higher risk for MS relapse following stressful life events (effect size d = 0.53, 95%CI 0.40, 0.65). Other lifestyle factors such as obesity, prenatal smoke exposure, and daily physical activity were not available for risk comparison. Socioeconomic information would also be important to look at in the future, particularly given the higher prevalence of smoking families within the study population.

Summary and Significance

Although pediatric MS is rare, the prevalence seems to be increasing (Lavery et al., submitted manuscript). MS is a serious disease resulting in long-term disability, and people who acquire the disease in childhood have more long-term consequences such as earlier disability onset and a higher cost burden to families. A cost-burden analysis has shown the annual cost to families with MS to be around $41,000 (Kolasa, 2013).

Compared to adults who are diagnosed in their late 20s-mid 30s, pediatric MS patients will be forced to pay more out-of-pocket expenses due to the longer period of time with the disease. The cost burden including additional expenses for increased hospitalizations, the annual cost for medications, and regular follow-up appointments stresses the need to find the causes of MS and relieve families of this burden.

The results from this study have indicated that a modifiable risk factor, TSE could influence the odds for MS, particularly in relation to patients with ADS when other factors are also involved. If TSE influences disease burden in MS patients further into disease development, parents should know the effects of this modifiable exposure on their child's disease progression. Despite a decrease in population smoking rates over the last several decades, approximately 30% of children in this study have parents who smoke. Once MS is diagnosed, it cannot be changed since no cure for the condition currently exists.

Results of a priori hypotheses tests showed that TSE significantly contributed to the odds ADS compared to healthy controls, although TSE was not significantly related to an increased odds for MS compared to healthy controls or compared to ADS patients. However, there was an increase in odds of MS observed when both TSE and HLA-DRB1*15 alleles were present (compared to ADS patients). Importantly, this finding suggests that some people may be genetically susceptible to MS, and their risk for MS is further increased if they are also exposed to tobacco smoke. An interaction effect was also present between TSE and vitamin D levels, indicating that TSE may influence immune system regulation through multiple mechanisms. Both interactions could have

significant impact on why a person only has one demyelinating attack while others have multiple sclerosis.

Findings from this study could have direct influence on creating intervention strategies to focus on helping parents to quit smoking. These interventions could engage parents and inform them through multiple communication channels about their child's risk for MS and other disease burdens. Studies are currently underway which utilize multi-level intervention strategies, focusing on the pediatricians as the primary source for anti-smoking assistance with families (Lepore et al., 2013). Research has shown that advice from pediatricians regarding smoking cessation can be an effective way to help motivate parents to quit smoking, although they do not always use clinic time to discuss this with families (Collins, Levin, & Bryant-Stephens, 2007). Perhaps the course of the disease could be significantly impacted by smoking cessation and parents should know this information. This modifiable risk factor could potentially impact thousands of families who have loved ones with this disease.

Results from the cotinine analysis could also be shown to families who indicate that they only smoke outside. As a sub-analysis in this study, parents who indicated only smoking outdoors still had children with detectable levels of cotinine exposure in their blood (data not shown). Part of the multi-level intervention could use this information to help inform parents that although smoking away from their children helps reduce exposure, it does not eliminate the exposure entirely and still puts the child at risk for negative health outcomes, particularly as exposure may lead to worsening of their child's disease burden later on.

The interaction results could also be important in other autoimmune diseases since some of these diseases may also be caused be an interaction of genes and environmental risk factors. Additionally, the NIH estimates that there are over 80 autoimmune diseases that attack healthy tissue within the body including multiple sclerosis, type I diabetes, and rheumatoid arthritis (NIH, 2002). These diseases affect between 14-22 million Americans (including children), negatively impacting the quality of life for these individuals and raising the costs of healthcare (NIH, 2002) . More research on the interactive effects between TSE and genetic markers in these other disease populations may help to impact millions who will be diagnosed with these diseases in the future.

CHAPTER 6

PROPOSED FUTURE RESEARCH

This study provided interesting findings and presented several key areas that future research could address. First, although pediatric MS is rare, future studies could examine larger populations of patients with age- and sex-matched controls and perform more detailed assessments of exposure at disease presentation. Future studies could also assess the risk of MS in children, particularly with the interaction of multiple exposures. Although the interactive effect of HLA-DRB1*15 alleles and TSE shows an increased risk associated with the combination of these factors, not all patients with MS express HLA-DRB1*15 or are exposed to tobacco smoke, therefore other factors must be at play.

Additionally, while this study had some unique findings on the risk of MS due to TSE, future studies could better classify a child's exposure to tobacco smoke. This study was able to utilize blood samples at baseline to help confirm parent self-report status, but a quantification of the exposure over a child's lifetime could not be analyzed because of limited data. Future studies should attempt to assess the amount of TSE a child has been exposed to over their lifetime, including possible prenatal exposure, and should analyze other potential sources of exposure. Cotinine should also be measured at specific time points longitudinally for patients to see how exposure levels may vary over time.

