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Association between Immunological Reactivity with Tetrabromobisphenol-A and Autoimmune Target Sites of the Nervous System

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The Association between Immunological Reactivity with Tetrabromobisphenol-A and

Autoimmune Target Sites of the Nervous System

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Dissertation for PhD in Health Sciences

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We hereby certify that this dissertation, submitted by Datis Kharrazian conforms to acceptable standards and is fully adequate in scope and quality to fulfill the dissertation requirement for the degree of Doctor of Philosophy in Health Science.

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Abstract

Tetrabromobisphenol-A (TBBPA) is the most widely used flame retardant. Flame retardants are sprayed on furniture, mattress beds, children's pajamas, car seats, upholstery, carpets, and rugs in the United States. Chemical immune reactivity may play a role in the epidemic of autoimmune disease. The goal of this research is to investigate whether any correlation exists between immunological reactivity to TBBPA, a key chemical used in most flame retardants, and neurological autoimmune target sites that are associated with neurological autoimmune diseases with a diverse and specific list of antibodies that include myelin basic protein, myelin-associated glycoprotein, alpha-synuclein, aquaporin receptors, and S100B antibodies with human serum samples. The outcomes of this research can be used to support the development of safety regulations and for identifying potential health concerns for current mandatory flame-retardant legislation. Additionally, this research may support the decisions made in respect of those suffering from neurological autoimmune diseases, as to whether removing flame retardant chemicals is a factor for consideration.

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Chapter 1: Introduction

Autoimmunity is a condition in which the immune system erroneously attacks and destroys tissues or compounds in the body (hormones, enzymes, etc.). Although it is estimated to affect 50 million Americans, a count that surpasses the number of cancer and heart disease cases combined, autoimmunity remains largely misunderstood, under-diagnosed, and poorly managed, if at all, according to the American Autoimmune Related Disease Association (2015, April).

It has been estimated that between 23.5 million and 50 million Americans suffer from autoimmune disease; further, one in five women and one in seven men are diagnosed with autoimmune disease, according to the American Autoimmune Related Disease Association (2015, April). These figures do not consider the millions of other people who have not been diagnosed with autoimmune disease, or whose condition has not advanced enough to be called a disease, even though they suffer from the symptoms. Researchers have found that chemical exposure, combined with an activated immune system or genetic risk, creates a recipe for the development of autoimmunity with disorders such as type I diabetes, multiple sclerosis, systemic lupus erythematous, and various idiopathic autoimmune diseases (Pollard & Kono, 2013; Barragan-Martinez *et al*., 2012).

Only a minority of the synthetic compounds introduced to our environment has been researched individually, leave alone compounds in conjunction with each other. The Environmental Protection Agency (EPA) doesn't require testing chemicals introduced in the market, unless evidence of potential harm exists, which means testing seldom happens. The EPA approves about 90% of the new chemicals and only a quarter of more than 80,000 have been tested for toxicity (Canor, 2008). Regulations relating to environmental toxins in the United

States are lax and outdated, allowing tens of thousands of untested chemicals into our environment and our bodies. A lab test on any of us would probably show levels of more than 200 different chemicals, some of them dangerously high (Houlihan, Kropp, WilSes, Gray, & Campbell, 2005).

American children are born with high levels of environmental chemicals in their systems. For example, a 2005 study of cord blood from newborns found almost 300 environmental compounds, including mercury and dichlorodiphenyltrichloroethane (DDT) (Houlihan *et al*., 2005). At the other end of the life spectrum, environmental toxins have been linked with neurodegenerative conditions such as Alzheimer's and Parkinson's disease (Stein, Schettler, Rohrer, & Valenti, 2008; Huang *et al*., 2005; Kelly *et al*., 2015). Researchers can predict an increased risk for Autism before a child is even born, by screening for antibodies in the brain of the fetus in the mother, during the third trimester (Bauman *et al*., 2013). Autism, which is increasingly being found to be autoimmune in nature, is the fastest growing developmental disability today; its prevalence is increasing from 10 to 17%each year, and it is linked to environmental triggers (Cavagnaro, 2007). It has been estimated that one in fifty children in the United States suffers from Autism Spectrum Disorder (Center for Disease Control, 2013). However, a more comprehensive study in South Korea shows its rate to be one in every 38 children. Researchers suggest that South Korean children aren't necessarily more prone to Autism, but that the study was actually more thorough and that autism rates are much higher in the United States as well (Kim *et al*., 2011). The rates of Autism, neurodegenerative diseases, and autoimmunity may be associated with environmental chemicals.

Research shows that Americans are now born with increasingly high levels of chemical and toxin burdens, and new exposure may occur as early as at the time of breastfeeding. (Lunder,

2003). Chronic exposure to arsenic in human drinking water has been shown to alter the lung barrier and hinder healing of wounds (Olsen *et al.*, 2008). Pesticides have been found in food and drinking water and are now considered a major route of exposure to the general population—the organo-photothionates in pesticides have been found to directly cause intestinal permeability, potentially leading to autoimmunity (Choi *et al.*, 2007). Polychlorinated biphenyls (PCBs) are no longer used but are still commonly found in the environment and have also been linked to impaired immune tolerance (Choi *et al.*, 2010). It appears that many chemicals, commonly found in our environment, may contribute to the growing epidemic of autoimmunity.

On the other hand, there are conflicting views and concerns about the role chemicals may play in the development of autoimmune diseases. There are a limited number of human studies that show a direct causal link between the two. Safety and ethical concerns for chemical risks in research designs have limited most of the research in this field and experimental studies on animals. Human studies are void of clinical trials, and retrospective and prospective studies have limitations due to the difficulty in determining when exposure to a chemical may have occurred. An exception to this is pharmaceutical drug studies that lead to autoimmunity, as patients are closely monitored with respect to dosage, clinical outcomes, and the development of autoimmune reactions. Two drugs, in particular, have been linked to chemical/drug induced autoimmunity. The first drug in procainamide for the treatment of cardiac arrhythmia and the second drug is hydralazine used to treat high blood pressure (Pollard *et al.*, 2010). Despite the observed role that these medications/chemicals may play in causing autoimmunity in susceptible patients, the design of the clinical trial was not designed to directly answer the role of these medications in autoimmune disease development, and therefore, there is little direct evidence that these chemicals are causative in a statistically significant relationship. Outside of these two

drugs closely monitored in a clinical trial setting, most of the research on chemical associations

with autoimmune disease in human subjects have been derived from epidemiological studies,

occupational studies, and correlational studies (Schmidt, 2011).

Statement of the Problem

Chemical immune reactivity may play a role in the epidemic of autoimmune disease. A

major concern in this regard is the group of chemicals that are flame retardants. Flame retardants

are sprayed on all pieces of furniture, mattress beds, children's pajamas, car seats, upholstery,

carpets, and rugs in the United States. This practice was driven by the California Flammability

Standard, Technical Bulletin 117 (TB117), which was instituted in 1975. TB117 states that a

manufacturer is not permitted to sell items such as furniture, upholstery, or mattresses in

California unless they are sprayed with flame retardants. As California is such a large consumer

market, the guidelines of TB117 have been adopted by all major manufacturers throughout the

country, leading to widespread use of flame retardants. As a consequence of TB117, studies have

found that these chemicals can be detected in household dust and serum concentrations in the

population (Zota, Rduel, Morello-Frosh, & Brody, 2008). A study showed that first-time mothers

in the United States had levels of flame retardants in their breast milk, 75 times higher than in

similar European studies (Lunder, 2003). Testing breast milk from women throughout the

country showed that every subject demonstrated very high levels of flame retardants (Mazdai *et

al*., 2003). High levels of flame retardants have also been identified in baby products such as

nursing pillows, strollers, and baby carriers (Stapleton, Eagle, Sjödin, & Webster, 2012).

Researchers have also found that infants may receive greater exposure to these chemicals than

adults and that individuals, in general, are likely to be exposed to a higher than the acceptable

daily intake of retardants(Staphletion *et al*., 2011). The incorporation of TB117 has led to an

influx of flame retardants into the human biota and the environment, and the effect they have on human health is a concern (Tung *et al.*, 2016).

There are more than 175 different flame retardants added to consumer products; however, tetrabromobisphenol-A (TBBPA) is the most widely used flame retardant. The potential for TBBPA to be a contributing factor to autoimmune disease is very high, due to known immunological influences of a structurally similar chemical found in plastic products called Bisphenol-A (BPA). BPA has been shown to promote many autoimmune-promoting reactions, such as cytokine activation, TH-17 activation, T-cell polarization, T-regulatory suppression, and activation of hydrocarbon receptors (Kharrazian, 2014). Immunoreactivity to BPA, measured with BPA bound to albumin antibodies, was recently shown to demonstrate a high degree of correlation with antibodies against neuron-specific antigens (Kharrazian & Vojdnai, 2016). Due to the molecular similarity of TBBPA to BPA and its wide use in household and consumer products, research is needed to identify whether TBBPA can be a chemical risk factor in the development of autoimmune disease. Furthermore, recent research has found that approximately 8% to 13% of healthy blood donors demonstrate immunological reactivity to TBBPA by producing excess antibodies to TBBPA (Vojdnai, Kharrazian, & Mukherjee, 2014).

Relevance and Significance of the Study

The outcomes of this study will contribute to understanding the impact of Tetrabromobisphenol-A (TBBPA), a chemical found in flame retardants, on autoimmune and inflammatory target sites found with conditions such as autism, Parkinson's disease, learning developmental disorders, and neurological autoimmune diseases. Evaluation of immune reactivity to autoimmune target sites, such as myelin basic protein (MBP) and myelin oligodendrocyte protein (MOG), have been associated with neuroinflammatory and neurological

autoimmune diseases. Anti-MBP and anti-MOG were found in 78.5% of autistic children and insignificantly in normal subjects. (Mostafa & Al-Ayadhi, 2013). Anti-MBP was also found to be significantly higher in 100 mothers of children with autistic disorder, compared to 100 age-matched, unaffected children leading to the possibility that there may be a placental transfer of maternal antibodies in Autism (Singer *et al*., 2008). In addition to autism, anti-MOG and anti-MBP are key serum biomarkers used to identify multiple sclerosis, and anti-MBP and anti-MOG have also been found to be key predictive biomarkers for identifying future demyelinating event after the onset of the disease (Berger *et al*., 2003). Anti-MOG is not only a useful biomarker in multiple sclerosis, but it is also a significant biomarker found in other inflammatory neurological diseases (Reindl, Lington, Brehm, & Egg, 1999).

In addition to acute autoimmune and inflammatory conditions, there is evidence that anti-MOG and anti-MBP are also found in chronic neurodegenerative disease. One study found increasing anti-MBP levels in patients suffering from Parkinson's disease and that anti-MBP may be used as a valuable marker to monitor the progression of the disease (Papuc, Kurzepa, Kurys-Denis, & Grabarska, 2014). This study has found that between 4 % and 9% of our sampled healthy serum demonstrated elevations of MBP and MOG that may predict future disease process and determine active neuroinflammation.

No current research demonstrates TBBPA impact on neurological autoimmune target sites such as MBP and MOG. TBBPA can act as a neurotoxin and induce cellular toxicity as well as disturb cellular dopamine secretion and alter acetylcholinesterase enzymatic activity (Liu *et al.*, 2016). TBBPA was recently found to cause neurotoxic and apoptotic responses in cultured mouse hippocampal neurons *in vitro* (Szychowski & Wójtowicz, 2016). Additionally, TBBPA was found to have induced apoptotic and neurotoxic effects in mouse neocortical cells. Another

study found that TBBPA may produce neurotoxic effects, especially when challenged with oxygen-glucose deprivation (Ziemińska *et al*., 2012). Despite these limited neurotoxic animal studies, there has been no investigation of the role of TBBPA in neurological autoimmune reactivity.

If a statistically significant correlation is found between TBBPA and autoimmune target site protein antibodies, it may suggest is the existence of a potential underlying relationship between chemical exposure and autoimmune reactivity. Identifying potential triggers for neuroinflammatory diseases is a growing concern that our population faces today. These diseases are classified as incurable, and currently, there are no effective treatment options. Preventive strategies for identifying and removing risk factors are essential for addressing this growing epidemic of neuroimmunological disorders, which are an important and relevant area of research. The outcome of this research can be used for supporting the development of safety regulations and identifying potential health concerns for current mandatory flame-retardant usage. Additionally, this research may support the decisions made in respect of those suffering from neurological autoimmune diseases, as to whether removing flame retardant chemicals is a factor to be considered.

Research Questions and Hypothesis

The theoretical framework that will guide this proposed study is the social ecological theory. The knowledge that exposure to TBBPA chemicals may impact neurological autoimmunity may lead to reconstructive and transformative outlooks on social and environmental issues. This research study hypothesizes that chemical exposure to TBBPA can lead to chemical binding to human protein and the development of new haptens that may promote neurological autoimmunity. Ten fundamental research questions will be investigated in

this study: (1) Can TBBPA bind to human albumin and induce immunological chemical reactivity as identified in TBBPA bound to albumin specific antibodies in human serum? (2) Is there a correlation between TBBPA immunological reactivity with neurological autoimmunity to the nerve sheath proteins (myelin basic protein and myelin oligodendrocyte protein)? (3) What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of autoimmunity against nerve sheath proteins (myelin basic protein and myelin oligodendrocytic protein)? (4) Is there a correlation between TBBA immunological reactivity to S100B protein, the biomarker used to assess breakdown of the blood-brain barrier and neuroinflammation? (5) What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of neuroinflammation and breakdown of the blood-brain barrier (S100B)? (6) Is there a correlation between TBBPA immunological reactivity and alpha-synuclein, the protein aggregate marker for neurodegenerative diseases such as Parkinson's disease? (7) What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of protein aggregate antibody biomarkers for neurodegenerative diseases such as Parkinson's (alpha-synuclein)? (8) Is there a correlation with TBBPA immunological reactivity and aquaporin-4 water-channel receptors, the target protein for autoimmune reactivity for neuromyletis optica? (9) What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity against the autoimmune target protein of neuromyleitis optica (aquaporin-4 antibodies)? (10) Are there any differences in chemical immune responses between IgA, IgG, and IgM?

Definition of Terms

Autoimmunity: an immune condition in which the immune system erroneously attacks its host.

Autoimmune trigger: an immunologic insult that activates the immune response against the host, such as a pathogen, chemical, or dietary protein exposed to the host.

Agonist: a chemical that binds to a receptor and activates the receptor to produce a biological response

Alpha-Synuclein: an intracellular protein found in nerves that clumps together in Parkinson's disease.

Alzheimer's: a neurodegenerative disease that develops from the product of tau proteins and amyloid plaques, leading to cognitive decline and dementia.

Antibodies: immune cell products produced by B-cells to tag proteins for T-cell mediated destruction.

Antagonist: a chemical that interferes or inhibits the physiological action of another chemical on a receptor.

Arsenic: a compound toxic to humans found in soil, water, and food products

Aquaporin-4: a water-channel receptor of the central nervous system and the target protein for neuromyleitis optica.

Bisphenol A (BPA): a chemical used in polymers for making plastic products.

Brominated Flame Retardant (BFR): chemicals produced and sprayed on to objects to make them less flammable.

Chemical immune-reactivity: an immune response to chemical exposure.

Dichlorodiphenyltrichloroethane (DDT): a colorless, odorless, and tasteless insecticide.

Environmental compounds: chemicals and pollutants that are harmful to humans and animals.

Flame retardants: chemicals sprayed on furniture and clothing to reduce the spread of fire.

Tetrabromobisphenol A: the most widely used brominated fire retardant.

Immune system: the biological system used to fight pathogens and protect the body.

Immune tolerance: the immune system's ability to not react to dietary proteins, chemicals, or self-tissue proteins.

Mercury: a toxic compound found in dental and medical products, soil, water, and foods.

Myelin Basic Protein: a protein found to surround the nerve sheath that allows for faster nerve conduction.

Myelin Oligodenrocyte Protein: a protein found to surround the nerve sheath that allows for faster nerve conduction.

Neuromyleitis Optica: an autoimmune disease impacting the optic nerves and the spinal cord.

Parkinson's disease: a neurodegenerative disease that develops from protein aggregation of alpha synuclein, leading to the slowness of movement and resting tremor.

Polychlorinated biphenyls (PCBs): a synthetic chemical compound used commercially for coolant fluids.

Synthetic compounds: products that are made in the laboratory and are not found in nature.

S100B: a calcium-binding protein and a biomarker, used to determine the breakdown of the blood-brain barrier.

Summary

Autoimmunity is a condition in which the immune system erroneously attacks the body's own tissue. It is a devastating disease that impacts as much as 23 million Americans and leads to diseases such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis, lupus, and 21 other autoimmune diseases, according to the prevalence reported by the National Institute of Health (NIH Publication 05-5140). Chemicals exposed to humans in our environment are a known trigger of autoimmunity. One of the chemicals to which humans are most commonly exposed, found in various household items today, is TBBPA used in flame retardants. In this study, we will investigate the correlation between immune reactivity to TBBPA and neurological autoimmune reactivity to myelin and myelin oligodendrocytic glycoprotein, aquaportin-4, alpha-synclein, S100B.

Chapter 2: Literature Review

Introduction

This chapter will present the historical overview and theory involved with the use, safety, and potential role that TBBPA may play in the development of inflammatory and autoimmune diseases involving the nervous system. A detailed review of both immunological and neurological roles of TBBPA in both human and animal studies will be presented and concluded with a summary of both known and unknown health risks of TBBPA. This chapter will provide a research background to understand the importance of and need for this research study.

Historical Overview of the Theory and Research

TBBPA has been classified as a Brominated Flame Retardant (BFR), and since the beginning of its use in 1979, it has become the most widely used BFR worldwide, with annual production of more than 210,000 tons (Alaee, Arias, Sjodin, & Bergman, 2003). Research conducted in 1979 initially found that TBBPA was easily metabolized from the body, and therefore, it was considered a safe compound without active biological activity (Brady, 1979). However, subsequent research found that TBBPA is not completely metabolized upon exposure and that TBBPA can accumulate in human fluids over time (Jakobsson *et al*., 2002). Not only was TBBPA found to accumulate in human fluids, but it was also later shown to build up in the adipose tissue of both animals and humans (Johnson-Restrepo, Adams, & Kannon, 2008). In a French human monitoring study of random women volunteers and their newborns, 44% TBBPA was found in analyzed breast milk samples and 30% in both maternal and cord serum samples (Cariou, 2008). In a Japanese human mother-infant study, TBBPA was measured in maternal blood, maternal milk, cord blood, and umbilical cords. Researchers detected levels of TBBPA and concluded that the chemical can pass through the blood-placenta barrier and lead to perinatal

exposure (Kawashiro, 2008). Recently, in a prospective study of 304 mothers and their children, flame retardants were detected in breast milk at three months' post-partum and again in 36 months: more than 70% of the subjects had detectable levels (Adgent, 2016). It appears that TBBPA is not as easily metabolized as had been first theorized in 1979. This study will further investigate the potential theory that chemicals, such as TBBPA, may play a role in the worldwide epidemic of autoimmune disease.

The role that commonly encountered environmental chemicals play both in the development of autoimmune disease, and its immune reactivity has been published in numerous clinical studies (Bigazzi, 1997; McFadden *et al*., 2009; Wisnewski *et al*., 2010; Chipinda *et al*., 2011; Barragan-Martinez *et al*., 2012; Perricone *et al*., 2013). Research in the past decade has further demonstrated that toxicants can induce autoimmunity. In addition, evidence studies for the role of chemicals in the development and progression of autoimmunity continues to build acceptance (Bigazzi, 1997; McFadden *et al*., 2009; Wisnewski *et al*., 2010; Pollard, 2010; Chipinda *et al*., 2011; Barragan-Martinez *et al*., 2012; Perricone *et al*., 2013). A recent expert panel workshop titled "Workshop on the consensus statement on the role of the environment in the development of autoimmune disease consensus statement" evaluated existing data and concluded that critical advances in the field had been made in the last ten years; but much more research is necessary to understand the role that environmental toxicants and chemicals play in autoimmunity (Parks *et al*., 2014). Currently, there is a need to identify which chemicals pose a risk to autoimmune disease to manage the growing epidemic of autoimmune diseases that impact patient lives and increase health care costs. Interest in commonly exposed chemicals, especially chemicals found in our household products such as TBBPA, are of critical research significance,

as we are directly exposed to them daily. The frequent exposure to TBBPA may play a role in contributing to the worldwide rise and epidemic of the autoimmune disease.

The Theoretical Framework That Will Guide the Research Question

The theoretical framework that will guide this proposed study is the social ecological theory. The social ecological theory has been used to develop guidelines for community health promotion by considering variables such as corporate-decision makers, legislators, and environmental pollutants in promoting the well-being of others (Stokles, 1996). This theory emphasizes the complexity of relationships between environment, social, political, legal, and ecological influences (Stokles, 1996). It works toward transformative and reconstructive approaches to harmonize people and nature (Fox & Aldred, 2016). Developments in environmental pollution and toxicology that promotes man's dominance over nature are viewed as ecological crises (Weiss & Bellinger, 2006). The ideology is concerned with how factors such as individual economic motivations impact the world (Oishi, 2013). According to the social ecology theory, life and environment should be seen as complex systems that are interrelated (Grzywacz & Fuqua, 2000).

According to this theory, ecological problems are a result of social dysfunctions, which include industrial expansion, exploitation, deforestation, and environmental pollution (Cumming & Peterson, 2017). The social ecological theory is embraced by environmentalists who identify ecological problems such as preservation of wildlife, global warming, and reducing global toxicity (Alava *et al*., 2017). Identifying how toxic pollutants impact human health and society as a whole is necessary to initiate social consciousness and change (Stokols, 2000). These include changes in public policy (local, state, national, and global), relationships among organizations, institutions, and both interpersonal and intrapersonal factors (Fox & Aldred, 2016). According to

the social ecological view, it is necessary to have environmental protection programs to support societal health (Geller Winett, & Everett, 1982). Developing integrative models to address joint influences of personal and environmental factors in health promotion and disease etiology is a challenge faced by the social ecological theory (Christopherssen, 1989).

Currently, there is no public policy to limit the use or determine the safety of industrialized chemicals such as fire-retardants. Current U.S. legislation requires chemicals risk to humans to be proven before changes can be made to the current health policy and regulations. The social ecology theory purposes that legislative interventions have a powerful and necessary impact on public health (McKinlay, 1975). Determining the potential risk of fire retardants (TBBPA) to human health, as proposed in this doctoral proposal, is a necessary step to increase social consciousness, legislative actions, and public health policy, as promoted by the social ecology theory. The knowledge that exposure to TBBPA chemicals may impact neurological autoimmunity may lead to reconstructive and transformative outlooks on social and environmental issues. Social consciousness that TBBPA may be harmful to humans may lead to changes in social and political issues and manufacturing practices that can impact human health and environmental pollution and thereby promote changes in manufacturing legislation.