The current study was unable to asses multiple measures of disease burden and disease progression in this population. Future studies would benefit from examining TSE effects on patient's relapse rate, MRI metrics, and measures of quality of life. Findings

could help to influence intervention strategies, assuming that exposure to smoke worsens these factors in children, as is seen in adults.

Furthermore, future studies could examine biological mechanisms that relate why TSE may be associated with increased risk for MS. Further exploration into the interacting effects of MS risk factors would be ideal. The relationship between increased odds for MS when vitamin D is low and TSE is present should be examined further. If better timing of measurements can be performed, this relationship can be further interrogated.

Oxidative stress has also been hypothesized as a potential initiator of MS because of the influence that increased radicals have on the immune system. One of the proposed mechanisms behind tobacco smoke exposure and MS is that smoke may increase oxidative stress in the body, particularly in the lungs (Gonsette, 2008; Naik et al., 2014). Once the body undergoes oxidative stress, and notably when it undergoes oxidative stress many times, it may cause immune system dysfunction or changes in DNA markers within the immune regulatory system (Jana & Pahan, 2007; Lu et al., 2000; Naik et al., 2014; van Horssen et al., 2008). Several biomarkers have been explored for assessing oxidative stress within the body such as nitric oxide and nitrates (Naik et al., 2014; Ortiz et al., 2009). Future studies could assess these biomarkers at the time of disease onset to determine if the patients are in fact in a period of oxidative stress when suffering from a demyelinating attack. Several blood tests should be analyzed over the months following the demyelinating attack to determine if a person with MS goes through a period of oxidative stress fluctuation. Perhaps these levels are elevated when a patient is in a

relapse. Studies should also examine whether these biomarkers of oxidative stress correlate with TSE or other environmental exposures to assess whether oxidative stress is part of the causal pathway.

Lastly, a closer examination of psychosocial risk factors that may influence MS risk should be pursued in future studies. These principles can be guided by the biopsychosocial model which combines elements of biological health with factors in the social and psychological environment. Of particular interest is the effect of stress on immune system function and factors in the person's social and physical environment that may contribute to this. Prior research has shown that stressful life events are associated with increased MS relapses (Mohr, Hart, Julian, Cox, & Pelletier, 2004). Research in MS could be improved through use of theory driven approaches, which could establish more potential sources of risk.

The model in figure 7 shows the interplay of potential factors on the risk for MS. Of interest are these factors which could possibly interact with smoke exposure or influence the higher rate of TSE in the population seen in this study.

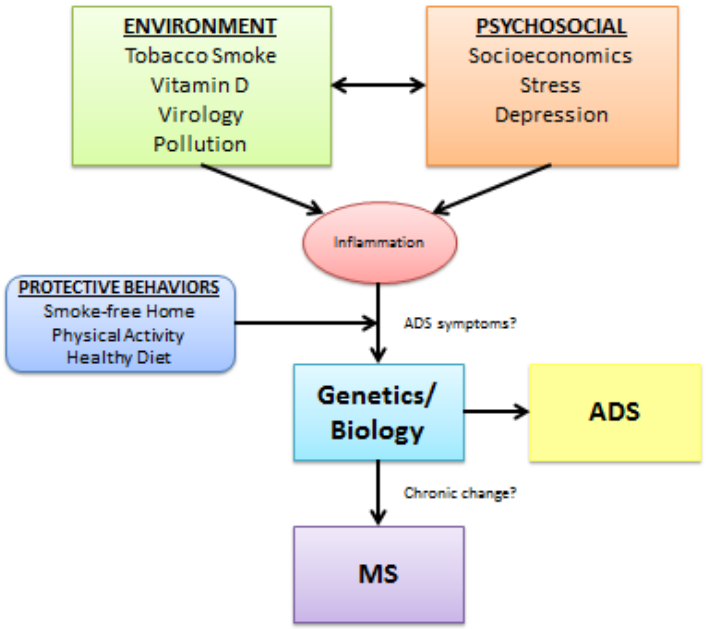

Figure 4. Logic model for MS disease progression relating biological, environmental, and psychosocial factors on the risk for MS.

For example, perhaps there is a psychosocial factor which is pre-existing that causes parents in these families to smoke. Additionally, maybe these factors relate to other risk factors for MS risk such as low vitamin D exposure or obesity. These are a few of the questions that should be answered with a more thorough assessment of these exposures to help determine the cause of MS, and hopefully, to help to prevent and/or treat patients around the world.

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APPENDIX A

MISSING DATA TABLES

Further analysis of the multiple imputation data was performed for Aims 2b&c. The following section offers a side-by-side comparison of these results with the complete case analysis presented in Chapter 4.

Aim 2b - Multiple Imputation Comparison

After MI a slight additive interaction was still found between TSE and vitamin E, although the effect was less than observed in the CCA (IRR=1.63). Odds for MS were the highest when the patient was exposed to both tobacco smoke in the home and low serum vitamin D levels (OR=2.76; 95%CI 1.18, 6.44) (Table A1). Logistic regression models also did not change significantly with the MI analysis and interaction terms did not make the model stronger (Table A2).