The Review of the Literature

A detailed literature review was conducted to identify any published research regarding the association between immunological reactivity with TBBPA and autoimmune target sites of the nervous system. Data sources included PubMed, EMBASE, and clinicaltrials.gov from inception until December 2016. At this time, no research on the specific associations between TBBPA and neurological autoimmunity has been published; however, a limited number of *in vitro* and animal studies have been published, demonstrating that TBBPA may have some potential neurotoxic

activity. In one *in vitro* study, low micromolar concentrations of TBBPA induced cerebellar granule cell damage by inducing oxidative stress in an environment challenged with oxygen-glucose deprivation to stimulate the synergistic effects of TBBPA combined with cerebral ischemia (Ziemińska *et al.*, 2012). In another *in vitro* study, TBBPA was found to potentially induce intracellular Ca^+ release, which leads to cytotoxic and neurotoxic changes from glutamate influx in cultured cerebellar granule cells (Zieminska, 2014). In an *in vitro* study using rats, the neurotoxicological potential for brominated flame retardants was also shown to potentially occur from neurotransmitter uptake in brain synaptosomes and vesicles (Mariussen, 2002). Finally, in the only *in vivo* animal study, pregnant rats were exposed to dietary TBBPA postnatally, and the offspring were found to exhibit aberrant neuronal development immunohistochemically (Seagusa, 2012). The impact of TBBPA on the nervous system or neurological autoimmunity has not been extensively studied at this time; however, the literature illustrated several studies in which TBBPA can promote immunological mechanisms that may play a role in autoimmunity and/or neurological autoimmunity.

TBBPA was shown to stimulate immune cells *in vitro*. Specifically, TBBPA was shown to increase MHC class II and CD86, CD 80, and CD 11 expression and interleukin and increase T-cell receptor expression (Koike, Yanagisawa, Takigami, & Takano, 2013). Similar immunological responses have been reported with toxic lead exposure, and it has been suggested that these reactions promote Th-2 mediated autoimmunity (Gao, Mondal, & Lawrence, 2007). TBBPA was found to trigger MAP kinases and protein kinase C *in vitro* with mussel hemocytes (Canesi *et al.*, 2005). Activation of these kinase pathways has been found to induce lupus-like autoimmunity in mice (Gorelik, 2015). TBBPA was found to disrupt immune regulation by IFN-γ production and signaling pathways (Almughamsi & Whalen, 2016). The IFN-γ mediated

signaling pathways have been found to be key mechanisms in the primary inflammatory pathway of autoimmunity (Strassner & Harris, 2016). TBBPA has been found to enhance the release of interleukin (IL)-6, IL-8, and prostaglandin E2, and suppress TGF-beta in HTR-8/SVneo cells (Park, Kamau, Korte, & Rita Loch-Caruso, 2014). The expression of these cytokine pathways in conjunction with suppression of regulatory T cells is a key mechanism in the promotion of autoimmunity (Zhang *et al*., 2015). TBBPA has been shown to target MAPK pathways (ERK1/2 and p38) and lead to increased IL-1β secretion from immune cells (Anisuzzaman & Whalen, 2016). Increased IL-1β secretion leads to the activation of T-helper-17 cells (TH-7). The activation of TH-17 is the key inflammatory pathway in the expression of autoimmune disease (Lasigliè *et al.*, 2011). In summary, TBBPA exposure in animals *in vitro* has been shown to activate immunological pathways involved in autoimmune disease promotion.

In addition to the impact TBBPA has on neurological and immunological pathways, some research illustrates that TBBPA can disrupt thyroid metabolism. TBBPA has been shown to function as a thyroid endocrine disruptor of homeostasis (Guyot, 2014). It has been found to share structural similarity with thyroid hormones and have the ability to interfere with thyroid hormone physiology (Grasselli *et al*., 2014). Additionally, TBBPA has been found to alter thyroid hormone gene expression (Guyot, 2014). In animals, TBBPA has been shown to affect neurobehavioral development and thyroid hormone levels (Darnerud, 2003).

Summary of What is Known and What is Unknown about the Topic

TBBPA has been classified as a Brominated Flame Retardant (BFR), and since the beginning of its use in 1979, it has become the most widely used BFR worldwide. TBBPA use has led to the accumulation of the chemical in both animals and humans worldwide (Decherf & Demeneix, 2011). TBBPA was initially thought to be easily metabolized from the body, and

therefore, a safe compound without active biological activity. However, subsequent research has found that TBBPA is not completely metabolized upon exposure, that TBBPA can accumulate in human fluids over time, and that there are potential health concerns with TBBPA exposure. Research in the past decade has further demonstrated that chemicals can induce autoimmunity. At this time, no research on the specific associations between TBBPA and neurological autoimmunity or autoimmunity, in general, has been published. However, a limited number of *in vitro* and animal studies have been published, demonstrating that TBBPA may have some potential neurotoxic activity. Additionally, TBBPA can promote immunological mechanisms that may play a role in autoimmunity and/or neurological autoimmunity. Lastly, some research illustrates that TBBPA can disrupt thyroid metabolism.

The Contributions this Study Will Make to the Field

This correlative research is a pioneer study, and it could possibly be one of the earliest research studies investigating the correlation of the role of TBBPA immune reactivity with the neurological autoimmune disease in humans. This study may contribute to the knowledge base of TBBPA as a potential threat to human health and may play a role as an autoimmune trigger for individuals suffering from autoimmune and inflammatory diseases of the nervous system. Additionally, this study may provide further evidence that TBBPA, as a flame retardant, may need to be removed and replaced with safer options.

Summary

Autoimmune diseases are a growing worldwide epidemic, as are neurodevelopment disorders and neurodegenerative diseases. Increased use of environmental chemicals may play a potential role in neuroinflammatory and autoimmune disorders of the nervous system. TBBPA is one of the most common chemicals to which humans are exposed, due to its presence in and

chemical contamination of food products. The current literature has limited research investigating the role of TBBPA and autoimmunity. Correlative investigation of TBBPA immune reactivity and neurological autoimmunity in human subjects may contribute data that may impact individuals suffering from autoimmunity or inflammatory diseases of the nervous system. In the next chapter, the detailed methodology and study design will be presented.

Chapter 3: Methodology

This chapter will review the research design, specific research methods, statistical methodology, resource requirements, reliability, and validity of the research methodology, timeline, and the limitations and delimitations used in this study. A specific step-by-step breakdown of all laboratory procedures using ELISA methodology will be presented in addition to each specific descriptive and inferential statistical analysis used for each correlative analysis.

Research Methods to Be Employed

A quantitative cross-sectional correlation research study was conducted to assess the relationships between human subjects with TBBPA bound to albumin antibodies with myelin basic protein (MBP) antibodies, myelin oligodendrocytic glycoprotein (MOG) antibodies, aquaporin-4 antibodies, alpha-synuclein antibodies, and S100B antibodies. The goal of this study was to investigate whether there is an association between chemical immune reactivity to TBBPA, found in flame-retardants, in human subjects and biomarkers used to diagnose inflammatory and autoimmune diseases of the nervous system. A power analysis was conducted to determine the number of subjects required for the study to achieve statistical significance. Sample size power calculations for correlation analysis with r0 at 0.3 and ra at 0.6 - 0.8 indicated that 57 samples would be sufficient to determine the population effect size to power this study properly (Appendix B).

Specific Procedures to Be Employed

The specific methodology for the research design was divided into five steps and received institutional approval from the director of the laboratory (Appendix A). Step 1 was the preparation of laboratory supplies and equipment calibration. Step 2 was the development of the ELISA plates necessary for conducting the laboratory investigation. In Step 3, the laboratory

methodology was employed to detect antibodies using direct enzyme-linked immune assay (ELISA) laboratory techniques. In Step 4, an optical density reader was used to quantify antibody reactions. Step 5 was the final step that involved evaluating the data and performing statistical analysis to evaluate research outcomes.

Step 1: Preparation and Supplies

Non-identifiable blood samples from 100 blood donors were purchased from Innovative Research Inc. (Southfield, MI, USA). The blood samples were certified as healthy blood donor samples, according to the Center for Disease Control (CDC) criteria. The blood was collected in a sterile blood collection bag containing anticoagulants. Before shipping, each blood sample was tested, according to FDA guidelines, to detect Hepatitis B surface antigen, antibodies to HIV, antibodies to hepatitis C, HIV-1 RNA, Hepatitis C RNA and syphilis. All units were required to yield non-reactive/negative results for each test performed. No medical examinations or additional lab tests were conducted to determine the health status of the donors otherwise.

Tetrabromobisphenol-A, myelin oligodendrocytic glycoprotein (MOG), alpha-synuclein, aquaporin-4, and S100B purchased from Sigma Aldrich (St. Louis, MO) were used to develop antigen-coated ELISA plates. Tools and supplies for ELISA were purchased from major laboratory suppliers; they included blank polystyrene microplates, pipetting reagent reservoirs, plate-sealing tape, racked tube systems for use with multi-channel pipettes, and unique vacuum-manifold apparatus for filter-based ELISA.

Lastly, the equipment was calibrated to ensure the accuracy of data. A computerized ELx808 absorbance microplate reader was used to measure enzyme-linked optical density, and a computerized Elx405 microplate washer was used to clean the ELISA plates throughout ELISA preparation and after each enzyme and sera application. Certification for annual calibration was

satisfied before the study. Six samples were used in the study during calibration and for ELISA

control wells, leading to 94 samples out of 100 to be used in the actual study.

Step 2: Developing ELISA Plates

Preparations of Tetrabromobisphenol-A ELISA plates were conducted by measuring 1

gram of human serum albumin (HSA) that was dissolved in 100mL of 0.01 M phosphate

buffered saline (PBS) pH 7.4, to which 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)

carbodimide HCL was added and kept on the stirrer for 10 minutes. In a separate tube, 100 mg of

N-hydroxysulfosuccinimide sodium salt was dissolved in 10 mL distilled water and was added

drop wise to the mixture. In 10 ml of 0.01 M PBS pH7.4, 100 mg of Tetrabromobisphenol-A will

be dissolved; each was separately added dropwise to the protein mixture. The mixtures were kept

for one hour at room temperature, and then, for 4 hours at 4 degrees Celsius. The unreacted small

molecules were removed by dialysis, using a molecular cutoff of 8,000 Dalton. The conjugation

of haptenic chemicals was confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis and a

shift in band configuration. In addition, spectrographic analysis of the conjugate was undertaken

until there was an increase in absorption from 230 to 260 nM, which indicated that haptenic

chemicals become covalently linked to the HSA or protein carrier.

Step 3: Quantitative Antibody Reaction

Antigens and peptides were dissolved in PBS or methanol at a concentration of 1.0

mg/mL, then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer at a pH of 9.5 and 100 uL were

added to each well of the polystyrene flat bottom ELISA plate. Plates were incubated overnight

at 4 degrees Celsius, and then, they were washed three times with 200 uL Tris-buffered Saline

(TBS) containing 0.05% Tween 20 at a pH of 7.4. The non-specific binding of immunoglobulins

was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in

TBS and incubated overnight at 4 degrees Celsius. Plates were washed, as described above, and then, the serum samples were diluted in the ratio 1:100 in 0.1 M PBS Tween containing 2% BSA were added to duplicate wells and incubated for an hour at room temperature. The plates were washed, and then, alkaline phosphatase goat anti-human IgG, IgM, and IgA F(ab')2 fragments with an optimal dilution of 1:400-1:2000 in 1% HSA-TBS were added to each well; the plates were incubated for an additional one hour at room temperature. The plates were then washed five times with TBS-Tween buffer, and the enzyme reaction was started by adding 100 uL of paranitrophenylphosphate (PNPP) in 0.1 mL diethanolamine buffer 1 mg/mL containing 1 mM $MgCl_2$ and sodium aside at a pH of 9.8. The reaction was stopped 45 minutes later with 50 uL of 1 N NaOH, and the samples were then ready for quantitative analysis using an optical density reader.

Step 4: Quantify Antibody Reaction

A computerized ELx808 absorbance microplate reader was used to measure enzyme-linked optical density. The antibody reacted ELISA plates were inserted into the optical density reader. The optical density was recorded at 405nm by the microtiter reader to provide quantitative antibody reactivity levels, and then, it was compared with control wells. Measurements of optical density above control wells were used to determine increased antibody reactivity. This data was then evaluated for statistical significance.

Step 5: Evaluation of Data for Statistical Correlation and Relative Risk

Statistical analysis was performed using STATA software. The data was imported into STATA and coded. A descriptive analysis was conducted using histograms and scatter plots to identify any outliers or missing data. Statistical analysis was conducted to determine the correlation between the following: (1) Tetrabromobisphenol-A (TBBPA) bound to albumin antibodies and

myelin basic protein (MBP) antibodies, (2) correlation between TBBPA bound to albumin antibodies and myelin oligodendrocytic glycoprotein (MOG) antibodies, (3) correlation between TBBPA bound to albumin antibodies and alpha-synuclein antibodies, (4) correlation between TBBPA bound to albumin antibodies and aquaporin-4 antibodies, (5) and correlation between TBPA bound to albumin antibodies and S100B antibodies. A separate analysis was conducted for three immunoglobulins: IgG, IgA, and IgM. It is possible for the relationships to exist for some immunoglobulins and not for others. The presence of statistically significant correlative relationships was conducted with Person's coefficients, Kendall's tau, and Spearman's rho, which are parametric and non-parametric association measures for each of the relationships described above for IgG, IgA, and IgM (Appendix B). A significant p-value of 0.01 was determined to adjust for multiple comparisons using a Bonferroni correction, and a confidence interval of 95% was used. STATA software package was used to conduct all inferential and descriptive analysis.

Relative risk was assessed by determining the risk ratios of exposure leading to chemical, immunological reactivity (TBBPA antibodies) and the development of neurological autoimmune disease biomarkers (MOG, MBP, alpha-synuclein, S100B, and AQP-4 antibodies) compared to non-exposed and non-disease samples. The 15 risk ratio calculations for this study include the following: (1) TBBPA IgA exposure and non-exposure with MBP IgA disease and non-disease outcomes, (2) TBBPA IgA exposure and non-exposure with MOG IgA disease and non-disease outcomes, (3) TBBPA IgA exposure and non-exposure with alpha synuclein IgA disease and non-disease outcomes, (4) TBBPA IgA exposure and non-exposure with aquaporin-4 IgA disease and non-disease outcomes, (5) TBBPA IgA exposure and non-exposure with S100B IgA disease and non-disease outcomes, (6) TBBPA IgG exposure and non-exposure with MBP IgG

disease and non-disease outcomes, (7) TBBPA IgG exposure and non-exposure with MOG IgG disease and non-disease outcomes, (8) TBBPA IgG exposure and non-exposure with alpha synuclein IgG disease and non-disease outcomes, (9) TBBPA IgG exposure and non-exposure with aquaporin-4 IgG disease and non-disease outcomes, (10) TBBPA IgG exposure and non-exposure with S100B IgG disease and non-disease outcomes, (11) TBBPA IgM exposure and non-exposure with MBP IgM disease and non-disease outcomes, (12) TBBPA IgM exposure and non-exposure with MOG IgM disease and non-disease outcomes, (13) TBBPA IgM exposure and non-exposure with alpha synuclein IgM disease and non-disease outcomes, (14) TBBPA IgM exposure and non-exposure with aquaporin-4 IgM disease and non-disease outcomes, and (15) TBBPA IgM exposure and non-exposure with S100B IgM disease and non-disease outcomes. The calculation of risk ratios involved transforming the continuous optical density antibody variables into binary variables. One standard deviation from the mean was used to classify a positive exposure to both TBBPA and disease development for MOG, MBP, S100B, AQP-4, and alpha-synuclein. Risk ratio calculations were conducted using STATA software and included 95% confidence intervals and p-values set at 0.05 for statistical significance.

Formats for Presenting Results

Scatter plots will be produced to demonstrate bivariate linear relationships. The following scatterplots were produced for IgA, IgG, and IgM. The 15 scatter plots have been listed as follows: (1) TBBPA IgA with MBP IgA, (2) TBBPA IgA with MOG IgA, (3) TBBPA IgA with alpha synuclein IgA, (4) TBBPA IgA with aquaporin-4 IgA, and (5) TBBPA IgA with S100B IgA, (6) TBBPA IgG with MBP IgG, (7) TBBPA IgG with MOG IgG, (8) TBBPA IgG with alpha synuclein IgG, (9) TBBPA IgG with aquaporin-4 IgG, and (10) TBBPA IgG with S100B IgG, (11) TBBPA IgM with MBP IgM, (12) TBBPA IgM with MOG IgM, (13) TBBPA IgM

with alpha synuclein IgM, (14) TBBPA IgM with aquaporin-4 IgM, and (15) TBBPA IgM with

S100B IgM. A list of the variables that were used for the 15 individual scatterplots has been

presented in Table 1. In addition to the 15 individual bivariate scatterplots, three scatterplot

matrix graphs were produced for each specific immunoglobulin (IgA, IgG, and IgM).

Table 1 – List of Correlations		
Correlation Analysis	Variable #1	Variable #2
1	TBBPA bound to human albumin IgA	Myelin basic protein IgA
2	TBBPA bound to human albumin IgG	Myelin basic protein IgG
3	TBBPA bound to human albumin IgM	Myelin basic protein IgM
4	TBBPA bound to human albumin IgA	Myelin oligodendrocytic glycoprotein IgA
5	TBBPA bound to human albumin IgG	Myelin oligodendrocytic glycoprotein IgG
6	TBBPA bound to human albumin IgM	Myelin oligodendrocytic glycoprotein IgM
7	TBBPA bound to human albumin IgA	Aquaporin-4 IgA
8	TBBPA bound to human albumin IgG	Aquaporin-4 IgG
9	TBBPA bound to human albumin IgM	Aquaporin-4 IgM
10	TBBPA bound to human albumin IgA	Alpha-synuclein antibodies IgA
11	TBBPA bound to human albumin IgG	Alpha-synuclein antibodies IgG
12	TBBPA bound to human albumin IgM	Alpha-synuclein antibodies IgM
13	TBBPA bound to human albumin IgA	S100B IgA
14	TBBPA bound to human albumin IgG	S100B IgG
15	TBBPA bound to human albumin IgM	S100B IgM

Table 2 illustrates the outcomes of the 15 risk ratio calculations. The table includes

exposure variables, disease variables, risk ratios, 95% confidence intervals, and p-values for each

of the following risk ratios: (1) TBBPA IgA exposure and non-exposure with MBP IgA disease

and non-disease outcomes, (2) TBBPA IgA exposure and non-exposure with MOG IgA disease

and non-disease outcomes, (3) TBBPA IgA exposure and non-exposure with alpha synuclein

IgA disease and non-disease outcomes, (4) TBBPA IgA exposure and non-exposure with

aquaporin-4 IgA disease and non-disease outcomes, (5) TBBPA IgA exposure and non-exposure

with S100B IgA disease and non-disease outcomes, (6) TBBPA IgG exposure and non-exposure

with MBP IgG disease and non-disease outcomes, (7) TBBPA IgG exposure and non-exposure with MOG IgG disease and non-disease outcomes, (8) TBBPA IgG exposure and non-exposure with alpha synuclein IgG disease and non-disease outcomes, (9) TBBPA IgG exposure and non-exposure with aquaporin-4 IgG disease and non-disease outcomes, (10) TBBPA IgG exposure and non-exposure with S100B IgG disease and non-disease outcomes, (11) TBBPA IgM exposure and non-exposure with MBP IgM disease and non-disease outcomes, (12) TBBPA IgM exposure and non-exposure with MOG IgM disease and non-disease outcomes, (13) TBBPA IgM exposure and non-exposure with alpha synuclein IgM disease and non-disease outcomes, (14) TBBPA IgM exposure and non-exposure with aquaporin-4 IgM disease and non-disease outcomes, and (15) TBBPA IgM exposure and non-exposure with S100B IgM disease and non-disease outcomes. In summary, there was a total of 15 scatterplots and three scatterplot matrix graphs to illustrate the outcome data of the study.

Table 2 - List of Risk Ratios

Exposure Antibody	Disease Antibody	Risk Ratio
TBBPA IgA	Myelin basic protein IgA	#1
TBBPA IgG	Myelin basic protein IgG	#2
TBBPA IgM	Myelin basic protein IgM	#3
TBBPA IgA	Myelin oligodendrocytic glycoprotein IgA	#4
TBBPA IgG	Myelin oligodendrocytic glycoprotein IgG	#5
TBBPA IgM	Myelin oligodendrocytic glycoprotein IgM	#6
TBBPA IgA	Aquaporin-4 IgA	#7
TBBPA IgG	Aquaporin-4 IgG	#8
TBBPA IgM	Aquaporin-4 IgM	#9
TBBPA IgA	Alpha-synuclein IgA	#10
TBBPA IgG	Alpha-synuclein IgG	#11
TBBPA IgM	Alpha-synuclein IgM	#12
TBBPA IgA	S100B IgA	#13
TBBPA IgG	S100B IgG	#14
TBBPA IgM	S100B IgM	#15

Resource Requirements

Several resources were needed to conduct this study. Laboratory analysis was conducted in a state-licensed laboratory certified by the CLIA (Clinical Laboratory Improvement Amendments) and American College of Pathologists. The laboratory contained a computerized optical density absorbance microplate reader. The tools and supplies for ELISA methodology included blank polystyrene microplates, pipetting reagent reservoirs, plate-sealing tape, racked tube systems for use with multi-channel pipettes, and unique vacuum-manifold apparatus for filter-based ELISA. Laboratory reagents used in this study included human serum albumin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCL, N-hydroxysulfosuccinimide sodium salt,

carbonate-bicarbonate buffer, bisphenol A, bisphenol A binding protein, alkaline phosphatase goat anti-human IgG, IgM, and IgA F(ab')2 fragments, paranitrophenylphosphate, myelin basic protein, myelin oligodendrocytic glycoprotein, alpha-synuclein, aquaporin-4, S100B, and diethanolamine buffer.

Reliability and Validity

ELISA assay is the gold-standard current technology used by all immunological laboratories to measure antibody reactions (Dobrovolskaia, Gam, & Slater, 2006). The test uses the basic concept of an antigen binding to specific antibodies. The assay uses antibodies and enzyme-mediated color change to detect the presence of either antigen (proteins, peptides, hormones, etc.) or antibody in a given sample. The color change is measured with computerized optical density evaluation, thereby providing accurate immune reactivity even with detections of extremely low concentrations (Crowther, 1995). ELISA testing is highly sensitive to compositional differences in complex antigen mixtures when the specific detecting antibody is present in relatively small amounts (Dobrovolskaia, Gam, & Slater, 2006). ELISA testing values are calculated by multiple-point parallel-line comparison and when performed with endpoint analysis, demonstrate good correlations when total antibodies are tested compared to radioimmunoassay (RIA) testing (Lagergard, Trollfors, Claesson, Schneerson, & Robbins, 1988). Additionally, the ELISA methodology has the advantage of significantly lower time requirement than the RIA for performing a typical assay (Garvey, Thomas, & Linton, 1987). The laboratory methodology used in this study was the gold standard test to evaluate antibody reactions; this method has good reliability and validity.

Timeline

The Institutional Review Board (IRB) application was approved by Nova Southeastern University on August 18, 2017 (IRB#: 2017-508). Supplies and regents necessary for the research were attained after the IRB approval. The laboratory analysis took approximately five weeks to complete. Data analysis was promptly completed upon completion of the laboratory analysis and submitted on September 23 2017. The first draft of the dissertation proposal was completed on January 1, 2018.

Limitations and Delimitations

There are several limitations and delimitations to this study. This study used correlative data analysis. Correlation is not causative, and at this point, there is no clear understanding whether immune reactivity to TBBPA is causative of neurological autoimmunity. Overzealous immune reactivity may occur due to loss of overall immunological tolerance, leading to both neurological and chemical immune reactivity. Correlative statistical analysis with both parametric (Pearson's coefficients) and non-parametric analysis (Spearman's rank and Kendall's tau) used to analyze data in this study does not permit to statistically account for confounding and effect modification factors in statistical data analysis. This study had anonymous healthy blood donors, and there was no information regarding their medical history. Additionally, this study is lacking in some degree of external validity. A random sample size does not reflect the population diversity accurately.

Chapter 4: Results

Introduction

This chapter will present both descriptive and inferential statistical results of the study. The data analysis of 94 subjects included scatter matrix charts and scatter plots to illustrate the correlation patterns of the data. Tables will be presented to exhibit the risk ratio outcomes. A review of data analysis methods will also be presented. The findings of the study will be presented with a detailed summary of the results. The data analysis of this study included both correlation analysis and measures of association with risk ratios. The correlation analysis includes 15 paired relationships that have been listed in Table 1. The risk ratio analysis includes 15 paired measures of association that have been listed in Table 2.

Data Analysis

STATA software version 14.2 was used to conduct all inferential and descriptive analysis. The initial steps of the analysis were performed to avoid any violations of measurement error. Data analysis was conducted by importing three independent data sets into STATA that included the optical density measurements from ELISA for IgA, IgG, and IgM. The first step in the analysis was to build histograms and scatter plots to identify any outliers or missing data. The researcher conducted a careful review of all the data points and compared it to the original data to confirm that there were no errors in measurement between the IgA, IgG, and IgM data sets. A matching comparison of the STATA data editor feature was conducted with the original data set to ensure further that there were no errors in the importation of the data into the software. All of the steps were repeated twice to ensure accurate data.

Correlational analyses were conducted using Pearson's correlation for the parametric variables and Kendall's tau and Spearman's rho for the non-parametric variables to measure for IgG, IgA,

and IgM independently. A Bonferroni correction was conducted to adjust the p-value to decrease the likelihood of a type I error, given the large number of analyses. The adjusted alpha for statistical significance was set to 0.01. This value was determined by dividing an alpha value of 0.05, which was divided by five, as five correlations were tested for each independent immunoglobulin.

Scatter plots were produced to visualize the bivariate linear relationships for all 15-correlation analysis. Pearson's correlation coefficient value of r and the p-value have been noted on the scatter plots to provide the statistical significance and strength of the relationship to the graphic representation of the data. Additionally, three scatterplot matrix graphs were built to graphically summarize the associations for each specific immunoglobulin (IgA, IgG, and IgM).

The measurement of risk ratios was conducted by converting each of the optical density continuous variables into binary variables. One standard deviation above the mean for each continuous variable was classified as a significant binary value of one. Any value below one standard deviation of the mean was classified as a non-significant binary value of 0. Standard deviations above one are commonly used to categorize abnormal reference ranges for medical laboratory ranges. Risk ratio analysis was conducted labeling the TBBPA antibodies as exposure variables and the neurological target protein antibodies as the disease outcome variable for each of the 15 relationships.

Findings

Relationships between Tetrabromobisphenol-A and Myelin Basic Protein

The two-way scatter plot evaluation for TBBPA and MBP for IgA immunological reactive demonstrates a positive monotonic relationship (Figure 1). Statistical analysis using Pearson's correlation coefficient was statistically significant ($p < 0.0001$) with a moderate

correlation of 0.61. Spearman's rank correlation was statistically significant (p-value < 0.0012) with a mild correlation of 0.33. Kendall tau rank correlation was statistically significant (p-value <0.0033) with a mild correlation of 0.23. The risk ratio for TBBPA IgA antibody production as an exposure variable and MBP IgA antibodies as an outcome variable was 13.3 (CI: 3.90, 45.50) and statistically significant (p-value <0.0001).

Figure 1 - Linear Relationships with IgA TBBPA and MBP IgA
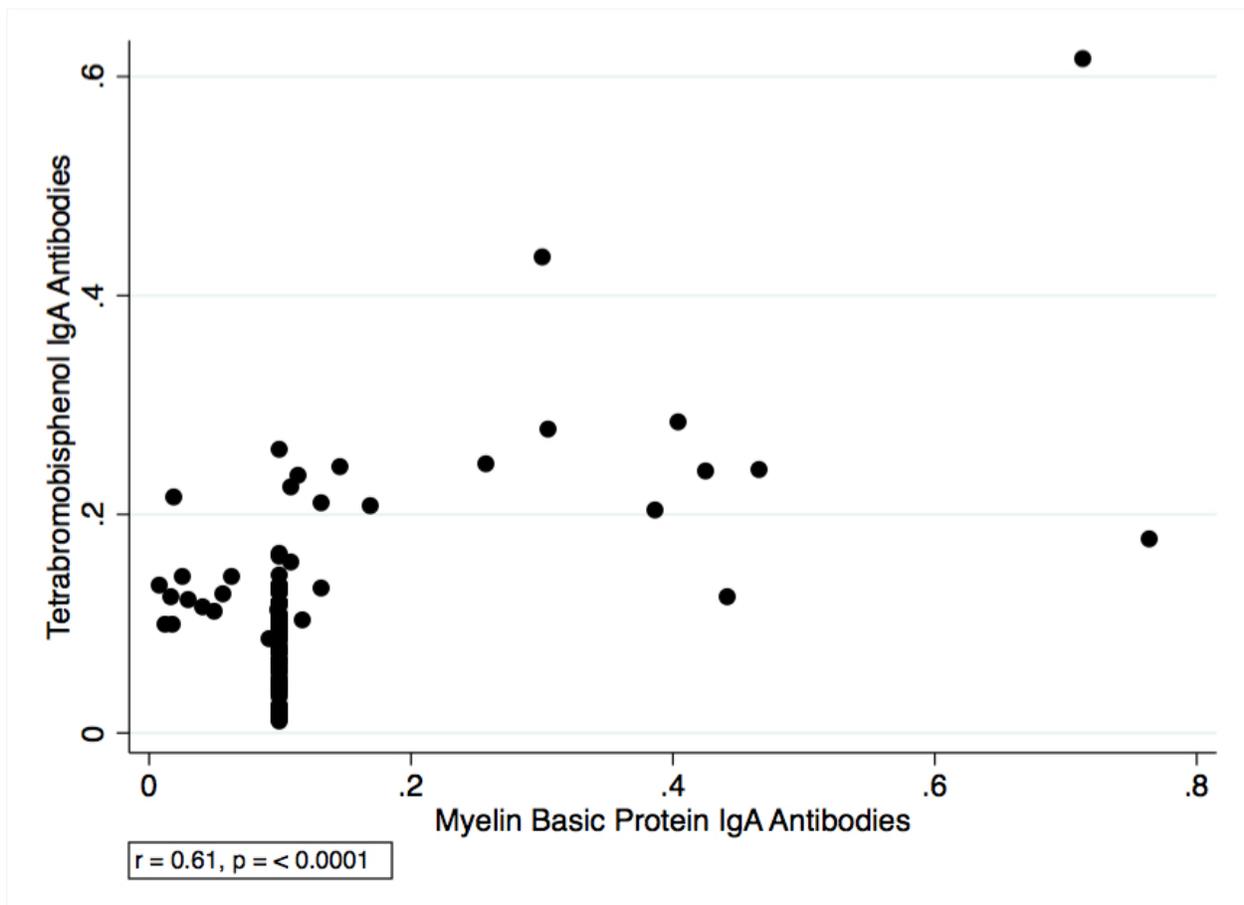
The two-way scatter plot evaluation for TBBPA and MBP for IgG immunological reactive demonstrates a positive monotonic relationship (Figure 2). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a

substantial correlation of 0.81. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.75. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.58. The risk ratio for TBBPA IgG antibody production as an exposure variable and MBP IgG antibodies as an outcome variable was 13.58 (CI: 5.55, 33.21) and statistically significant (p-value <0.0001).

Figure 2 - Linear Relationships with IgG TBBPA and MBP IgG

The two-way scatter plot evaluation for TBBPA and MBP for IgM immunological reactive demonstrates a positive monotonic relationship (Figure 3). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a

substantial correlation of 0.87. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.84. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.67. The risk ratio for TBBPA IgM antibody production as an exposure variable and MBP IgM antibodies as an outcome variable was 22.4 (CI: 7.01, 70.10) and statistically significant (p-value <0.0001).

Figure 3 - Linear Relationships with IgM TBBPA and MBP IgM

Relationships between Tetrabromobisphenol-A and Myelin Oligodendrocytic Glycoprotein

The two-way scatter plot evaluation for TBBPA and MOG for IgA immunological reactive demonstrates a positive monotonic relationship (Figure 4). Statistical analysis using

Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a moderate correlation of 0.68. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.71. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.54. The risk ratio for TBBPA IgA antibody production as an exposure variable and MOG IgA antibodies as an outcome variable was 14.29 (CI: 5.20, 39.30) and statistically significant (p-value <0.0001).

Figure 4 - Linear Relationships between TBBPA IgA and MOG IgA

The two-way scatter plot evaluation for TBBPA and MOG for IgG immunological reactive demonstrates a positive monotonic relationship (Figure 5). Statistical analysis using

Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a moderate correlation of 0.50. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a moderate correlation of 0.55. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.39. The risk ratio for TBBPA IgG antibody production as an exposure variable and MOG IgG antibodies as an outcome variable was 5.40 (CI: 2.06, 14.08) and statistically significant (p-value <0.0005).

Figure 5 - Linear Relationships with IgG TBBPA and MOG IgG

The two-way scatter plot evaluation for TBBPA and MOG for IgM immunological reactive demonstrates a positive monotonic relationship (Figure 6). Statistical analysis using

Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.88. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.86. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.68. The risk ratio for TBBPA IgM antibody production as an exposure variable and MOG IgM antibodies as an outcome variable was 16.8 (CI: 6.15, 45.91) and statistically significant (p-value <0.0001).

**Figure 6 - Linear Relationships with IgM TBBPA and MOG IgM**

r = 0.88, p = < 0.0001
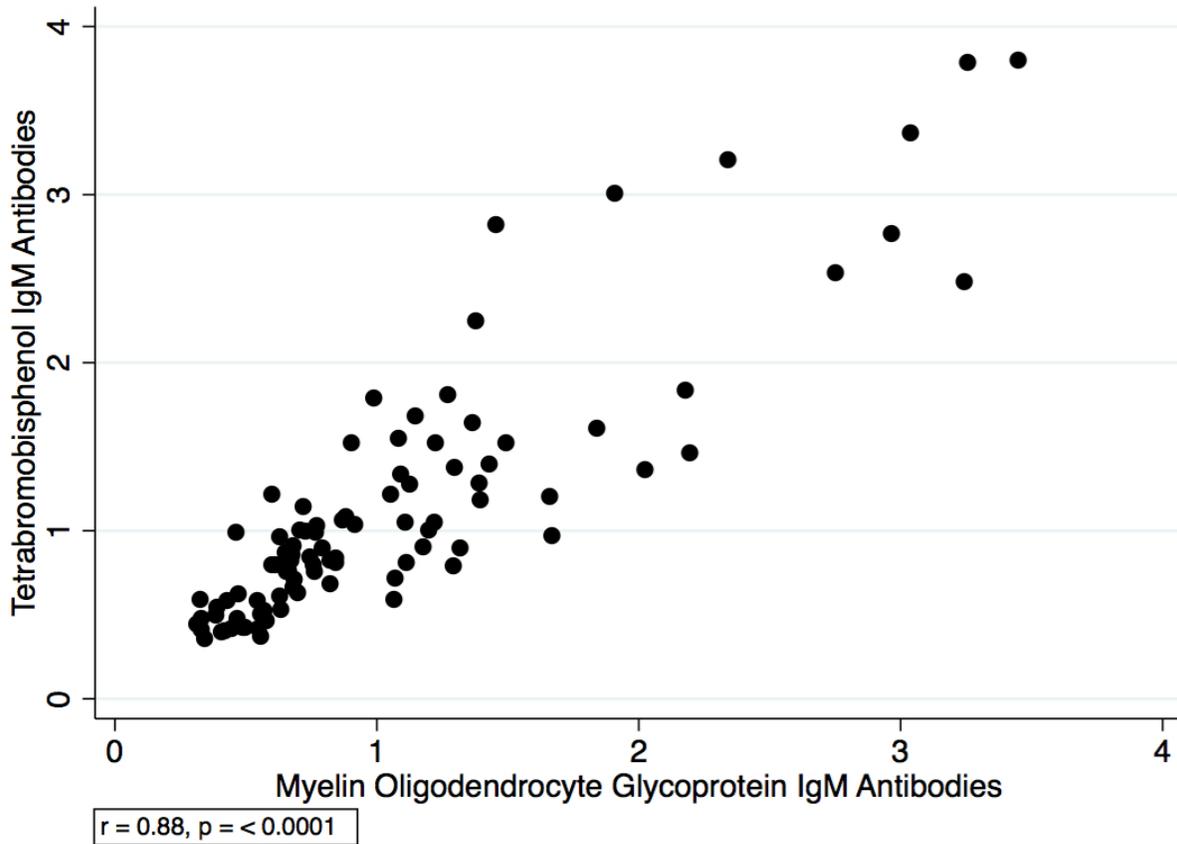
Relationships between Tetrabromobisphenol-A and Aquaporin-4

The two-way scatter plot evaluation for TBBPA and AQP4 for IgA immunological reactive demonstrates a positive monotonic relationship (Figure 7). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a moderate correlation of 0.70. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a moderate correlation of 0.67. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.50. The risk ratio for TBBPA IgA antibody production as an exposure variable and AQP4 IgA antibodies as an outcome variable was 10.29 (CI: 4.04, 26.18) and statistically significant (p-value <0.0001).
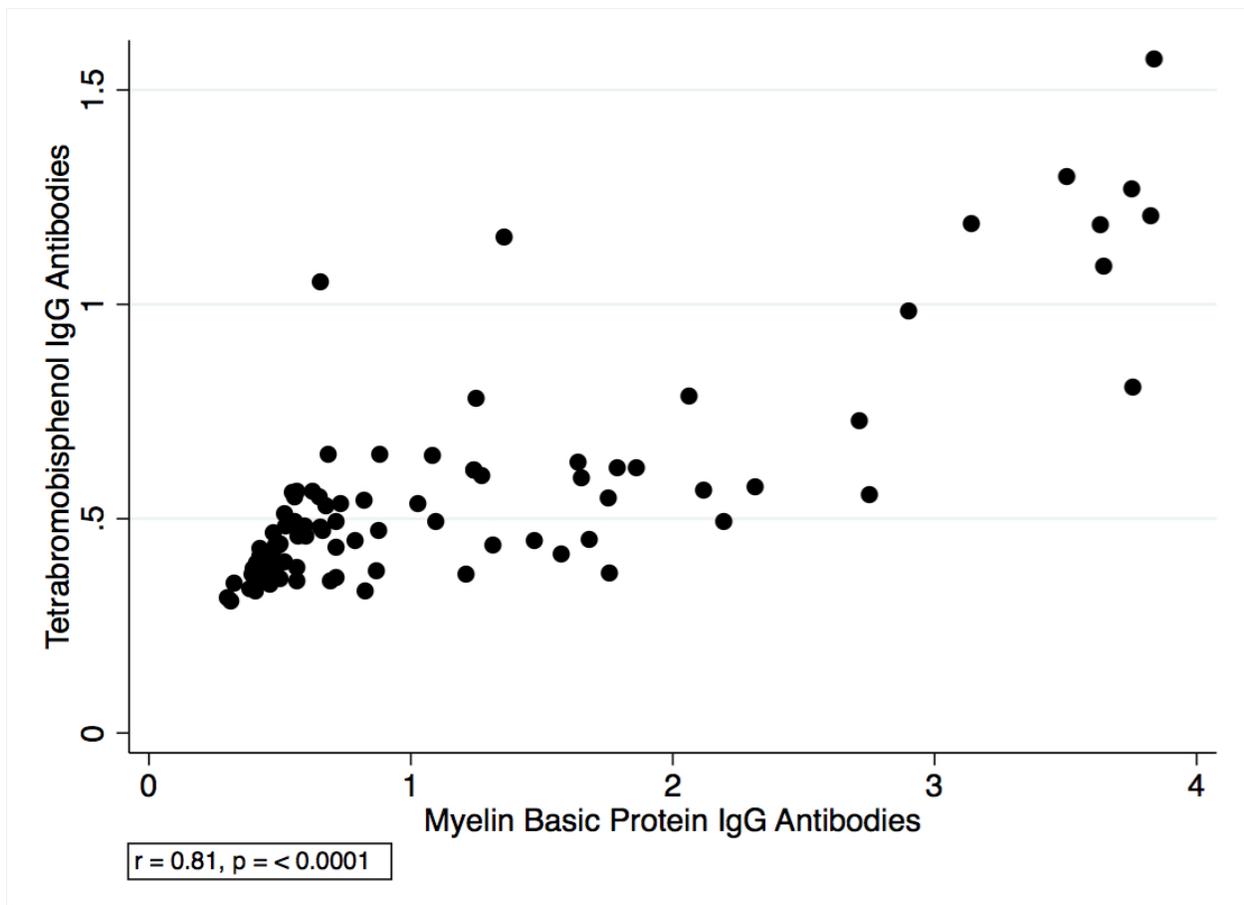
Figure 7 - Linear Relationships between TBBPA IgA andAQP4 IgA

The two-way scatter plot evaluation for TBBPA and AQP4 for IgG immunological reactive demonstrates a positive monotonic relationship (Figure 8). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.83. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.77. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.59. The risk ratio for TBBPA IgG antibody production as an exposure variable and AQP4 IgG antibodies as an outcome variable was 60.36 (CI: 8.32, 437.83) and statistically significant (p-value <0.0001).
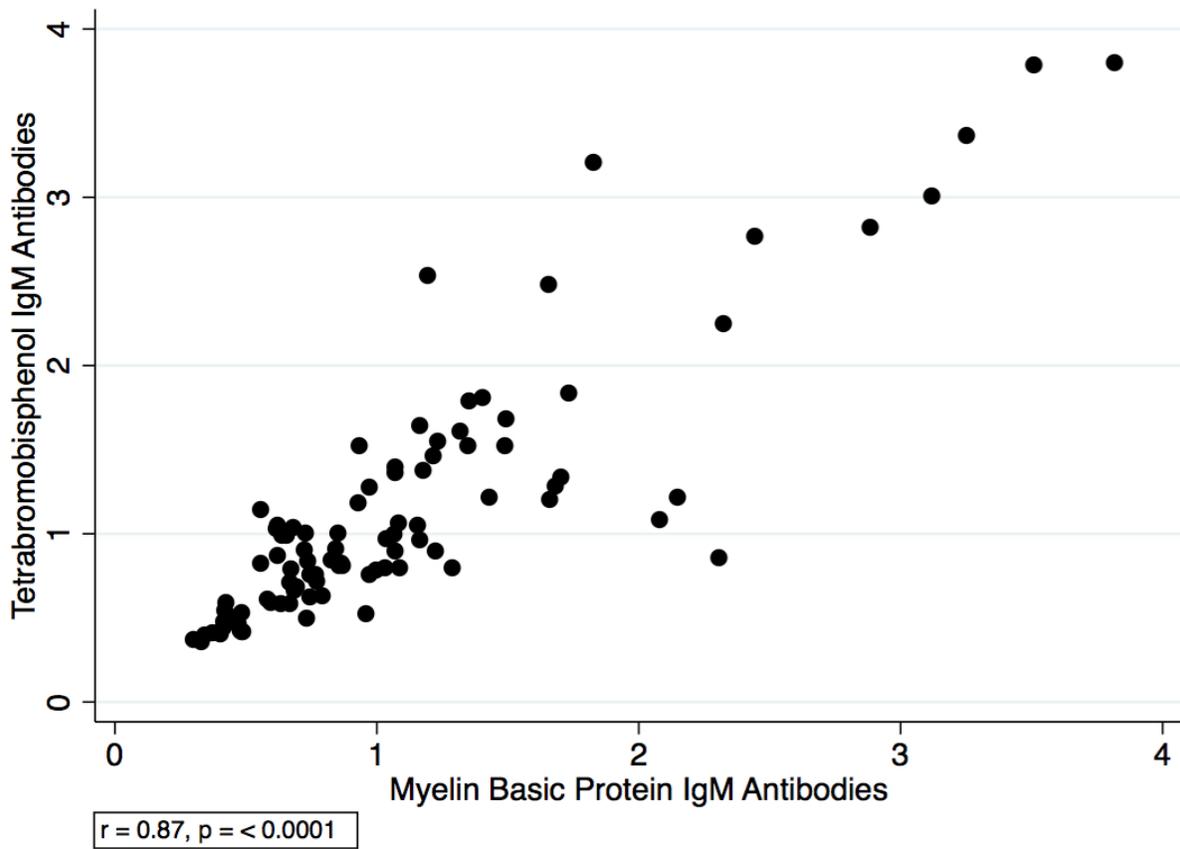
Figure 8 - Linear Relationships with IgG TBBPA and AQP4 IgG

The two-way scatter plot evaluation for TBBPA and AQP4 for IgM immunological reactive demonstrates a positive monotonic relationship (Figure 9). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.88. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.84. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.67. The risk ratio for TBBPA IgM antibody production as an exposure variable and AQP4 IgM antibodies as an outcome variable was 75.6 (CI: 10.66, 536.27) and statistically significant (p-value <0.0001).