Table A1 – Aim 2b Comparisons

Multiple imputation analysis for Aim 2b- Stratified analysis assessing the effects between TSE and low serum vitamin D at disease onset and odds for MS using imputed data for vitamin D exposure.

		Complete Case Analysis		*Multiple Imputation Analysis*	
Passive Smoking	**Vitamin D (<50)**	**OR (95%CI)**	**p-Value**	**OR (95%CI)**	**p-Value**
-	-	1	--	1	--
-	+	2.03 (0.90, 4.54)	0.087	2.09 (0.95, 4.59)	0.067
+	-	0.98 (0.37, 2.55)	0.963	1.04 (0.46, 2.32)	0.933
+	+	2.89 (1.12, 7.46)	*0.028	2.76 (1.18, 6.44)	*0.019

*Significant at the 0.05 level

Table A2 – Aim 2b Comparisons

Multiple imputation analysis for Aim 2b- Results of logistic regression models with and without the presences of the TSE*Vitamin D interaction term.

	Complete Case Analysis		*Multiple Imputation Analysis*	
	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	0.93 (0.51, 1.70)	0.70 (0.25, 1.95)	0.91 (0.50, 1.68)	0.79 (0.33, 1.88)
Vitamin D	1.29 (0.65, 2.53)	1.37 (0.57, 3.30)	1.83 (0.92, 3.67)	1.63 (0.69, 3.84)
Age	1.28 (1.17, 1.39)*	1.29 (1.16, 1.43)*	1.27 (1.17, 1.39)*	1.27 (1.17, 1.39)*
Sex	1.80 (1.01, 3.22)*	1.87 (0.91, 3.86)	1.72 (0.96, 3.09)	1.73 (0.96, 3.12)
TSE*Vitamin D	--	1.70 (0.38, 7.74)	--	1.40 (0.34, 5.68)

*Significant at the 0.05 level

Similarly, MI did not alter the effects observed between TSE and prior EBV infection, although standard errors were slightly smaller. Prior EBV infection still showed the most notable increase of odds for MS compared to ADS patients (OR=3.99; 95%CI 1.67, 9.55) (Table A3). Although a further increase in odds of MS was observed for patients with both TSE and prior EBV infection (OR=4.75; 95%CI 1.91, 11.8), an additive interaction was not confirmed.

Table A3 – Aim 2c Comparisons

Multiple imputation analysis for Aim 2c- Stratified analysis assessing the effects between TSE and prior EBV exposure and odds for MS using imputed data for EBV exposure.

		Before Imputation		*After Imputation*	
Passive Smoking	**Prior EBV Exposure**	**OR (95%CI)**	**p-Value**	**OR (95%CI)**	**p-Value**
-	-	1		1	--
-	+	4.13 (1.62, 10.9)	*0.001	3.99 (1.67, 9.55)	*0.002
+	-	0.38 (0.01, 3.07)	0.360	0.68 (0.15, 3.12)	0.618
+	+	5.13 (1.79, 14.9)	*0.043	4.75 (1.91, 11.8)	*0.001

*****Significant at the 0.05 level

Additionally, logistic regression models using an interaction term between TSE and EBV were not changed using MI versus CCA, and models did not show a significant multiplicative interaction effect (Table A4).

Table A4 – Aim 2c Comparisons

Multiple imputation analysis for Aim 2c- Results of logistic regression models with and without the presences of the TSE*EBV interaction term.

	Complete Case Analysis		*Multiple Imputation Analysis*	
	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)	Model 1 No interaction term OR (95%CI)	Model 2 Interaction term OR (95%CI)
TSE	0.78 (0.34, 1.76)	0.28 (0.03, 2.50)	0.81 (0.43, 1.54)	0.53 (0.01, 2.64)
EBV Remote Infection	3.85 (1.70, 8.72)	2.99 (1.19, 7.52)	3.85 (1.70, 8.70)	3.31 (1.30, 8.45)
Age	1.25 (1.12, 1.40)	1.25 (1.12, 1.40)	1.26 (1.16, 1.38)	1.26 (1.16, 1.38)
Sex	1.74 (0.80, 3.78)	1.71 (0.79, 3.72)	1.76 (0.95, 3.25)	1.75 (0.95, 3.25)
TSE*EBV	--	3.51 (0.33, 37.3)	--	1.80 (0.25, 12.8)

APPENDIX B

IRB ACKNOWLEDGEMENT LETTER

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Research Administration

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Committee: A1 Protocol Number:

23524

Project Title: Tobacco Smoke Exposure and Pediatric Multiple Sclerosis

Date: 12-Feb-2016

The above new study was administratively closed because the IRB determined that the proposed activity is not human subjects research as defined by DHHS or FDA regulations. Consequently, Temple IRB approval is not applicable and the study was given the status of "closed/never opened." You are welcome to pursue the activity, obtaining any applicable administrative or departmental (non-IRB) approvals.

Please contact the IRB at (215) 707-3390 if you have any questions.