Figure 9 -Linear Relationships with IgM TBBPA and AQP4 IgM
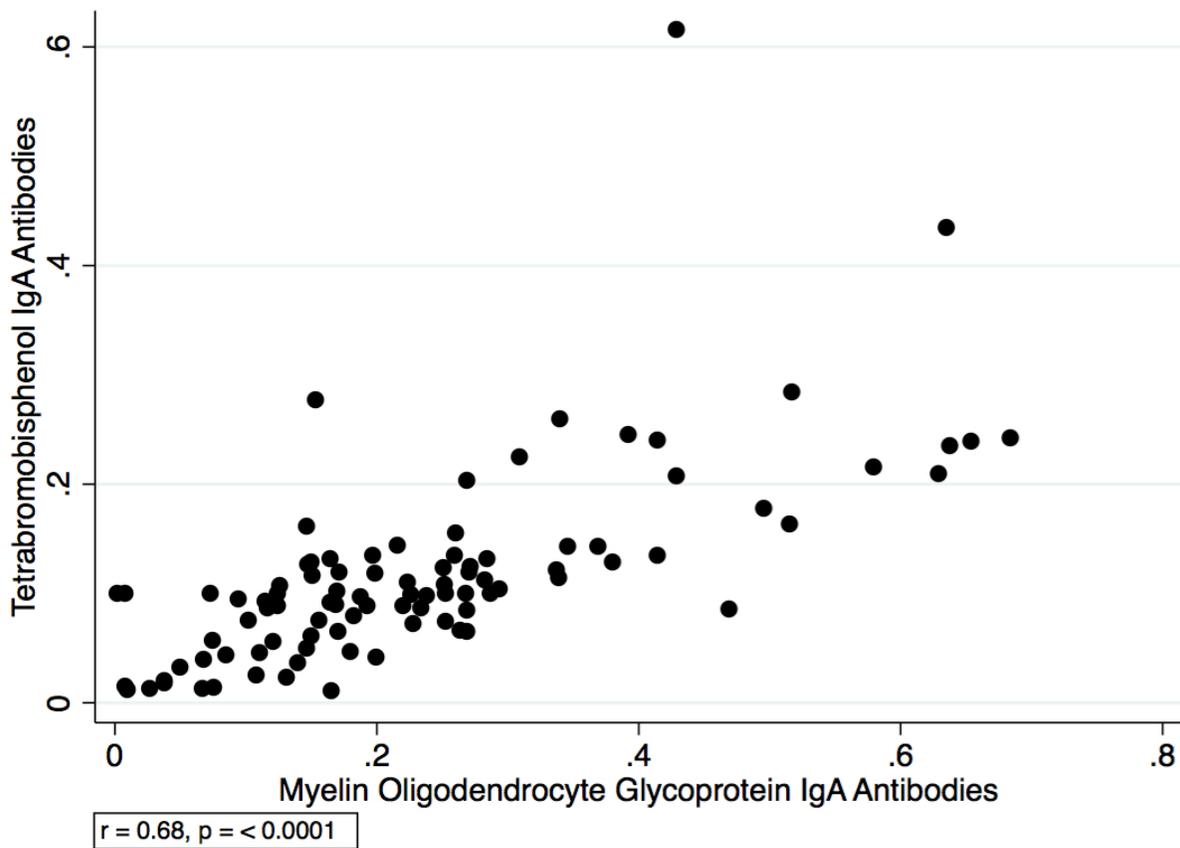
Relationships between Tetrabromobisphenol-A and Alpha-Synuclein

The two-way scatter plot evaluation for TBBPA and Alpha-Synuclein for IgA immunological reactive demonstrates a positive monotonic relationship (Figure 10). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.72. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.72. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.53. The risk ratio for TBBPA IgA antibody production as an exposure variable and Alpha-Synuclein IgA antibodies as an outcome variable was 18.57 (CI: 7.07, 48.80) and statistically significant (p-value <0.0001).

Figure 10 - Linear Relationships between TBBPA IgA and Alpha-Synuclein IgA
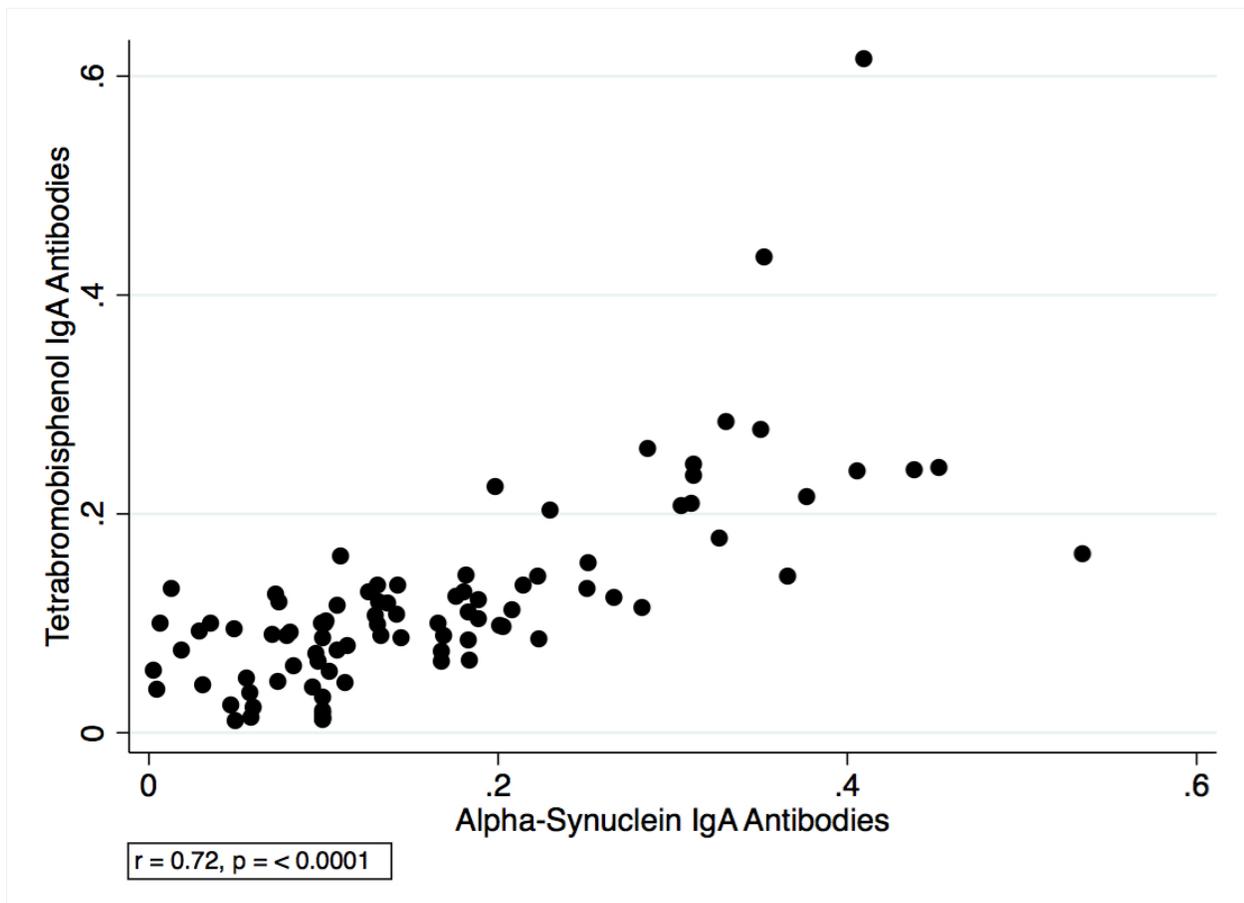
The two-way scatter plot evaluation for TBBPA and Alpha-Synuclein for IgG immunological reactive demonstrates a positive monotonic relationship (Figure 11). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.76. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a moderate correlation of 0.63. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.47. The risk ratio for TBBPA IgG antibody production as an exposure variable and Alpha-Synuclein IgG antibodies as an outcome variable was 18.22 (CI: 5.60, 59.33) and statistically significant (p-value <0.0001).
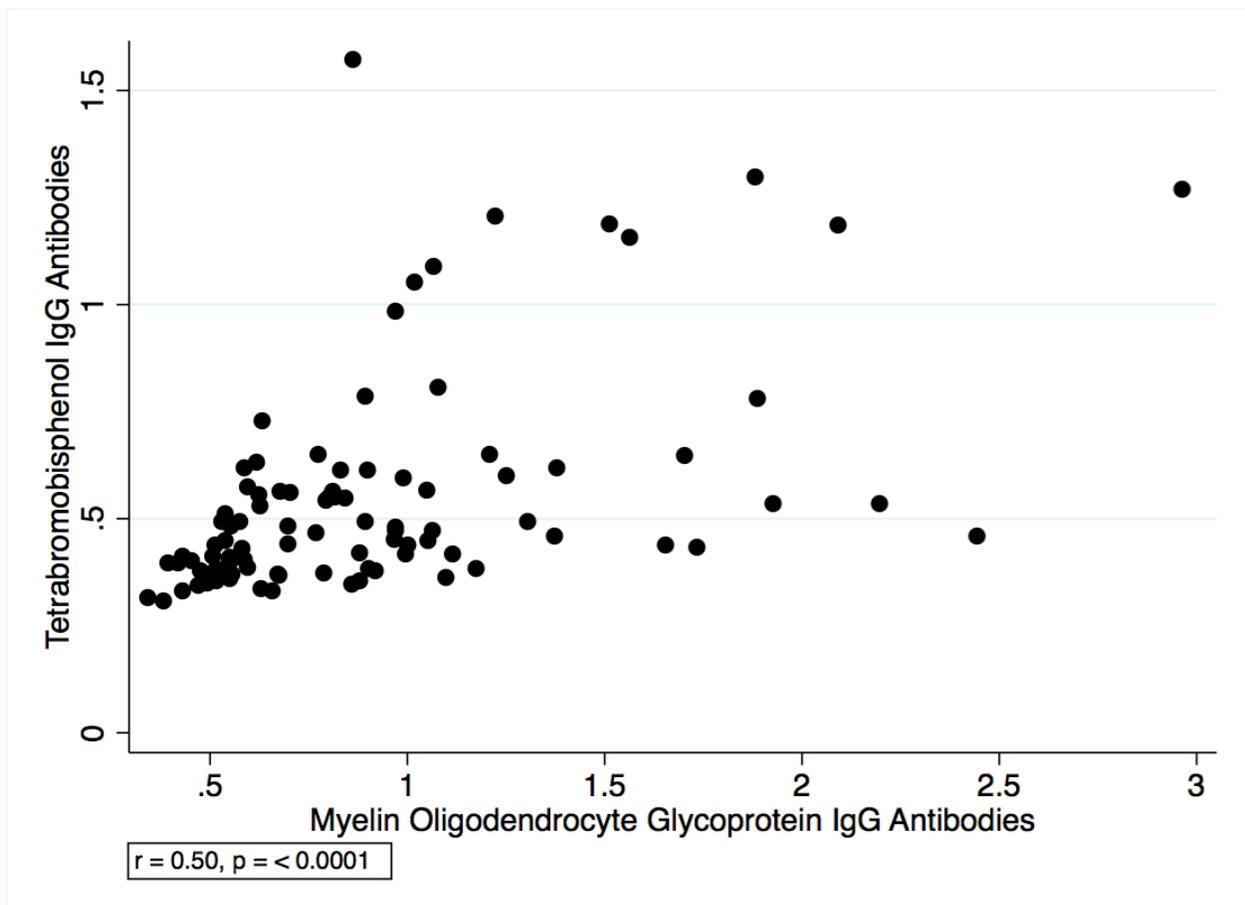
Figure 11 - Linear Relationships with IgG TBBPA and Alpha-Synuclein IgG
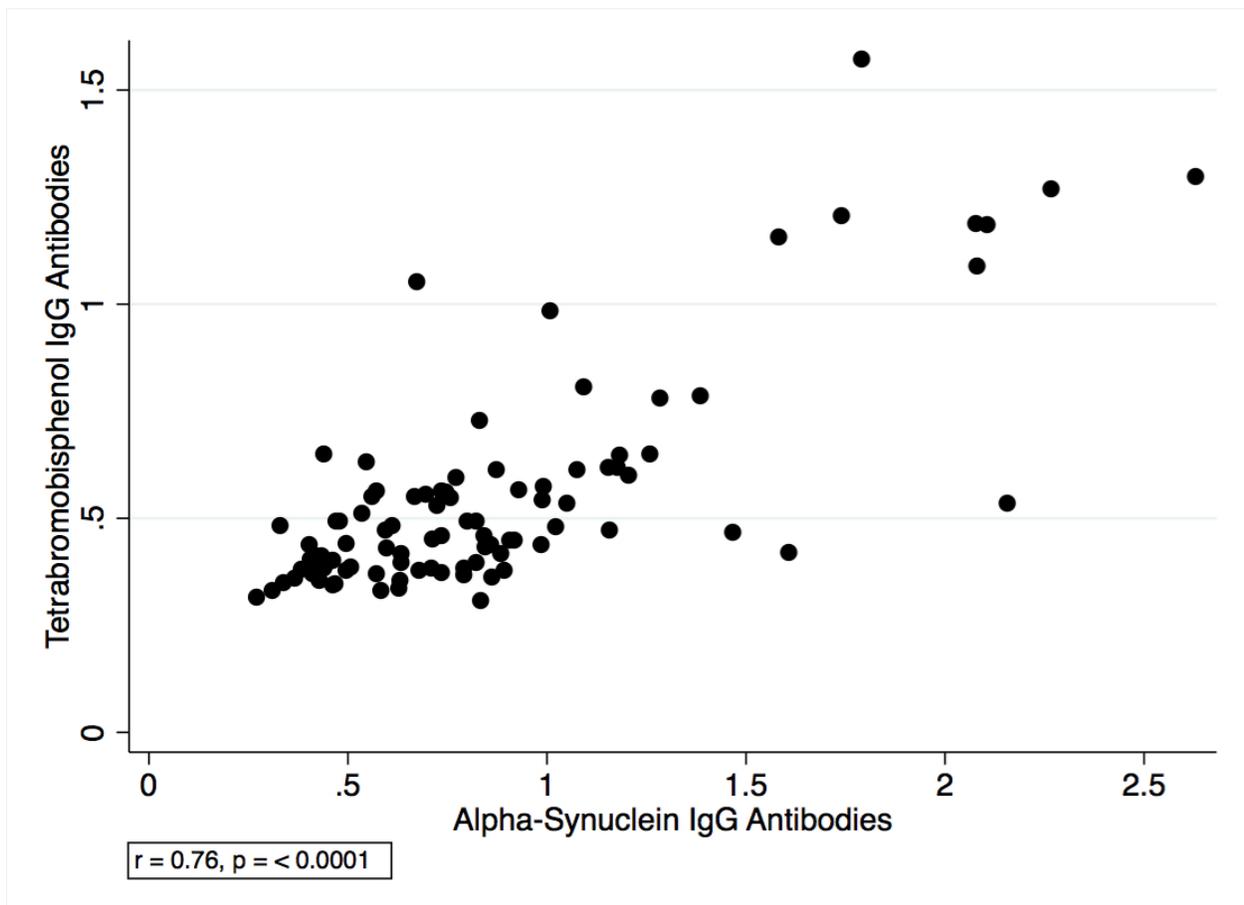
The two-way scatter plot evaluation for TBBPA and Alpha-Synuclein for IgM immunological reactive demonstrates a positive monotonic relationship (Figure 12). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.85. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.85. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.66. The risk ratio for TBBPA IgM antibody production as an exposure variable and Alpha-Synuclein IgM antibodies as an outcome variable was 75.6 (CI: 10.66, 536.27) and statistically significant (p-value <0.0001).

Figure 12 - Linear Relationships with IgM TBBPA and Alpha-Synuclein IgM

r = 0.85, p = < 0.0001
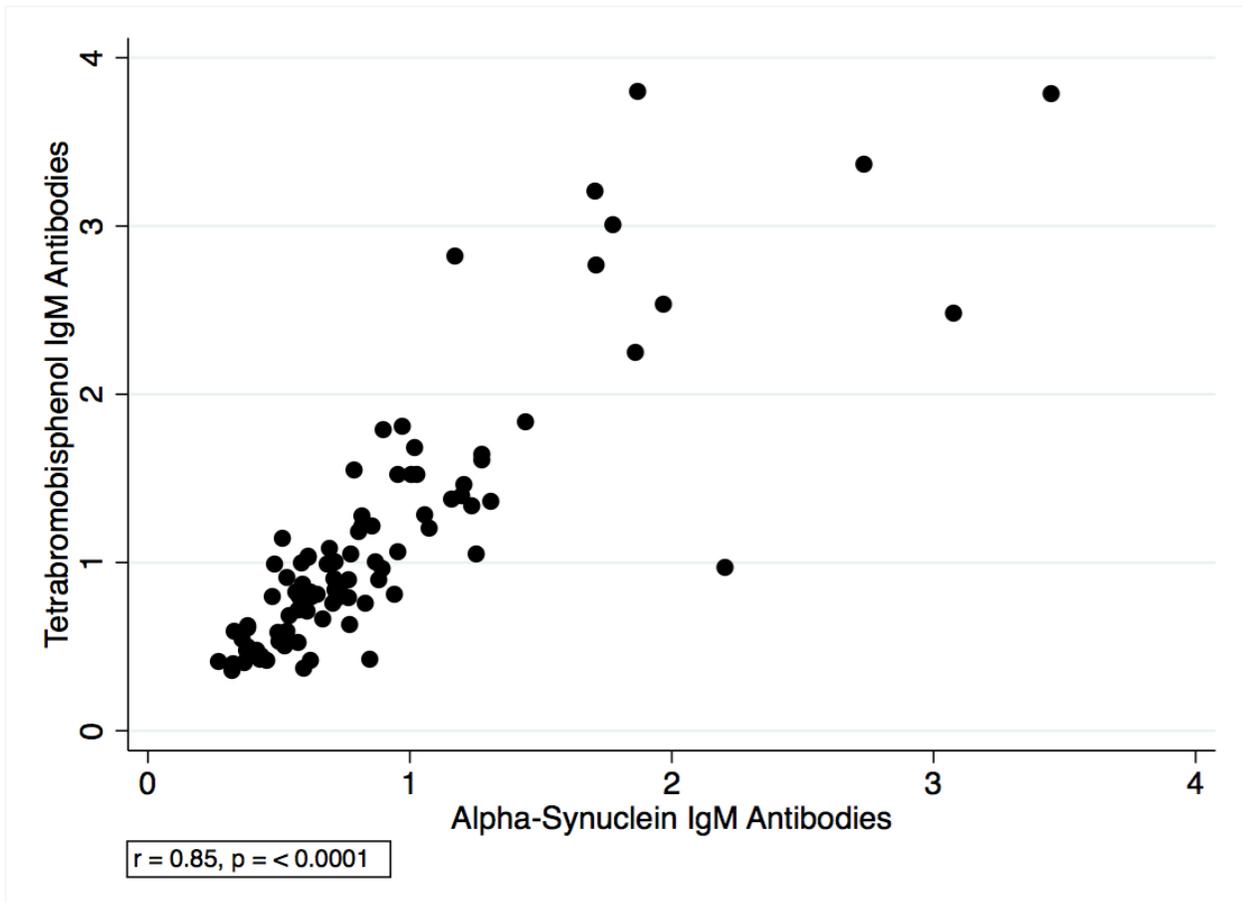
Relationships between Tetrabromobisphenol-A and S100B

The two-way scatter plot evaluation for TBBPA and S100B for IgA immunological reactive demonstrates a positive monotonic relationship (Figure 13). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a moderate correlation of 0.61. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a moderate correlation of 0.63. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.46. The risk ratio for TBBPA IgA antibody production as an exposure variable and S100BIgA antibodies as an outcome variable was 20.96 (CI: 6.68, 65.73) and statistically significant (p-value <0.0001).
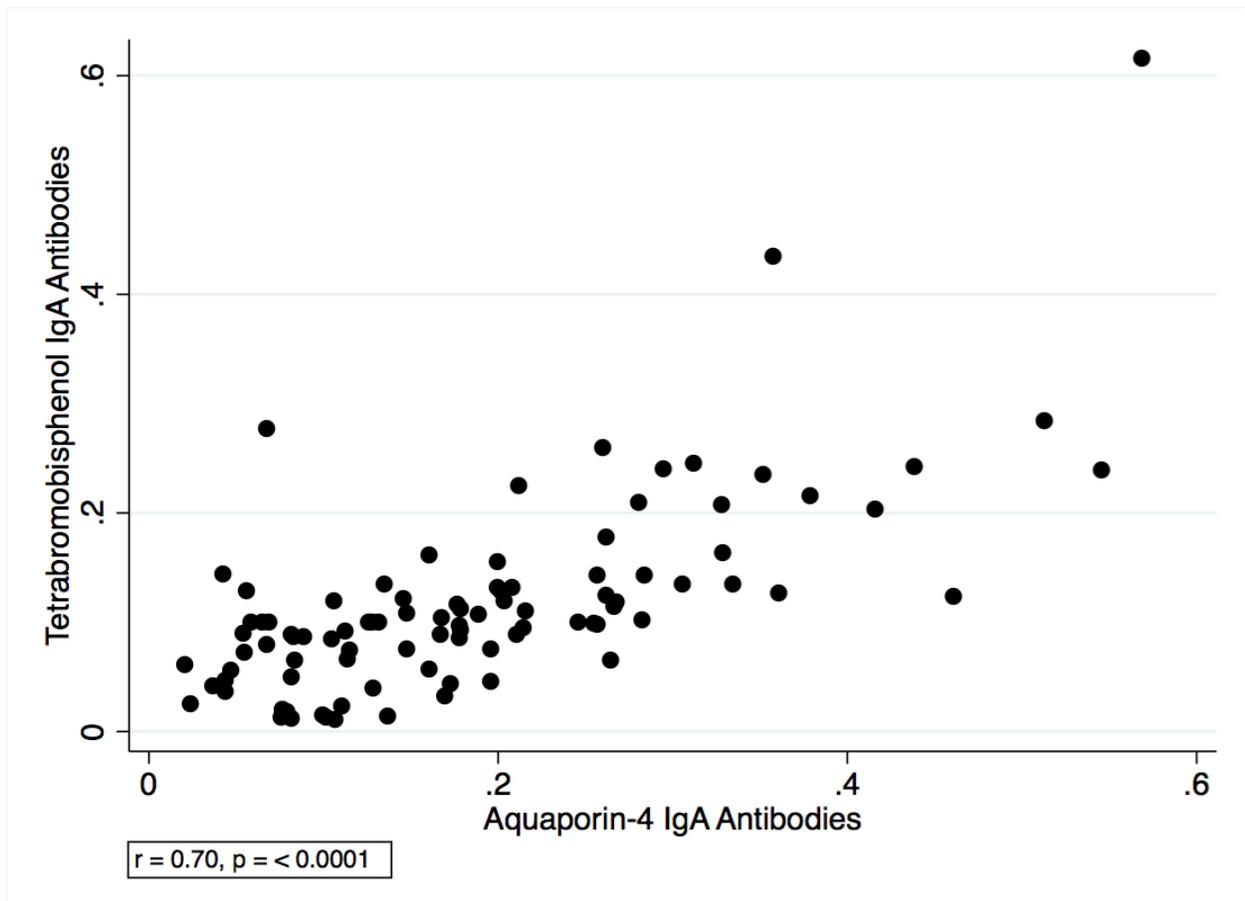
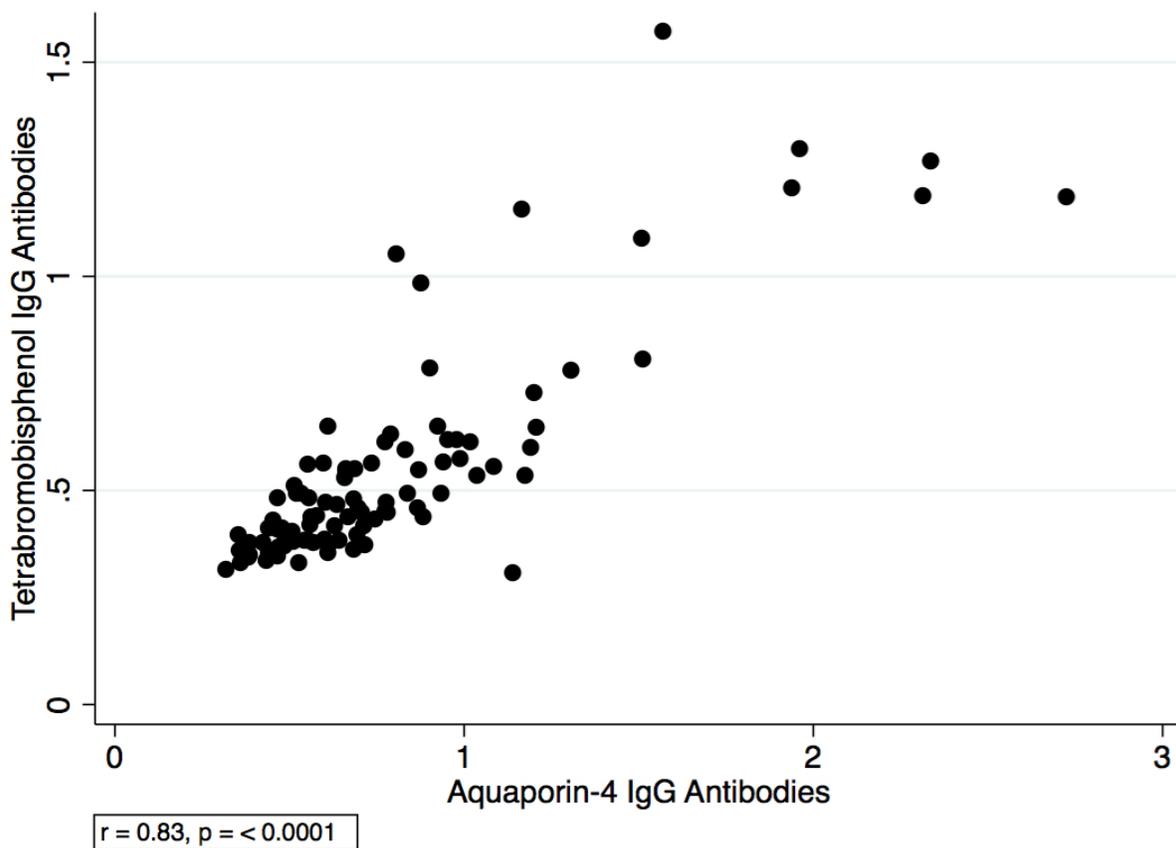**Figure 13 - Linear Relationships between TBBPA IgA and S100B IgA**
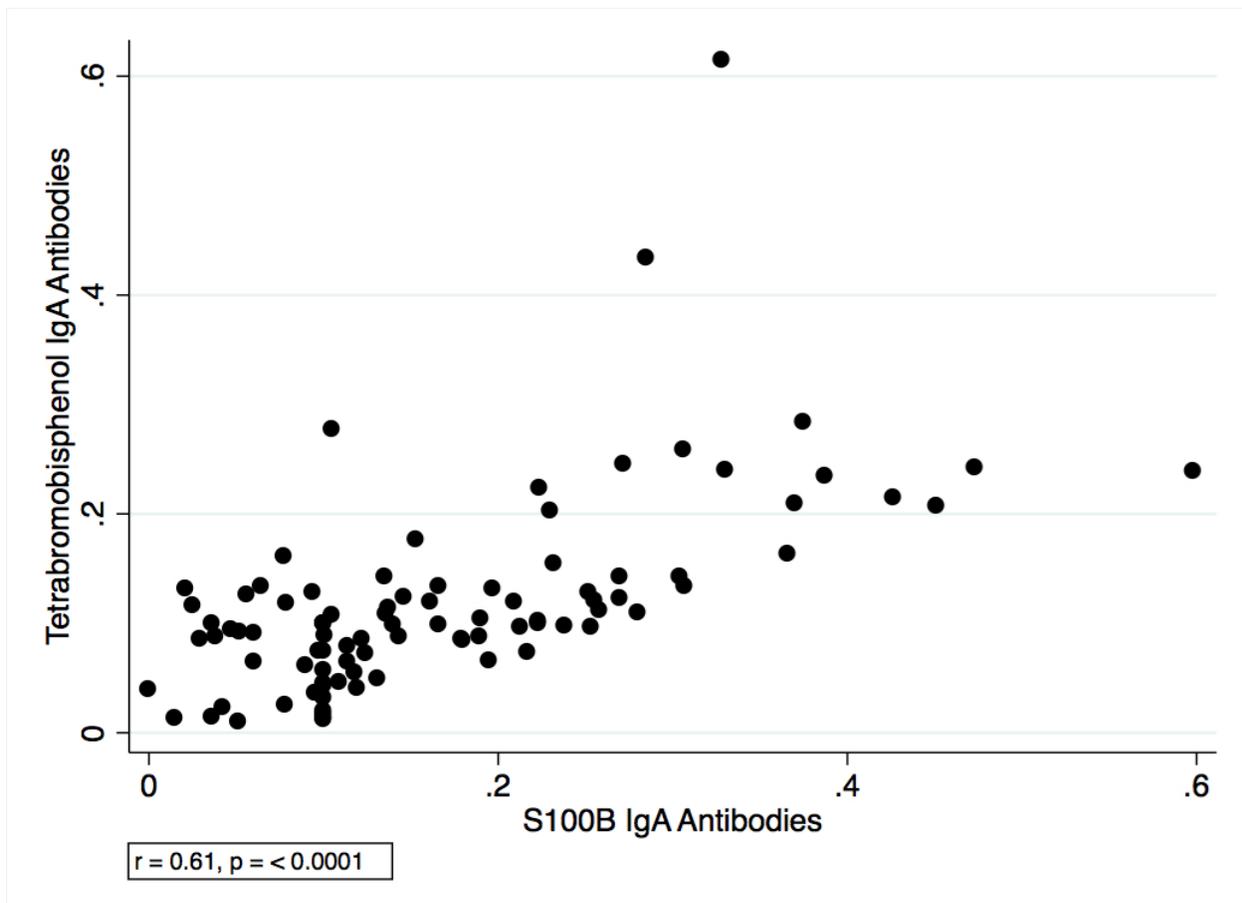
The two-way scatter plot evaluation for TBBPA and S100B for IgG immunological reactive demonstrates a positive monotonic relationship (Figure 14). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a mild correlation of 0.35. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a moderate correlation of 0.53. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a mild correlation of 0.39. The risk ratio for TBBPA IgG antibody production as an exposure variable and S100B IgG antibodies as an outcome variable was 7.5 (CI: 2.60, 21.96) and statistically significant (p-value <0.0001).

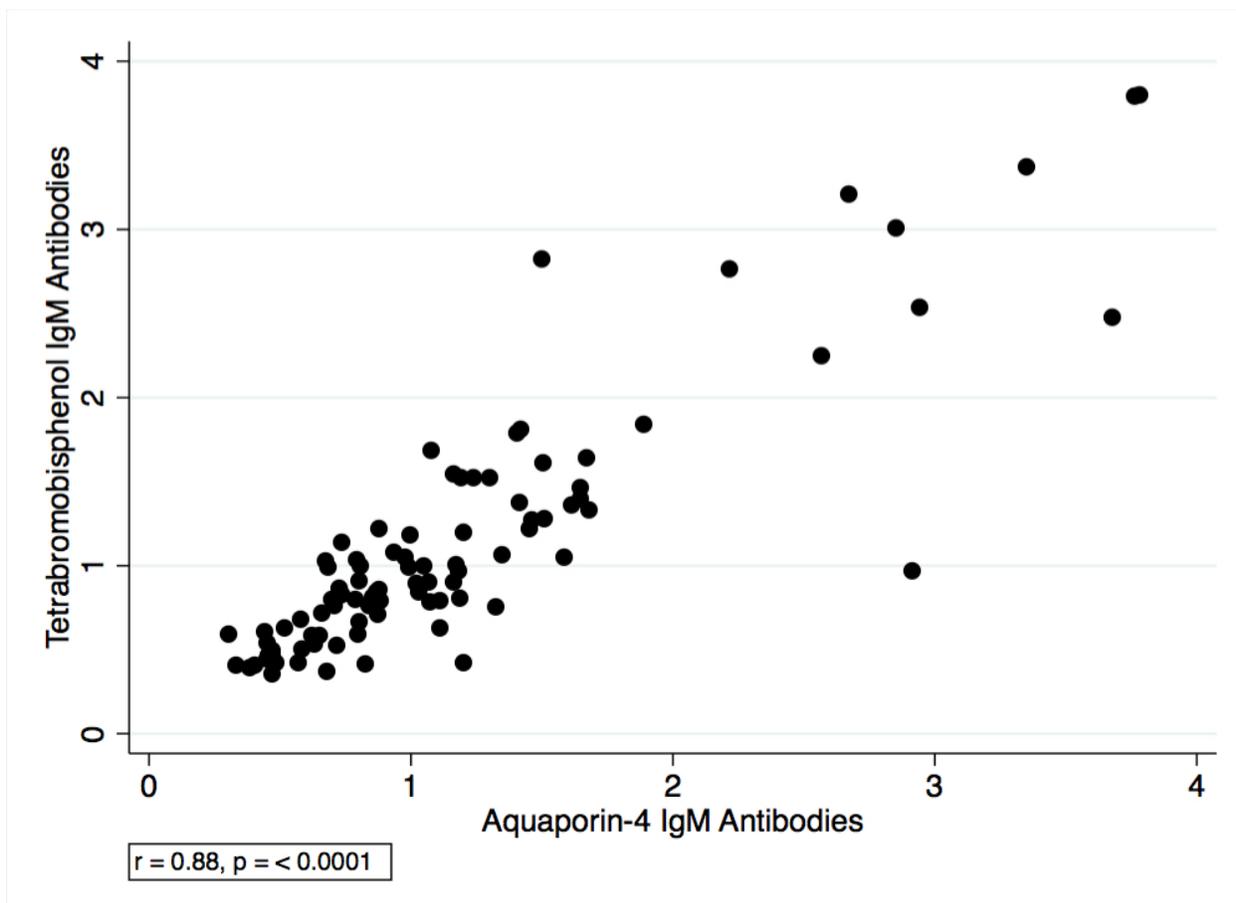**Figure 14 - Linear Relationships with IgG TBBPA and S100B IgG**
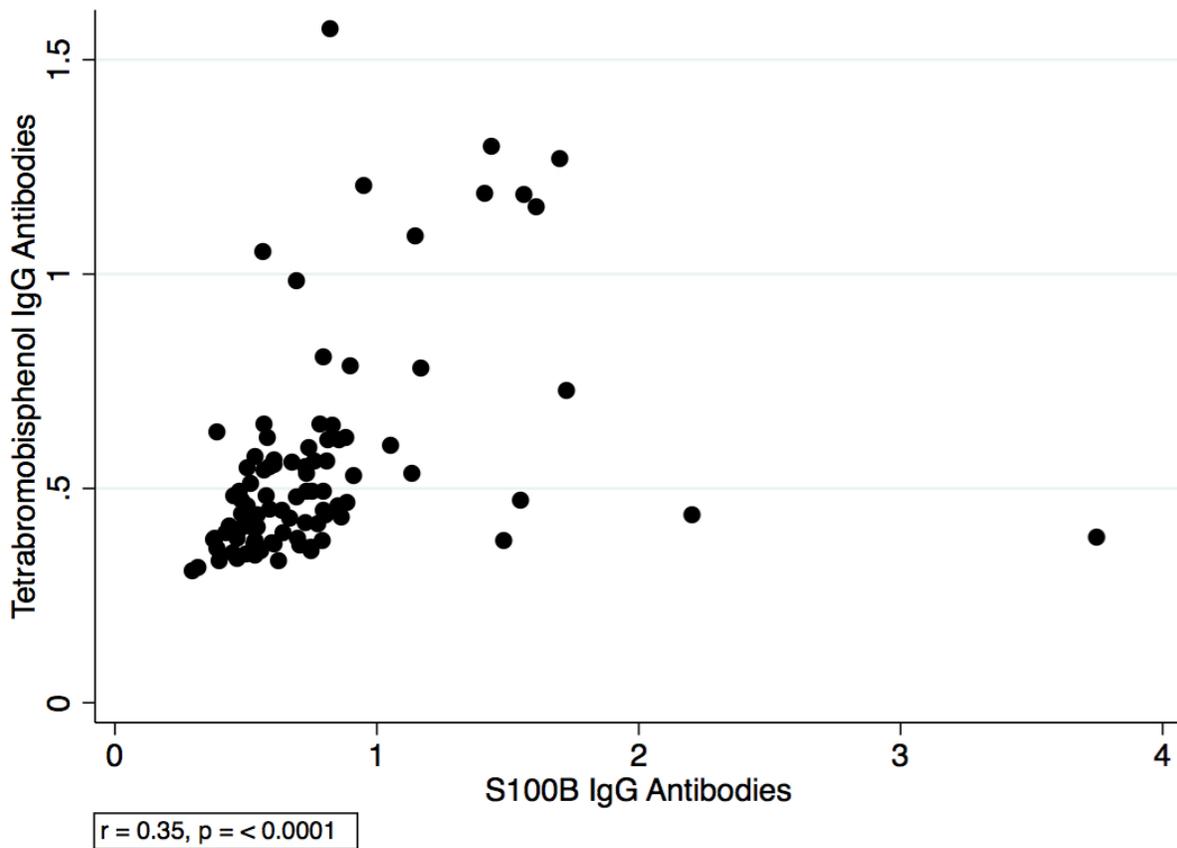
The two-way scatter plot evaluation for TBBPA and S100B for IgM immunological reactive demonstrates a positive monotonic relationship (Figure 15). Statistical analysis using Pearson's correlation coefficient was statistically significant (p-value < 0.0001) with a substantial correlation of 0.92. Spearman's rank correlation was statistically significant (p-value < 0.0001) with a substantial correlation of 0.82. Kendall tau rank correlation was statistically significant (p-value <0.0001) with a moderate correlation of 0.65. The risk ratio for TBBPA IgM antibody production as an exposure variable and S100B IgM antibodies as an outcome variable was 75.6 (CI: 10.66, 536.27) and statistically significant (p-value <0.0001).

Figure 15 - Linear Relationships with IgM TBBPA and S100B IgM
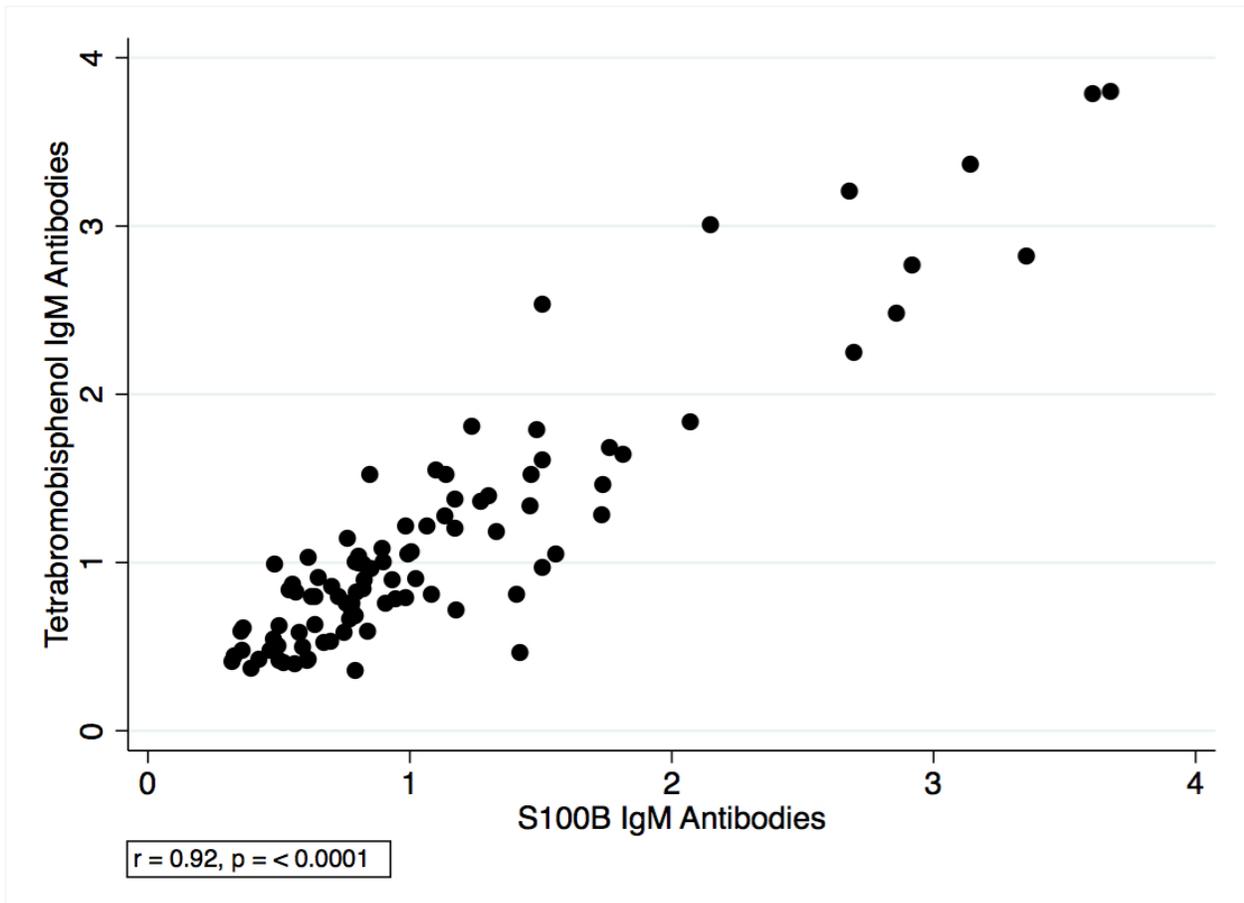
Summary of Results

There is a clear positive linear relationship that is statistically significant (p-value <0.0001) between TBBPA bound to human protein antibodies and myelin basic protein, myelin associated glycoprotein, aquaporin, and alpha-synuclein with all three forms of immunoglobulins: IgA, IgG, and IgM. The degree of correlation ranges from moderate to significant (Table 3). The highest degree of association is with IgM. These correlations coefficients ranged from 0.85–0.92.

Table 3 - Summary of Results Risk Ratio and Correlation Coefficients			
Exposure Antibody	**Disease Antibody**	**Risk Ratio of lowest tail of the 95% confidence interval**	**r**
TBBPA IgA	Myelin basic protein IgA	3.90	0.68
TBBPA IgG	Myelin basic protein IgG	5.55	0.80
TBBPA IgM	Myelin basic protein IgM	7.01	0.87
TBBPA IgA	Myelin oligodendrocytic glycoprotein IgA	5.20	0.68
TBBPA IgG	Myelin oligodendrocytic glycoprotein IgG	2.06	0.50
TBBPA IgM	Myelin oligodendrocytic glycoprotein IgM	6.14	0.88
TBBPA IgA	Aquaporin-4 IgA	4.04	0.70
TBBPA IgG	Aquaporin-4 IgG	8.32	0.83
TBBPA IgM	Aquaporin-4 IgM	10.65	0.88
TBBPA IgA	Alpha-synuclein IgA	7.06	0.72
TBBPA IgG	Alpha-synuclein IgG	5.6	0.76
TBBPA IgM	Alpha-synuclein IgM	10.66	0.85
TBBPA IgA	S100B IgA	6.69	0.61
TBBPA IgG	S100B IgG	2.6	0.35
TBBPA IgM	S100B IgM	10.67	0.92

All data is statistically significant (p-value <.001 - <.0001)

There is a significant risk for the development of neurological antibodies with subjects that exhibited antibodies to TBBPA bound to human albumin. The study was powered for

correlation analysis (Appendix E) and not for a risk ratio analysis; thus, the data represents wide confidence intervals (Table 4). However, despite these wide confidence intervals, the risk ratio analysis was statistically significant (p-values <0.001 – 0.0001) and the lowest tail of the confidence interval identified a major risk. These risks ranged from a two-fold to a ten-fold increase in risk even when using the lowest tail of the 95% confidence interval. In summary, there is a significant linear association and risk in human subjects that exhibit antibodies to TBBPA with antibodies to a diverse list of neurological autoimmune target protein sites that include myelin basic protein, myelin-associated glycoprotein, aquaporin, and alpha-synuclein for IgA (Figure 16), IgG (Figure 17), and IgM (Figure 18). An analysis of correlations between IgA, IgG, and IgM immunoglobulins identified that IgM antibodies have the most significant reactions (Tables 5–7)

Table 4 – Results of Risk Ratios				
Exposure Antibody	**Disease Antibody**	**Risk Ratio**	**95% CI**	**P value**
TBBPA IgA	Myelin basic protein IgA	13.33	3.90, 45.50	<0.0001
TBBPA IgG	Myelin basic protein IgG	13.58	5.55, 33.21	<0.0001
TBBPA IgM	Myelin basic protein IgM	22.4	7.01, 70.10	<0.0001
TBBPA IgA	Myelin oligodendrocytic glycoprotein IgA	14.29	5.20, 39.30	<0.0001
TBBPA IgG	Myelin oligodendrocytic glycoprotein IgG	5.40	2.06, 14.08	0.0005
TBBPA IgM	Myelin oligodendrocytic glycoprotein IgM	16.8	6.15, 45.91	<0.0001
TBBPA IgA	Aquaporin-4 IgA	10.29	4.04, 26.18	<0.0001
TBBPA IgG	Aquaporin-4 IgG	60.36	8.32, 437.83	<0.0001
TBBPA IgM	Aquaporin-4 IgM	75.6	10.66, 536.27	<0.0001
TBBPA IgA	Alpha-synuclein IgA	18.57	7.07, 48.80	<0.0001
TBBPA IgG	Alpha-synuclein IgG	18.22	5.60, 59.33	<0.0001
TBBPA IgM	Alpha-synuclein IgM	75.6	10.66, 536.27	<0.0001
TBBPA IgA	S100B IgA	20.95	6.68, 65.73	<0.0001
TBBPA IgG	S100B IgG	7.5	2.60, 21.96	0.0001
TBBPA IgM	S100B IgM	75.6	10.66, 536.27	<0.0001

Figure 16 – Scatter Matrix of IgA Correlations

IgA Pairwise Relationships
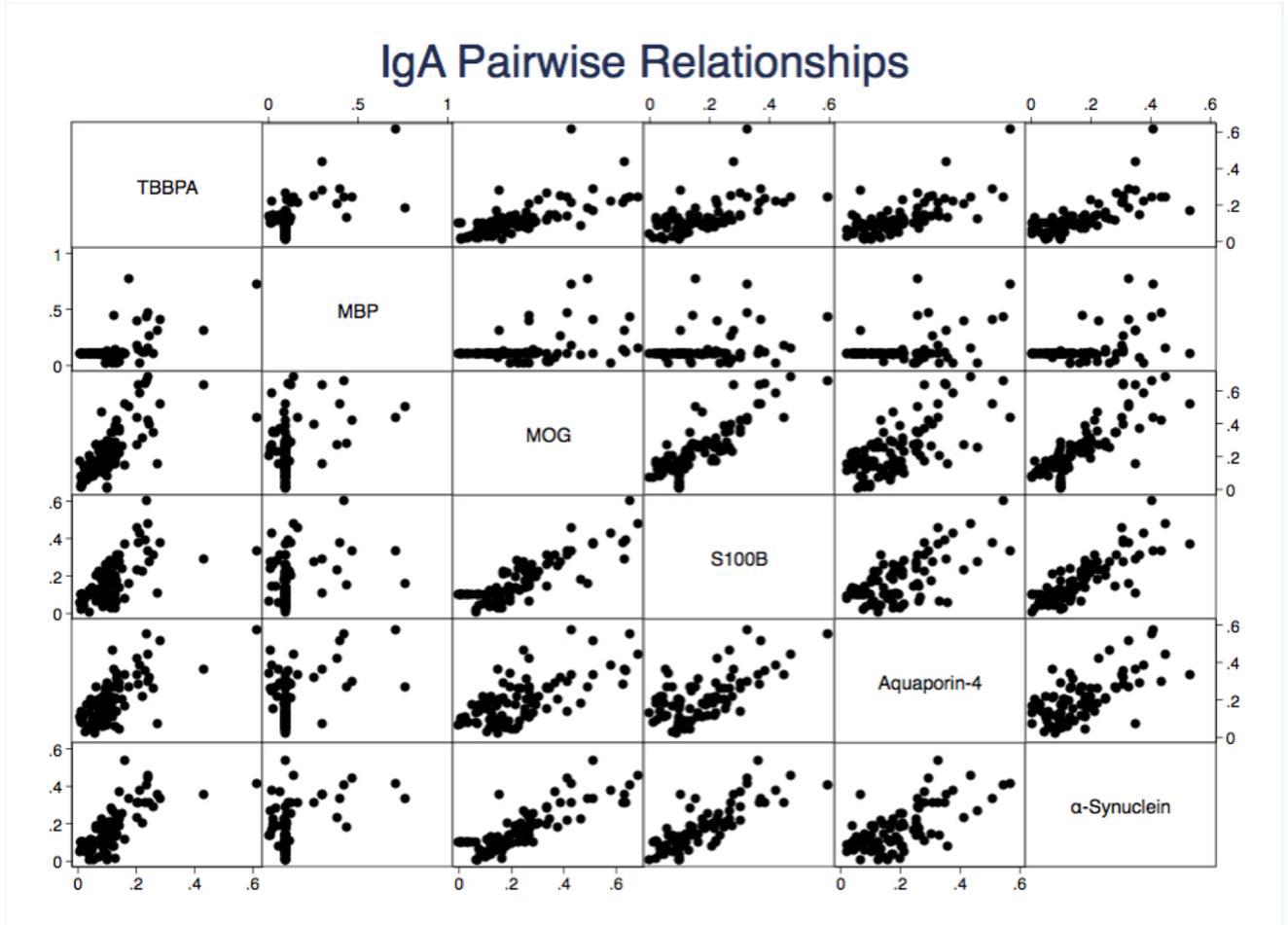
Figure 17 - Scatter Matrix Graph IgG Correlations

IgG Pairwise Relationships
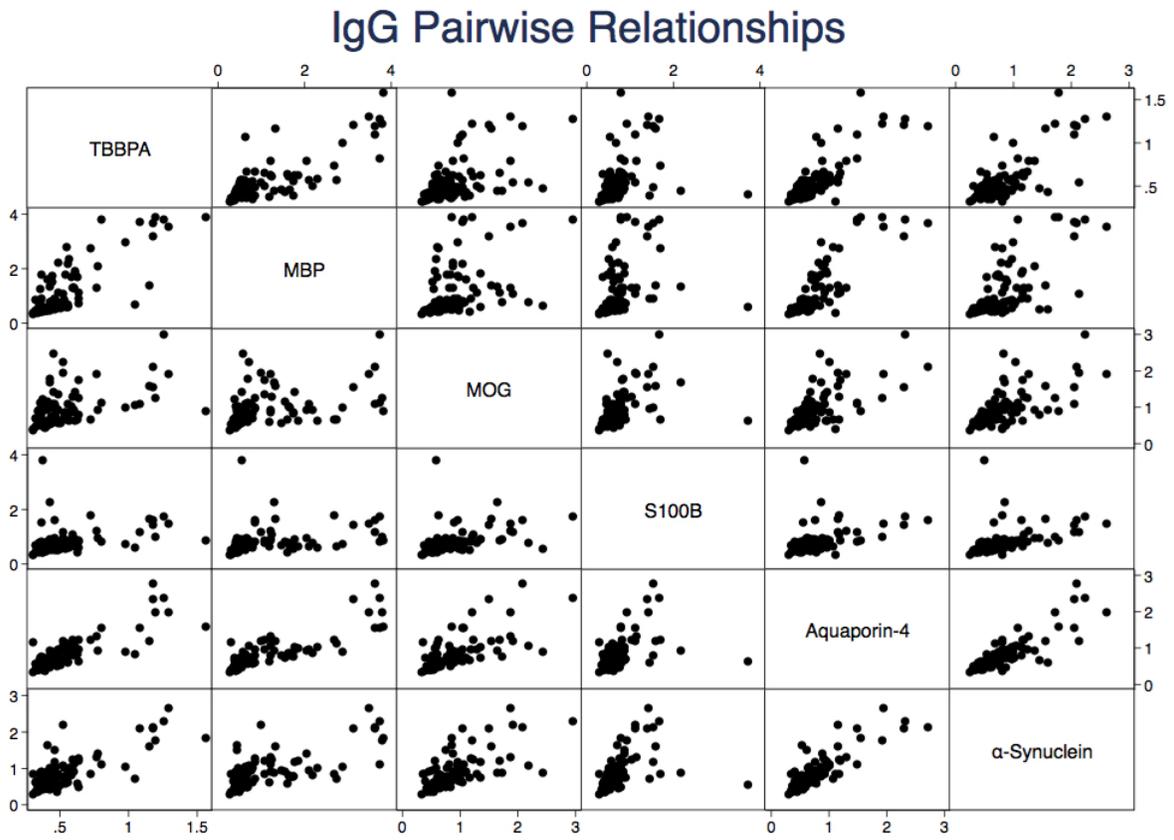
Figure 18 - Scatter Matrix Graph IgM Correlations
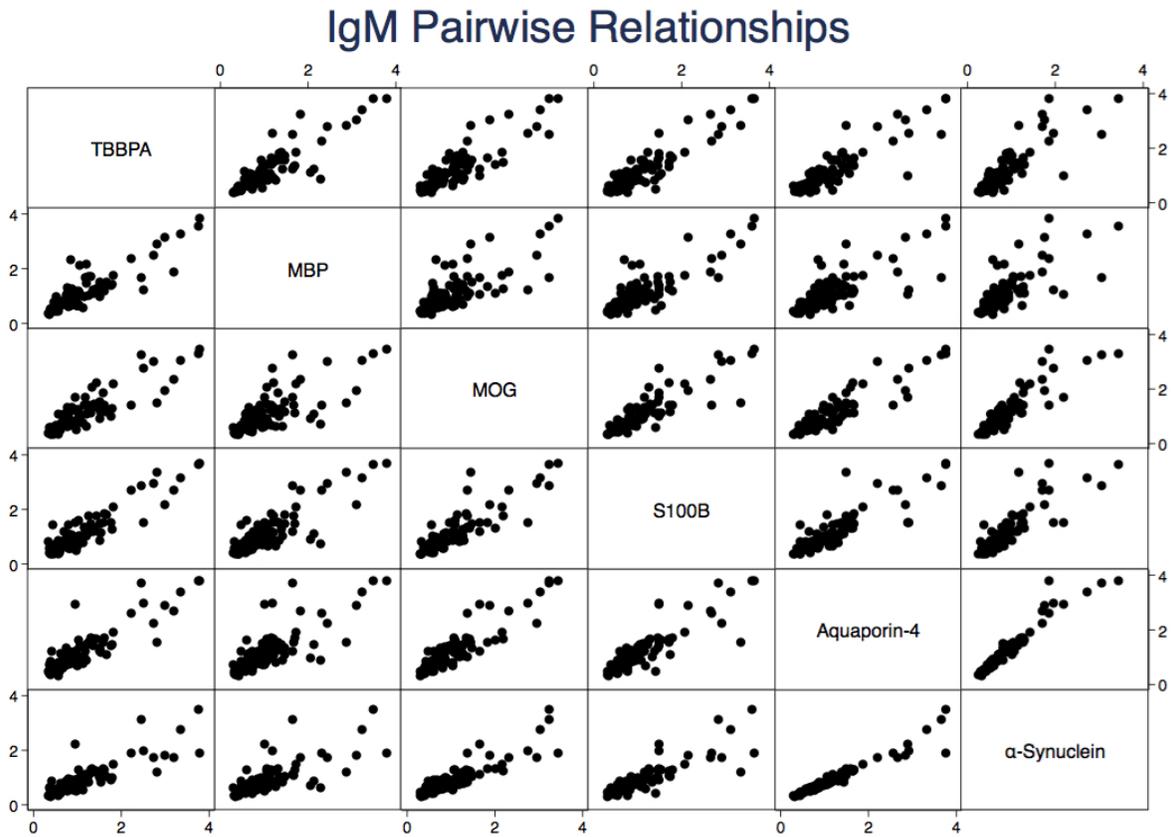
Table 5 - Summary of IgA Risk Ratios and Correlation Coefficients

Exposure Antibody	Disease Antibody	Risk Ratio of lowest tail of the 95% confidence interval	r
TBBPA IgA	Myelin basic protein IgA	3.90	0.68
TBBPA IgA	Myelin oligodendrocytic glycoprotein IgA	5.20	0.68
TBBPA IgA	Aquaporin-4 IgA	4.04	0.70
TBBPA IgA	Alpha-synuclein IgA	7.06	0.72
TBBPA IgA	S100B IgA	6.69	0.61

All data is statistically significant (p-value <.0001)

Table 6 - Summary of IgG Risk Ratios and Correlation Coefficients

Exposure Antibody	Disease Antibody	Risk Ratio of lowest tail of the 95% confidence interval	r
TBBPA IgG	Myelin basic protein IgG	5.55	0.80
TBBPA IgG	Myelin oligodendrocytic glycoprotein IgG	2.06	0.50
TBBPA IgG	Aquaporin-4 IgG	8.32	0.83
TBBPA IgG	Alpha-synuclein IgG	5.6	0.76
TBBPA IgG	S100B IgG	2.6	0.35

All data is statistically significant (p-value <.0001)

Table 7 - Summary of IgM Risk Ratios and Correlation Coefficients

Exposure Antibody	Disease Antibody	Risk Ratio of lowest tail of the 95% confidence interval	r
TBBPA IgM	Myelin basic protein IgM	7.01	0.87
TBBPA IgM	Myelin oligodendrocytic glycoprotein IgM	6.14	0.88
TBBPA IgM	Aquaporin-4 IgM	10.65	0.88
TBBPA IgM	Alpha-synuclein IgM	10.66	0.85
TBBPA IgM	S100B IgM	10.67	0.92

All data is statistically significant (p-value <.0001)

Chapter 5: Discussion

In this chapter, the results of the research study will be interpreted and examined, and inferences will be drawn from the data. The findings of the study will be stated, and a discussion on whether the outcomes of the study support the proposed hypotheses will be presented. The implications of the data and its contribution to the field of neurotoxicology, neuroimmunology, and environmental medicine will be evaluated. Implications for future research constructed on the output of the discovered data will be proposed. Scientific, clinical, and legislative recommendations will be formulated from the study outcomes and will be presented in this section. This chapter will conclude with an acknowledgement of the study's limitations and a summary of the entire research study.

Discussion

Tetrabromobisphenol-A (TBBPA) is the most widely used flame retardant. Flame retardants are generally sprayed on furniture, mattress beds, children's pyjamas, car seats, upholstery, carpets, and rugs in the United States of America. TBBPA may play a role in the epidemic of autoimmune disease. The goal of this research was to investigate whether any correlation or risk exists between the immunological reactivity to TBBPA, a key chemical used in most flame retardants, and the neurological autoimmune target sites that are associated with neurological autoimmune diseases with a diverse and specific list of antibodies with human serum samples.

Ten fundamental research questions were investigated to evaluate the hypothesis that chemical reactivity to TBBPA can promote neurological autoimmunity. In this study, TBBPA immunological reactivity was used to investigate any association with neurological autoimmunity by looking at antibodies associated with five separate neurological target sites that

included myelin basic protein, myelin oligodendrocyte protein, S100B protein, alpha-synuclein, and aquaporin-4. These antibodies reflect the autoimmune target sites associated with multiple sclerosis, autoimmune demyelinating disorders, Parkinson's disease, neuromyelitis optica, and inflammatory conditions of the brain and the peripheral nervous system. The outcome of each research question will be discussed in this section starting with research question #1.

Research Question # 1: Can TBBPA bind to human albumin and induce immunological chemical reactivity as identified with TBBPA bound to albumin specific antibodies in human serum? Chemical molecules known as haptens can bind directly to self-proteins, thereby creating new antigens in the immune system known as hapten-protein-adducts. Haptens can also indirectly bind to proteins after hepatic or extra-hepatic biotransformation from pro-haptens to haptens, generating hapten-protein-adducts. These hapten-protein-adducts lead to neoantigen formations, resulting in systemic T-cell and antibody immune responses (Kubicka-Muranyi *et al.*, 2013). The results of this study found clear outcomes that TBBPA can bind to human albumin and develop neoantigens that can be objectively captured by quantifying the TBBP bound to albumin antibodies with enzyme-linked immunosorbent assay (ELISA) methodology. Additionally, the spectrographic analysis of the conjugate was undertaken until there was an increase in absorption from 230 to 260nM. This was performed to confirm that haptenic chemicals were covalently linked to the protein carrier during the development of the ELISA plates. Furthermore, the development of specific antibodies was captured and quantified through optical density analysis.

This finding provides a novel understanding of how fire-retardant chemicals may impact human health. It is understood that that immunological antibody response can occur only with proteins and not with chemical adjuncts alone. The novel concept that the commonly used

chemicals in our day-to-day lives, which were investigated in this study, all have the ability to bind to human proteins and change the primary structure of the protein provides further insight into how these chemicals may induce inflammation and autoimmune reactivity. The role that environmental xenobiotics and toxic agents play in the development of autoimmune diseases has been found with various mechanisms, such as the mechanisms of oxidative stress and mechanisms that induce immunological deletion, modification of gene expression, and immunological dysregulation (Rao & Richardson, 1999). The primary focus in the past was on known toxic agents, such as heavy metals, poisons, pesticides, and volatile compounds, that impact oxidative stress pathways (Lehmann, 2017). In this study, a specific investigation of how commonly encountered TBBPA may act as a trigger in the normal immunological response with the development of hapten-protein adducts independent of previous models of disease development was conducted.

Research Question #2: Is there a correlation between TBBPA immunological reactivity with neurological autoimmunity to the nerve sheath proteins (myelin basic protein and myelin oligodendrocyte protein)? This study found a statistically significant correlation between TBBPA immunological reactivity and neurological autoimmunity to nerve sheath proteins, myelin basic protein (MBP), and myelin oligodendrocyte protein (MOG) for IgA, IgG, and IgM. Myelin is a phospholipid membrane that surrounds the axons of nerve cells to allow for the increased speed of nerve propagation in both the central and peripheral nervous system. MBP isoforms are essential for the formation of multi-lamellar sheaths of myelin that surround axons in combination with MOG (Vassall *et al*., 2015).

Antibodies to MBP and MOG are associated with demyelinating diseases of the nervous system that include neuropathy, multiple sclerosis, ataxia, transverse myelitis, and other

inflammatory and autoimmune diseases of the nervous system (Peschl *et al*., 2017). Injury to the myelin sheath may lead to diverse clinical symptoms, such as weakness of muscles, loss of sensation, balance disorders, double vision, nerve pain, loss of bowel control, and involuntary urination. The clinical presentations from demyelination depend on the specific regions of the nervous system that have been damaged (Kinzel *et al*., 2017). MOG and MBP antibodies have been found to be an accurate biomarker for predicting multiple sclerosis progression from early patterns to progressed patterns of the disease (Berger *et al*., 2003). These antibodies have also been found to be predictive for determining the relapse in patients diagnosed with relapsing-remitting multiple sclerosis (Rauer *et al*., 2006).

In addition to the inflammatory demyelinating diseases of the nervous system, such as neuropathy and multiple sclerosis, some recent publications have found that the antibodies to MOG and MBP may represent a subtle inflammatory response in the brain and that they are associated with the impairment of general functions of the brain, such as focus, attention, concentration, and memory. In a study of rheumatoid arthritis patients, their levels of MOG and MBP antibodies and their association to cognitive function were compared. The study found that the levels of MOG and MBP antibodies were negatively associated with the delayed verbal recall, Stroop Color-Word, and N-Back Total scores and positively with Trail Making Test B. The study concluded that elevation of these antibodies' levels is associated with an impaired cognitive function (Baptista *et al*., 2017). Another study examined the MOG and MBP antibody levels in the serum of 26 patients diagnosed with Alzheimer's disease (AD) and 26 healthy controls. The study found that these antibodies were significantly higher in AD patients and suggested that the antibodies to myelin could serve as an early biomarker of AD in human subjects before the onset of dementia (Papuc *et al*., 2015).

The role that chemicals play in the destruction of myelin is an area of investigation in the field of neuroimmunology. The exposure to environmental toxins and heavy metals are a known cause of peripheral nerve diseases associated with the destruction of myelin (Katona *et al.*, 2017). In one study, 50 subjects exposed to pesticides and 25 subjects not exposed to pesticides were evaluated for the development of neurological symptoms and the presence of autoantibodies against neural proteins that included MOG and MBP. The study found that the subjects who were exposed to pesticides had developed neurological signs and symptoms of neural injury, and there was a 7.67-fold and 5.89-fold increase in antibody levels to MBP and MOG, respectively, in subjects who were chronically exposed to chemicals compared to subjects who were not. The correlation between MOG and MBP antibodies with the chemical bisphenol-A found in plastic products and its target protein, protein disulphide isomerase, has been reported in the literature (Kharrazian & Vojdani, 2016).

Studies specific to TBBPA have found that the chemical can act as a neurotoxin and induce cellular toxicity as well as disturb cellular dopamine secretion and alter acetylcholinesterase enzymatic activity (Liu *et al.*, 2016). TBBPA was also recently found to cause neurotoxic and apoptotic responses in cultured mouse hippocampal neurons *in vitro* (Szychowski & Wójtowicz, 2016). Additionally, TBBPA was found to have induced apoptotic and neurotoxic effects in mouse neocortical cells. Another study found that TBBPA can induce neurotoxic effects, especially when challenged with oxygen-glucose deprivation (Ziemińska *et al.*, 2012).

This study identified an association between TBBPA and MOG and MBP antibodies, which had not been reported previously. The antibodies to myelin lead to the destruction of nerve sheaths that are necessary for healthy nerve transmission. These nerve sheath proteins were

originally thought of as a biomarker for demyelinating diseases, such as multiple sclerosis (Peschl *et al*., 2017). The elevation of the levels of these antibodies is used as a diagnostic and predictive biomarker for diseases, such as multiple sclerosis (Berger *et al*., 2003). However, further research has found that antibody-level elevations of MOG and MBP also occur in less devastating and clinically obvious diseases, such as subtle neuroinflammation and the cognitive decline found with subtle patterns of neurodegenerative disease (Papuc *et al*., 2015). In this study, the association with chemical, immunological reactivity to TBBPA with both MOG and MBP antibodies suggests that fire-retardant chemicals may play a role in the inflammation of the nerve sheath of neurons. These reactions may play a role in a diverse list of diseases on the rise that includes autism, multiple sclerosis, dementia, AD, and peripheral neuropathy.

Research Question #3: What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of autoimmunity against nerve sheath proteins (myelin basic protein and myelin oligodendrocytic protein)? The production of antibodies to TBBPA, MOG, and MBP are an acquired immune response. TBBPA antibodies may only occur from environmental exposure to TBBPA and are not intrinsically found in human serum. Additionally, autoantibodies to MOG and MBP do not occur until the immune system targets these myelin sheath proteins due to an autoimmune disease response. A risk ratio calculation was conducted with TBBPA antibodies as the exposure antibody and MOG and MBP antibodies as the disease antibodies. This study found that the relative risk for developing autoimmunity against nerve sheath proteins by individuals who exhibit TBBPA immunological reactivity ranges from 5.4 to 22.4 times the risk when compared to individuals who did not exhibit TBBPA immunological reactivity, depending on whether immunoglobulins A, G, or M were assessed. These findings were statistically significant with p-values <0.0001. The

confidence intervals were large for this risk ratio calculation. To conservatively estimate the relative risk using only the lower tail of the 95% confidence interval, the relative risk ranged from 2.06 to 7.01 times the risk when compared to individuals who did not exhibit TBBPA immunological reactivity, depending on whether immunoglobulins A, G, or M were assessed.

These findings suggest that the exposure to TBBPA, leading to antibody production against TBBPA bound to albumin, promotes a significant risk of developing autoimmunity against nerve sheath proteins. Therefore, individuals who have elevated levels of TBBPA antibodies should be carefully evaluated for clinical signs and symptoms of demyelinating diseases. The symptoms of demyelinating diseases include numbness, pain, dizziness, loss of movement, sensory impairment, and cognitive decline. The signs of demyelination include nystagmus, upper and lower motor neuron signs, ataxia, pallanesthesia, and loss of brainstem and spinal cord reflexes, among others. The presence of these signs and symptoms suggest injury to myelin sheath nerve proteins, and this response can be confirmed by elevated levels of MOG and/or MBP antibody (Reindl *et al.*, 2013). Additionally, for those who have confirmed the demyelinating disease and elevated levels of MBP and MOG antibodies, the investigation for environmental reactivity to TBBPA can be conducted with serum antibody testing and appropriate lifestyle modification to reduce fire retardant exposure.

Research Question #4: Is there a correlation between TBBA immunological reactivity to S100B protein, the biomarker used to assess breakdown of the blood-brain barrier and neuroinflammation? This study found a statistically significant correlation between TBBPA immunological reactivity and S100B protein, the biomarker used to assess breakdown of the blood-brain barrier (BBB) and neuroinflammation with IgA, IgG, and IgM antibodies. Several researchers have labelled S100B as a candidate biomarker for blood-brain

barrier permeability and central nervous system damage (Sun *et al.*, 2013). S100B protein is located in the central nervous system; it can pass through the blood-brain barrier and enter peripheral circulation upon injury to the brain or blood-brain barrier (Bloomfield *et al.*, 2007). When the blood-brain barrier is destroyed, the astrocytic protein called S100B enters the bloodstream and over time, S100B antibodies develop in those individuals who have a persistent breakdown of the BBB (Choi *et al.*, 2016). S100B is normally segregated in the brain and has a sequence of 10 amino acids that are not present in any other human protein, and therefore, has unique auto-antigenic properties. Hence, when the BBB breaks down, and S100B enters the blood, dendritic cells are likely to perceive these proteins as non-self-antigens and promote an autoimmune response to S100B (Marchi *et al.*, 2013*)*. S100B antibody is a highly sensitive marker for detecting the breakdown of the BBB and brain metastases in patients with lung cancer, using brain imaging (Choi *et al.*, 2016).

S100B is a calcium-binding protein that is expressed primarily by astrocytes, and it functions both as an intracellular regulator and an extracellular signal located in the blood-brain barrier (Santamaria-Kisiel *et al.*, 2006). S100B has both neurotrophic and gliotrophic functions (Yardan *et al.*, 2011). As an intracellular regulator, S100B promotes cell proliferation and migration and also acts to inhibit apoptosis and differentiation during cellular development and neuronal repair. As an extracellular regulator and extracellular factor, S100B engages receptors for advanced pro-proliferative or pro-differentiative responses, depending on the concentration attained by the protein and the surrounding microenvironment (Donato *et al.*, 2009). Nanomolar concentrations of S100B *in vitro* enhance the survival of neurons and stimulates neurite branching (Donato, 2001). However, micromolar concentrations of S100B *in vitro* promote a neuroinflammatory response and activate the receptor for advanced glycation end products

(RAGE), inducing apoptosis by promoting reactive oxygen species, cytochrome C release, and activating the caspase cascade (Sen & Belli, 2007).

Additionally, the levels of S100B have been shown to potentially determine both the extent of the injury and predict the prognosis of individuals who have suffered a traumatic brain injury (Wiesmann *et al.*, 2010). In fact, S100B has been found to increase in serum within six hours of traumatic brain injury (Vos et al., 2010). In one study, the sensitivity of determining the prognosis of recovery from traumatic brain injury using serum S100B was 80% (Rainey *et al.*, 2009). S100B serum levels have also been found to reflect the significance of brain injury from acute stroke and predict the prognosis of acute stroke patients (Beer *et al.*, 2010). In addition, the release of S100B can activate the surrounding microglia and promote neuroinflammatory damage to the brain, which may serve as a contributing factor to neurodegenerative diseases (Rothermundt *et al.*, 2003).

In addition to the elevation in levels of S100B in cases of acute stroke and acute traumatic brain injury, this protein may also play a role in chronic neurodegenerative diseases. Significantly increased levels of cerebrospinal fluid S100B have been identified in patients suffering from AD and frontotemporal lobe dementia (Fox & Freeborough, 1997; Green *et al.*, 1997). It has been shown that ß-amyloid found in AD stimulates the synthesis of both S100B mRNA and S100B protein in astrocytes (Pena *et al.*, 1995). Additionally, it has been suggested that extracellular S100B may play a neuroinflammatory role in activating astrocytes and surrounding microglia. Higher levels of S100B antibodies have been identified in the serums of patients diagnosed with Lewy-body associated dementias compared to healthy controls (Maetzler *et al.*, 2011). Furthermore, S100B has been found to correlate with brain atrophy in AD; this finding suggests that S100B may play a role in neurodegenerative diseases (Petzold *et al.*, 2003).

It has also been suggested that S100B is a marker for disease progression in Parkinson's disease (Schaf *et al*., 2005). In a mice study, where the neurotoxin 1-4-methyl-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was used to induce Parkinsonism, S100B was found to be elevated after neuronal damage (Muramatsu *et al*., 2003). Furthermore, S100B has been used as a biomarker for sleep-related neuroinflammation in Parkinson's disease (Carvalho *et al*., 2015). These findings may support the correlation that was also found in this study linking TBBPA with alpha-synuclein, which is the target protein of Parkinson's disease. When BBB loses its integrity, the brain becomes vulnerable to antigens, chemicals, and circulating antibodies from the peripheral immune response that have the potential to induce pathogenic insults to the brain, including autoimmune and inflammatory responses (Vincnet *et al*., 2011).

This study found an association between TBBPA immunological reactivity and S100B protein, the biomarker used to assess the breakdown of the BBB. S100B is the surrogate marker for the disruption of the BBB. The BBB is found to be compromised in many patterns, including traumatic brain injury, neurodegenerative diseases, and stroke. The underlying response to the production of S100B antibodies is the activation of the astrocytes in the BBB from destructive and inflammatory response, leading to the release of S100B into the bloodstream, and then, the development of antibodies to S100B. The association with S100B antibodies, in this study, and TBBPA antibodies suggests that the chemical found in flame retardants may have a role in the systemic inflammatory response of the brain that is associated with the activation of astrocytes and impairment of the BBB's integrity. The compromising of the BBB barrier may lead to significant exposure of the brain to toxic chemical insults and the promotion of diseases in the brain (Zheng, 2001). Although the association is not causation, it appears that the immunological reactivity to TBBPA may play some role with the biomarker S100B associated with the

impairment of the BBB. The breach of the BBB can lead to a further significant risk of chemical exposures to target proteins in the brain that have been found to correlate with immunological reactivity in this study, including MOG, MBP, AQP4, and alpha-synuclein. The exact pathophysiological mechanism of BBB breakdown is unknown from the study design of this investigation; however, research designs to further investigate the precise role of TBBPA and its impact on the BBB have been warranted due to the outcomes of this research.

Research Question #5: What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of neuroinflammation and breakdown of the blood-brain barrier (S100B)? The production of antibodies to TBBPA and S100B are an acquired immune response. TBBPA antibodies may only occur from environmental exposure to TBBPA; they are not intrinsically present in the human serum. Additionally, the autoantibodies to S100B do not occur until the immune system targets these BBB proteins due to an inflammatory response. A risk ratio calculation was conducted with TBBPA antibodies as the exposure antibody and S100B antibodies as the disease antibodies. This study found that the relative risk for developing neuroinflammation and breakdown of the BBB, which leads to S100 antibodies in individuals who exhibited TBBPA immunological reactivity, ranges from 7.5 to 75.6 times the risk when compared to individuals who did not exhibit TBBPA immunological reactivity, depending on whether immunoglobulins A, G, or M were assessed. These findings were highly statistically significant with p-values <0.0001. The confidence intervals were large for this risk ratio calculation. To conservatively estimate the relative risk using only the lower tail of the 95% confidence interval, the relative risk ranged from 2.6 to 10.66 times the risk when compared to individuals who did not exhibit TBBPA immunological reactivity, depending on whether immunoglobulins A, G, or M were assessed.

These findings suggest that exposure to TBBPA leads to antibody production against TBBPA bound to albumin and promotes a significant risk of developing neuroinflammation and breakdown of the BBB. Individuals who have S100B antibodies should be concerned about chemicals that have been found to be directly neurotoxic to the brain, such as TBBPA (Liu *et al.*, 2016). Furthermore, those who have TBBPA antibodies should consider having their S100B levels checked to determine if they have activated the astrocyte response involving S100B release. The findings of either elevated levels of S100B antibodies or elevated levels of TBBPA antibodies raise concern regarding the vulnerability of the brain to toxic agents despite the exact underlying mechanism of pathology.

Research Question #6 Is there a correlation between TBBPA immunological reactivity and alpha-synuclein, the protein aggregate marker for neurodegenerative diseases, such as Parkinson's disease? This study found a statistically significant correlation between TBBPA immunological reactivity and alpha-synuclein, the protein aggregate marker for neurodegenerative diseases, such as Parkinson's disease (PD) with IgA, IgG, and IgM. PD is the second most common neurodegenerative disease in the world, and the prevalence of PD is increasing rapidly. The majority of PD cases are sporadic and not associated with familial PD, which only accounts for 5% of the cases (Liu *et al.*, 2016).

The exposure to various industrial and environmental chemicals, including flame retardants, have been recognized in the etiopathogenesis of PD (Caudle, 2015). Mice were exposed to a brominated flame retardant called hexabromocyclododecane (HBCDD) for six weeks, and neurotoxic was reported to impact the dopamine circuits in the brain (Pham-Lake *et al.*, 2017). Another study exposed brominated flame retardants during the brain growth in mice, and a single oral dose of the chemical led to alterations of proteins, including alpha-synuclein, in

the brain involved with neurodegeneration (Alm *et al*., 2006). Additionally, the PD subjects were found to have compromised hepatic conjugation with glucuronic acid and statistically significant reduced levels of conjugated bisphenol-A compared to other controls (Landolfi, 2017). TBBPA shares the same hepatic biotransformation pathways as BPA, and the impaired conjugation of these chemicals lead to increased risk of oxidative stress and inflammation (Nakagawa *et al*., 2007). The impaired conjugation of these chemicals in subjects with PD can make them extremely vulnerable to TBBPA exposure.

Alpha-synuclein is an intracellular protein that supports neuron synaptic transmission and the release of neurotransmitters in the presynaptic vesicle under normal conditions (Cheng *et al*., 2011). Alpha-synuclein can misfold and aggregate in the extracellular regions under various pathological mechanisms; it is the hallmark protein aggregate in PD. The aggregation of alpha-synuclein can be neurotoxic by promoting oxidative stress and impairing vesicle trafficking, thereby promoting neurodegenerative changes in the brain (Xu *et al*., 2016). Once alpha-synuclein aggregates in the extracellular space, the humoral immune system produces antibodies against these protein targets, leading to the elevation in the levels of alpha-synuclein antibodies in the serum (Orr *et al*., 2005).

Alpha-synuclein antibodies play a protective role by attaching to these protein aggregates for immune cells to engulf them and limit their toxic effects on the surrounding neurons (Ingelsson *et al*., 2016). In studies done on mice, alpha-synuclein vaccinations induced reactive antibodies that reduced the level of alpha-synuclein aggregation associated with neurodegenerative changes (Masliah *et al*., 2005). Extracellular alpha-synuclein is cleared by microglial cells when alpha-synuclein antibodies specifically target them, and thus, reduce their neurotoxic actions on the surrounding cells (Lindstrom *et al*., 2014). Exogenously produced

antibodies to alpha-synuclein can reduce alpha-synuclein aggregate accumulation and reduce the oxidative and inflammatory response that they induce (Gruden *et al.*, 2012).

The identification of alpha-synuclein antibodies occurs with the accumulation of these proteins in the extracellular space associated with neurodegenerative disease (Lee *et al.*, 2016). Evidence continues to illustrate that the antibodies measured are identifiable in the serum, and they can be used as a sensitive biomarker in alpha-synucleinopathies (Yanamandra et al., 2001; Papachroni et al., 2007). In one study, PD patients exhibited a 400–800% increase in the measurements of alpha-synuclein antibodies compared to non-disease controls that changed during the progression of the disease. Non-disease subjects had a narrow distribution of antibody levels throughout the study (Yanamandra *et al.*, 2001). Another study found that 65% of the subjects with PD had elevated levels of alpha-synuclein antibodies (Papachroni *et al.*, 2007). A clear understanding of neurotoxicity and the guidelines for neurotoxicity laboratory tests are currently being developed to address the contribution of neurotoxic chemicals, including fire-retardant chemicals, to the development of neurodegenerative diseases, such as PD and AD (Giordano & Costa, 2012). The outcomes of this study may contribute to neurotoxic laboratory tests involving diseases such as PD in the future.

This study found an association between chemical, immunological reactivity to TBBPA in the form of serum antibodies and antibodies to alpha-synuclein, the target protein aggregate biomarker of PD. The development of PD involves several decades, and various stages of the disease have been identified. Although most healthcare providers associate a resting tremor as an early sign of PD, it is, in fact, a finding that occurs in the last stages of the disease. Early markers of the PD include impaired smell, muscle tightness, and constipation (Rietdijk, 2017). These patterns occur because the protein, alpha-synuclein, which is normally found in healthy neurons,

aggregates together and promotes neuronal death. Recent advances in biomarker testing have found that as alpha-synuclein aggregates, the immune system produces antibodies to these proteins that can be identified in serum samples as alpha-synuclein antibodies (Lee *et al.*, 2016) even before the clinical presentations can become evident. The strong linear correlation between alpha-synuclein antibodies and antibodies to TBBPA suggest that TBBPA may play some role in the neurodegenerative process. Therefore, individuals who have been diagnosed with PD should consider being tested with TBBPA antibodies, and if immunological reactivity to TBBPA is identified, lifestyle changes to reduce the exposure of fire retardants in their household and daily activities should be considered. As stated earlier, it is clear that TBBPA is directly harmful to dopaminergic centers of the brain in animal studies and that patients with PD have impaired biotransformation pathways to metabolize chemicals such as TBBPA from their bodies. These findings suggest that TBBPA exposure may be a risk factor in the development of PD. Further research is warranted to identify if a direct causal relationship exists, and if so, what is the exact pathophysiology of this relationship.

Research Question #7: What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and the development of protein aggregate antibody biomarkers for neurodegenerative diseases such as Parkinson's (alpha-synuclein)?
Antibodies to TBBPA and alpha-synuclein are an acquired immune response. TBBPA antibodies may only occur from environmental exposure to TBBPA and are not intrinsic to human serum. Additionally, autoantibodies to alpha-synuclein do not occur until the immune system targets aggregation of these proteins due to a neurodegenerative process (Ingelsson, 2016). A risk ratio calculation was conducted with TBBPA antibodies as the exposure antibody and alpha-synuclein

antibodies as the disease antibodies. This study found that the relative risk for developing alpha-synuclein antibodies with individuals that exhibit TBBPA immunological reactivity ranges from 18.22–75.6 times the risk when compared to individuals who do not exhibit TBBPA immunological reactivity depending on whether immunoglobulin A, G or M was assessed. These findings were statistically significant (p <0.0001). The confidence intervals were large for this risk ratio calculation. To conservatively estimate the relative risk using only the lower tail of the 95% confidence interval, the relative risk ranged from 5.6–10.66 times the risk when compared to individuals who do not exhibit TBBPA immunological reactivity depending on whether immunoglobulin A, G, or M was assessed.

These findings suggest that exposure to TBBPA, which leads to antibody production against TBBPA bound to albumin, promotes a significant risk of developing alpha-synuclein antibodies. Therefore, if an individual has developed antibodies to TBBPA, careful clinical examination for early patterns of PD should be conducted with a neurological exam that includes findings hypometric movements, loss of arm swing during ambulation, loss of smell, resting tremor, and loss of postural reflexes. Additionally, careful evaluation of early symptoms of PD, such as joint stiffness, depression, slowness, clumsiness, poor balance, and chronic constipation, should be evaluated. Immunological reactivity to TBBPA may induce risk for the development of PD, and these findings may be a useful marker in the early stages of the disease. Careful examination of early signs and symptoms of PD with those who have TBBPA antibodies may help identify early stages of PD and identify a potential risk factor.

Research Question #8: Is there a correlation with TBBPA immunological reactivity and Aquaporin-4 water-channel receptors, the target protein for autoimmune reactivity for neuromyletis optica?

This study found a statistically significant correlation between TBBPA immunological reactivity and aquaportin-4 (AQP4) water channel receptors antibodies, the target protein for autoimmune reactivity to neuromyelitis optica and neuromyelitis optic spectrum disorder with IgA, IgG, and IgM. AQP4 is a water-channel protein and functions to support fluid homeostasis, removal of waste, calcium signaling, osmosensation, water homeostasis, and regulation of extracellular space volume in central and peripheral nervous tissues (Nagelhus & Ottersen, 2013).

The autoimmune response against AQP4 with autoantibodies can impact both the central nervous system and bodily systems outside the brain. In extreme cases of AQP4 autoimmunity, there is severe destruction of the visual pathways and the spinal cord due to the high amount of AQP4 water channel proteins in these tissues. (Nagelhus & Ottersen, 2013). This pattern has been associated with the neurological autoimmune disease called neuromyleitis otpica (NMO), as the initial clinical presentation of the autoimmune response leads to the clinical presentation of visual and spinal cord clinical findings (Papadopoulos et al., 2010). Further understandings of the AQP4 autoimmune response has led to the realization that these water channel proteins are also found in other regions of the central nervous system. The autoimmune response to AQP4 target proteins is not just limited to the visual pathways and the spinal cord but also to a spectrum of clinical presentations due to the distribution of AQP4 throughout the nervous system. This diverse response has been termed neuromyeltis optical spectrum disorders (NMOSD) (Wingerchuck et al., 2007).

Antibodies to AQP4 cross the blood-brain barrier through endothelial transcytosis and bind the AQP4 water channel membrane proteins. This selective autoimmune binding induces destruction of the blood-brain barrier and injure myelin nerve sheaths by cytotoxic and complement immunological responses (Misu et al., 2007). Once the blood-brain barrier is

breached, there is massive infiltration of leukocytes into the brain, leading to aggressive nerve tissue destruction. Antibodies against the target protein AQP4 represent the key target protein for NMO and NMOSD and play a key role in the pathophysiology of the disease (Papadopoulos |& Verkman, 2012). There is a 73% sensitivity and 91% specificity for autoimmune destruction of the nervous system with the characteristic patterns of NMO with elevated AQP4 antibodies (Lennon *et al*., 2007). AQP4 antibodies are highly specific (85%–99%) for NMO when significantly elevated and can be detected in sera of most patients (68%–91%) (Papadopoulos *et al*., 2012). AQP4 antibodies are specific to the disease process, and the levels of the antibodies fluctuate as the severity of the clinical findings of the disease change (Takahashi *et al*., 2007). Furthermore, AQP4 antibodies can be used to monitor disease activity and determine the potential for relapse and the efficacy of disease treatment (Jarius *et al*., 2008).

AQP4 target proteins are also found outside the central nervous system in the peripheral tissues of the body, such as the organs, the intestinal mucosa, the respiratory airways, and muscles tissues. (Papadopoulos & Verkman, 2012). Antibodies to AQP4 induce immunological responses to these peripheral tissues as well and lead to a diverse list of subtle or progressed symptoms. (Wingerchuk *et al*., 2007). These reactions throughout the body have expanded the categorization of the AQP4 autoimmune reactivity to NMSOD (Rosales & Kister, 2016). Autoimmune reactions against AQP4 target proteins in the placenta have been found to play a role in miscarriages. Subjects with seropositive AQP4 IgG antibodies have been found to have increased rates of miscarriage (De Falco *et al*., 2007; Reuss *et al*., 2009; Saadoun *et al*., 2013). AQP4 antibodies also target specific neuroinflammation in the cerebral aqueduct and have been reported in cases of obstructive hydrocephalus (Owler *et al*., 2010). The AQP4 protein is also found to be a target for autoimmune responses in the organ of corti in the inner ear; it plays a role

in autoimmune induced hearing loss (Jarius *et al.*, 2013). Animal studies have found that AQP4 proteins play a role in osmotic water fluxes in the inner ear, and autoimmune responses against these water-channel proteins induce deafness (Li & Verkman, 2001). The diverse location of AQP4 water-channel proteins leads to a diverse list of clinical symptoms when antibodies are produced against them. These autoimmune reactions can vary in intensity and lead to spectrum that includes subtle or non-clinical symptoms all the way to progressed states of debilitation autoimmune diseases such as NMO and NMOSD.

Although significantly elevated AQP4 antibodies are the diagnostic laboratory marker for NMO and NOMSD, environmental chemicals such as TBPBPA may bind to serum albumin and have the potential to promote subtle immunological responses against water-channel proteins without expression into an end-stage disease. AQP4 antibodies vary according to the intensity of the autoimmune response (Takahashi *et al.*, 2007). It is likely that many of the healthy subjects in our study with varying elevations of AQP4 antibodies will have a subtle degree of neuroinflammation against aquaporin water-channels.

This study found an association between immunological reactivity to TBBPA and AQP4 antibodies. AQP4 antibodies, when extremely elevated, are the critical biomarkers used to diagnose NMO (Wingerchuck *et al.*, 2007). These antibodies may be accompanied by symptoms and signs of demyelination in the visual pathways and/or the spinal cord. The clinical presentation of demyelination and the elevated levels of AQP4 antibodies together fulfill the established diagnostic criteria for NMO (Patterson & Goglin, 2017). However, despite the typical criteria for the diagnosis of NMO, researchers have also found that AQP4 target proteins are located throughout the central and peripheral nervous system, and autoimmune reactivity to AQP4 can lead to a diverse list of symptoms unrelated to just demyelination of the spinal and

visual pathways associated with NMO (Kim *et al.*, 2017). Additionally, various levels of AQP4 antibodies can be associated with multiple degrees of neuroinflammation and not always associated with an extreme clinical presentation of demyelination disease (Takahashi *et al.*, 2007). The findings of this study show a strong, positive linear correlation between the degree of immunological reactivity to TBBPA and the degree of immunological reactivity to AQP4 target proteins. This suggests that that immune responsiveness to TBBPA may play some role in the neuroinflammatory, and autoimmune response found with diseases such as NMO and NMOSD. Further research is needed to determine if a causal role exists between TBBPA exposure and the development of NMO and NMOSD and what the mechanisms of pathophysiology of this flame-retardant chemical are to AQP4 associated autoimmune diseases.

Research Question #9: What is the relative risk (risk ratio) for exposure to TBBPA that leads to immune reactivity and against the autoimmune target protein of neuromyleitis optica (aquaporin-4 antibodies)? Antibodies to TBBPA and aquaporin-4 are an acquired immune response. TBBPA antibodies may only occur from environmental exposure to TBBPA and are not intrinsic to human serum. Additionally, autoantibodies to aquaporin-4 do not occur until the immune system targets these water-channel proteins in the blood-brain barrier (Ingelsson, 2016). A risk ratio calculation was conducted with TBBPA antibodies as the exposure antibody and aquaporin-4 antibodies as the disease antibodies. This study found that the relative risk for developing aquaporin-4 antibodies with individuals who exhibit TBBPA immunological reactivity ranges from 10.29–75.6 times the risk when compared to individuals who do not exhibit TBBPA immunological reactivity depending on whether immunoglobulin A, G, or M was assessed. These findings were highly statistically significant with p-values <0.0001. The confidence intervals were large for this risk ratio calculation. To conservatively estimate the

relative risk using only the lower tail of the of the 95% confidence interval, the relative risk

ranged from 8.32–10.65 times the risk when compared to individuals who do not exhibit TBBPA

immunological reactivity depending on whether immunoglobulin A, G, or M was assessed.

These findings suggest that exposure to TBBPA, which leads to antibody production

against TBBPA bound to albumin, promotes a significant risk of developing breach of water-

channel proteins of the blood-brain barrier and promote autoimmune reactions against the target

protein associated with NMO. The outcomes of this risk ratio suggest that individuals who have

elevated TBBPA antibodies should be carefully screened for symptoms of NMO, such as loss or

blurriness of vision, changes in color perception in one eye compared to the other eye, weakness

or paralysis of the limbs, uncontrollable vomiting or hiccups, and numbness in various regions of

the body. Individuals with elevations of TBBPA antibodies should also be evaluated for clinical

signs of NMO with a detailed neurological examination. Examination findings of NMO include

upper motor neuron signs, positive pathological reflexes, dysconjugate gaze, gaze-evoked

nystagmus, blindness, and scotomas. Immunological reactivity to TBBPA can be a substantial

risk factor for the target protein of NMO and NMOSD.

**Research Question #10: Are there any differences in chemical immune responses

between IgA, IgG, and IgM?** An antibody is also known as an immunoglobulin (Ig). There are

three forms of immunoglobulins or antibodies: immunoglobulin A (IgA), immunoglobulin G

(IgG), and Immunoglobulin M (IgM). These immunoglobulins all serve to tag foreign proteins

such as bacteria and viruses as part of the immune response against foreign organisms.

Antibodies can also occur against one's own body, which results in the development of

autoimmune disease. The functions of immunoglobulins are generally the same, but the three

different immunoglobulins occur in various tissues, and some forms of immunoglobulins are also

found more specifically with various diseases and immunological responses. IgA responses occur in the intestinal and oral mucosa tract and typically with interactions that happen in the gastrointestinal tract, oral cavities, or the respiratory passages. IgG responses are found in all bodily fluids and typically with delayed exposures. IgM antibodies are detected in the blood, and lymph fluid and typically reflect the initial response against antigens. A fundamental question in this study was to determine if there were any notable differences between IgA, IgG, or IgM for TBBA bound to human albumin.

The study results revealed moderate-to-high degrees of correlation between the IgM, IgG, and IgA antibody levels against TBBPA and neurological autoimmune target sites (Tables 5-7). However, the study also found that the IgM antibodies have the most notable correlation for all reactions. Earlier studies also offer evidence that immune response to haptenic chemicals brings about a higher production of IgM antibodies than IgG or IgA (de Guercio *et al.*, 1974; Onoue *et al.*, 1965). This confirms the previous findings and suggests that immunological reactivity with IgM may be the most sensitive antibody to evaluate chemical neoantigen associations, and these reactions typically occur in the blood or lymph fluid of the subjects.

Immunological testing of various forms of antibodies can be expensive; however, identifying which immunoglobulin has the highest degree or reactivity with neoantigens, such as TBBPA bound to albumin, can provide data that can help determine which immunoglobulin to test that will make cost restrictions of future research more feasible. This study generated similar results for all the three forms of immunoglobulins. However, IgM was most reactive, which suggests that the immunological response to the TBBPA and various neurological autoimmune target proteins involve the mucosal immune response and immune responses found in blood and

lymph regions of the body. However, if only one form of immunoglobulin can be tested due to feasibility, IgM testing would be preferred.

Furthermore, testing three different forms of immunoglobulins with the same variables provide an indirect form of independent analyses. Each enzyme-linked immunoassay analysis (ELISA) in the laboratory is entirely independent of the other type of immunoglobulin ELISA analysis. The design of this study to evaluate all three forms of immunoglobulins with the same variable provides further cross-validation of the correlations found within this investigation.

Implications

The outcomes of this research may support the decisions made in respect of those suffering from neurological diseases, as to whether reducing flame retardant exposure is an environmental factor for consideration. Additionally, this study can be used to support the development of safety regulations and identify potential health concerns for current mandatory flame-retardant legislation. The outcomes of this study demonstrated the statistically significant correlation of p-values <0.0001 and correlation coefficients ranging from 0.68–0.92 linking immune reactivity to TBBPA with various target sites of the nervous system, which are associated with a diverse list of neurological diseases. Although correlation analysis does not determine whether TBBPA is causative, it does demonstrate that individuals with neurological autoimmunity have associative immune reactivity to TBBPA.

The association between TBBPA and autoimmune reactivity to various neurological disease target sites may occur for three theoretical possibilities:

1. It is possible that TBBPA is, in fact, a causative agent in the development of neurological disease.

2. The association between TBBPA and neurological disease is not causative, but there are common underlying physiological mechanisms between autoimmune neurological diseases and chemical, immunological reactivity.

3. There is no physiological mechanism involved in the association between TBBPA and neurological disease, and there is no causative role.

If an assumption is made that the associations in this study are, in fact, causative, it would suggest that immunological reactivity to TBBPA is a key factor in the development of neurological disease. TBBPA can act as a neurotoxin and induce cellular toxicity as well as disturb cellular dopamine secretion and alter acetylcholinesterase enzymatic activity (Liu *et al*., 2016). TBBPA was recently found to cause neurotoxic and apoptotic responses in cultured mouse hippocampal neurons *in vitro* (Szychowski & Wójtowicz, 2016). Additionally, TBBPA was found to have induced apoptotic and neurotoxic effects on mouse neocortical cells. Another study found that TBBPA may produce neurotoxic effects, especially when challenged with oxygen-glucose deprivation (Ziemińska *et al*., 2012). The outcomes of this study cannot determine that the neurotoxic effects of TBBPA are causative in the development of antibodies to neurological disease target sites; however, the correlation outcomes identified in this study warrant further investigation.

The second possibility for the association between TBBPA and neurological disease is that the association is not causative, but rather there are common underlying physiological mechanisms between neurological diseases and chemical, immunological reactivity. Exposure to TBBPA leads to a distribution of the chemical throughout the body. Once the chemical enters the systemic circulation, it can be detected in serum samples. TBBPA is then converted into a water-soluble metabolite by phase I and phase II hepatic biotransformation pathways, after which it is

excreted through the urine. Chemicals, such as TBBPA, also have the potential to bind to various proteins in the body, such as albumin, while they are in circulation. When chemicals bind to a protein, they change its allosteric structure and can act as new antigens to the immune system. When this occurs, the humoral system produces antibodies against these chemical-bound neoantigens. There are two plausible mechanisms in which the association with TBBPA and neurological antibodies are due to associative physiological pathways and not from a causative relationship. First, subjects who have a neurological disease may have compromised hepatic biotransformation pathways, leading to increased TBBPA levels and neurological antibodies from impaired chemical clearance. This physiological mechanism will lead to a correlation between the variables in this study, but the association would not be causative.

Another possible non-causative physiological mechanism of this study would be related to concurrent loss of chemical tolerance found with neurological diseases. In this study, we did not measure the quantity of chemical in the serum, but we checked the levels of chemical antibodies. Antibodies to chemicals occur when the immune system reacts against chemicals. The reaction to chemicals is an immunological response. An exaggerated response to chemicals can occur with loss of oral tolerance. In this scenario, the immune system has an overzealous response to chemicals. Loss of oral tolerance is a co-occurrence with the neurological autoimmune disease. In this study, the association between both chemical and neurological antibodies may not be associated with a causative model but with the loss of oral tolerance that can occur in neurological disease, leading to the association outcomes found in this study.

It should be noted, even if TBBPA immunological reactivity is not a causative variable in the development of antibodies against neurological disease target sites, both impaired biotransformation activity or loss of tolerance activity found with non-causative pathways of

association are still clinically relevant. The immunological response to an environmental antigen is a concern for individuals who suffer from any form of autoimmune disease. Chemical responses to antigens promote destructive immunological responses. The strong correlation with immunological reactivity to TBBA and neurological autoimmune disease antibodies still suggest that exposure to TBBPA is a concern for subjects suffering from autoimmune neurological diseases, despite the fact the correlation may not be causative. Therefore, restriction of TBBPA exposure should be considered for individuals at risk. Immunologically reactivity to a specific chemical in a subset of individuals raises health and safety concerns.

The third possibility that may explain the associations found in this study is that no physiological mechanism is associated with the associations between TBBPA and neurological disease, and there is no causative role. It is possible that the actual findings of the study were null and type I errors were obtained. However, as we had made a Bonferroni adjustment to the p-value to account for a false discovery rate for multiple comparisons and the results of the p-value was <0.0001, it is unlikely, but still possible. The possibility for experimental error is also always a possibility; however, laboratory standard operations procedures was designed very carefully to avoid this possibility as much as possible. As in any study, the findings of this experiment should be repeated by other investigators to determine if the findings are reproducible.

Despite the clinical implications of this study for subjects suffering from a neurological disease associated with the target proteins used in this study, there are implications for determining chemical risks by measuring antibodies to chemicals. A novel feature of this study was the association between antibodies formed to TBBPA bound to protein and not merely the detection of TBBPA levels. Detectible levels of TBBPA in serum and urine samples are an

expected finding for most of the general population. Antibodies produced to TBBPA bound to protein, however, are an independent feature of only a subset of the population. In a previous study on blood donors, it was reported that antibodies to TBBPA bound to albumin only occur in 16% of the samples (Vojdani *et al*., 2015). The percentage of immunological reactivity to BPA in the form of antibodies is much lower than serum levels of BPA found in more than 80% of blood donors (Lu *et al*., 2017). These findings suggest that TBBPA exposure alone may not be pathogenic. Rather, immunological reactivity to TBBPA may be a key feature in the development of neurological disease. Another implication of this study is that measuring serum TBBPA levels may not be as important for determining diseases as measuring immunological reactivity to TBBPA as found with antibodies to TBBPA bound to proteins. It is likely that simply having chemicals occur in serum samples are not pathogenic, as they occur in large samples of the population without disease (Lu *et al*., 2017). This finding can invalidate safety studies that have only measured the occurrence of TBBPA serum levels.

The outcomes of this study may impact legislation. Flame retardants are sprayed on all pieces of furniture, mattress beds, children's pajamas, car seats, upholstery, carpets and rugs in the United States. California Flammability Standard drove this practice in Technical Bulletin 117 (TB117) that was instituted in 1975. TB117 states that a manufacturer is not permitted to sell items such as furniture, upholstery or mattresses in California unless they are sprayed with flame retardants. As California is such a large consumer market, the guidelines of TB117 have been adopted by all major manufacturers throughout the country, leading to widespread use of flame retardants.

Legislative concerns regarding the safety of TBBPA to humans compared to its protective fire safety role have been debated for some time. Previous debates have unfortunately

been one-sided. There was always the assumption that TBBPA added no risk to humans despite research on the safety of TBBPA before its widespread use. Legislation regarding the mandatory use of fire retardants may change as research continues to grow regarding their health concerns. The outcomes of this study provide additional evidence for human health concerns. The outcomes of this research can be used to support the development of safety regulations and for identifying the potential health concerns for current mandatory flame-retardant legislation.

The theoretical framework that guided this study was the social ecological theory. The social ecology theory emphasizes the complexity of relationships between environment, social, political, legal, and ecological influences (Stokles, 1996). Exposing the potential risk of fire retardants (TBBPA) to human health as determined by the outcomes of this study is a necessary step to increase social consciousness, legislative actions, and public health policy, as promoted by the social ecology theory. The knowledge that exposure to TBBPA chemicals may impact neurological autoimmunity may lead to reconstructive and transformative outlooks on social and environmental issues. Social consciousness that TBBPA may be harmful to humans may lead to changes in social and political problems and manufacturing practices that can impact human health and environmental pollution, and thereby, promote changes in manufacturing legislation.

The extensive use of TBBPA impacts both animal and human life, and these interactions should be carefully accounted for by environmental health scientists, legislators, urban and regional planners, and the diverse list of professional disciplines that are concerned with the interactive role of toxicant exposure to both social and environmental threats. The removal of toxic chemicals may have profound impacts on the society. For example, the removal of lead from gasoline increased the mean IQ of all American children and had generated an annual economic benefit of $213 billion, according to analysts (Landeigan & Fuller, 2016). There is

potential to gain both health and economic benefits after TBBPA is removed from consumer products.

Flame retardants have spread throughout nature, and high concentrations of the toxic chemical have been reported in the meat and liver of wildlife such as wild boar, deer, and moose (Zacs *et al*., 2017). A marine sampling of water pollution in various aquatic regions led to the discovery of high levels of TBBPA chemicals that have been reported to impact marine life (Gong *et a*l., 2017). The concentration of TBBPA has been reported at various levels of the food chain and include increased concentrations of the chemical in human blood, fat tissue, and mother's milk (Jarosiewicz & Bukowska, 2017). These widespread findings of TBBPA concentrations throughout environmental locations, animal, and human tissues are alarming, considering the outcomes of this study and the role it may play in the social ecology theory.

Identifying how toxic pollutants impact human health and society as a whole is necessary to initiate social consciousness and change, according to the social ecology theory (Stokols, 2000). These include changes in public policy (local, state, national, and global), relationships among organizations, institutions, and both interpersonal and intrapersonal factors (Fox & Aldred, 2016). Determining the potential risk of fire retardants (TBBPA) for human health is a necessary step to increase social consciousness, legislative actions, and public health policy, as promoted by the social ecology theory.

Recommendations

The results of this study provide scientific, legislative, and clinical recommendations. As with all research studies, it is recommended that the outcomes of this study be verified and duplicated by other scientists. Descriptive and detailed steps to duplicate this study have been presented in the methods section of this study. Furthermore, study designs that provide greater

causal inference regarding the role of TBBPA should be investigated. Although clinical trials provide the greatest evidence for causal relationships, they are ethically not permissible in toxicology studies with humans. However, other study designs, such as prospective studies and case-control studies, can provide greater evidence for the potential toxic role that TBBPA may play in autoimmune disease. Case-control studies examining the occurrence of TBBPA antibodies in sera of disease samples compared to healthy controls can provide greater evidence of their potential causative role in disease promotion than the cross-sectional correlative design conducted in this study. The occurrence of TBBPA antibodies may occur in sera of patients who have multiple sclerosis, neuromyelitis optica, PD, and autoimmune demyelinating diseases. Study designs should compare if immunological reactivity to TBBPA in the form of antibodies has statistically significant differences from non-disease controls and disease case samples. Study designs should account for confounders such as medications, exposures, lifestyle, occupation, age, sex, and other immunological variables such as intestinal permeability, regulatory T-cell function, and hepatic biotransformation integrity. These variables can be controlled for confounding and providing more details regarding the causative role of TBBPA in neurological autoimmune diseases associated with a multivariate analysis using methods such as logistic regression.

Additionally, prospective study designs evaluating the role that TBBPA antibodies may play in the development of autoimmune disease is recommended, if the cost of the study is feasible. Currently, the time-frame in which exposure or factors that impact immunological reactivity to TBBPA lead to the development of neurological tissue antibodies and neurological autoimmune diseases is unknown. Previous studies have suggested various timelines regarding the role of toxic chemicals and the onset of disease. An evaluation of the onset of neurological antibodies

and/or neurological autoimmune disease clinical presentation can be conducted in five-year intervals and compared to those individuals who do not exhibit TBBPA antibodies to further understand the potential toxic role of TBBPA in disease development.

In addition to designing research to further investigate the causative role of TBBPA in neurological autoimmune disease development, this study also found that the use of antibodies to TBBPA bound to human albumin provides a different biomarker for evaluating safety concerns for TBBPA than simply measuring quantitative serum levels of TBBPA. As discussed previously, only a small fraction of a sample population exhibits antibodies to TBBPA. The prevalence of these antibodies is significantly less than the prevalence of elevated TBBPA levels found in both human and animal life. No associations with quantitative levels of TBBPA have been found with health concerns in previous safety studies. However, this study found that TBBPA antibodies are highly correlated with antibodies associated with neuroinflammation and autoimmune diseases. The outcomes of this study suggest that measuring antibodies to TBBPA bound to human albumin may provide a different perspective regarding biomonitoring studies, which are used to evaluate the safety profile of TBBPA in humans and animal life. This study provides further evidence that immunological reactivity to TBBPA may be a critical biomarker for evaluating the role the TBBPA may have in inducing the risk of neurological autoimmune disease development. TBBPA bound to albumin antibodies can be considered for biomonitoring safety studies.

These safety concerns regarding the use of TBBPA in modern society provide further evidence for legislative safety concern recommendations for mandatory use of fire retardants. In 1974, California passed a mandatory flame-retardant regulation called TB117 on upholstered furniture. Other states passed similar legislations requiring various products, including drapery in

public places, baby pajamas, and various fabrics used in the upholstery in furniture, to be sprayed with fire retardants. In 2013, a legislation in California modified the mandatory use of some types of flame retardants on many products. However, there are still mandatory flame-retardant policies throughout the nation. Despite a gradual shift to remove mandatory guidelines, there is an ongoing need to publish research that identifies safety concerns for flame retardants. The outcomes of this study provide further evidence that flame-retardant chemicals containing TBBPA are associated with increased risk for the development of neurological autoimmunity and neuroinflammation.

Lastly, the findings of this study may impact the clinical management of patients suffering from neuroinflammatory and neuroautoimmune diseases that are found with the specific tissue antibodies used in this study (MOG, MBP, S100B, AQP4, and alpha-synuclein). Although there is not enough evidence from this study to suggest that these chemicals play a direct causative role in the development of the neurological autoimmune diseases, this study does provide evidence that individuals who have antibodies to the target proteins found in this study are strongly associated with immunological reactivity to TBBPA. Whether TBBPA is causative or not, the co-occurrence of these antibodies does have potential clinical considerations. The avoidance of toxic chemicals and environmental pollutants is a standard, general approach for reducing the toxic and immunological stimulating role of environment in neurological autoimmune diseases. Reducing exposure to TBBPA in the household by avoiding furniture, foam mattresses, and upholstery that is sprayed with TBBPA may reduce the exposure of an environmental immune trigger that has been found to be associated with diseases that have immunological reactions to the target proteins that were investigated in this study. In summary,

this research leads to scientific, legislative, and clinical recommendations regarding the impact of TBBPA health risks.

Limitations

Several limitations have been found within this research design. In this study, there was no information regarding the exact exposure of TBBPA. This limitation has been acknowledged in this study design. Although there are no specific details regarding the exposure of TBBPA to the study population, it is clear that TBBPA exposure occurs from various household products such as furniture, rugs, bedding, and the upholstery of automobiles. As the exposure to this chemical occurs in so many environments today due to the extensive use of fire retardants, it would be impossible to target a specific source of exposure. Furthermore, in our study, we did not measure the quantity of the TBBPA found in serum; rather, we investigated the immune reactive role of TBBPA when it binds to proteins. This measurement is not solely reflective of the degree of TBBPA exposure. Immunological reactivity to chemicals occurs from several factors associated with a complex interplay between exposure in combination with numerous immunological factors. This study was not designed to identify a toxicity index based on the degree of TBBPA exposure. Additionally, we neither had any information regarding the medical history of the study subjects nor was the study designed to determine the variables that lead to the development of chemical immune reactivity. Lastly, this study is limited in term of external validity. A random sample of 95 healthy blood donors may not accurately represent the general population or any subsets of the population that may have increased vulnerability to disease. Due to these limitations, further research would be required to determine the exact relationship between chemical reactivity to TBBPA and the development of neurological disease in human subjects. However, the outcome of the study provides novel findings that serve to assist in

understanding the potential role flame retardant chemicals, such as TBBPA, may have with respect to human health and disease.

Summary

Autoimmunity is a condition in which the immune system erroneously attacks the body's own tissue. It is a devastating disease that impacts as much as 23 million Americans, according to the prevalence reported by the National Institute of Health (NIH Publication 05-5140). Chemicals may play a role in the epidemic of autoimmune disease. Researchers have found that chemical exposure, combined with an activated immune system or genetic risk, creates a recipe for the development of autoimmunity (Barragan-Martinez et al., 2012; Pollard & Kono, 2013). A significant concern in this regard is the group of chemicals that are flame retardants.

Flame retardants are sprayed on furniture, mattress beds, children's pajamas, car seats, upholstery, carpets, and rugs in the United States. The widespread use of flame retardant spraying was driven by the California Flammability Standard, Technical Bulletin 117 (TB117), which was instituted in 1975. TB117 states that a manufacturer was not permitted to sell items such as furniture, upholstery or mattresses in California unless they were sprayed with flame retardants. As a consequence of TB117, studies have found that these chemicals can be detected in household dust and serum concentrations in the population (Zota, Rduel, Morello-Frosh, & Brody, 2008).

Tetrabromobisphenol-A (TBBPA) has been classified as a brominated flame retardant (BFR), and since the beginning of its use in 1979, it has become the most widely used BFR worldwide, with an annual production of more than 210,000 tons (Alaee, Arias, Sjodin, & Bergman, 2003). Research conducted in 1979 initially found that TBBPA was readily metabolized from the body, and therefore, it was considered a safe compound without active

biological activity (Brady, 1979). However, subsequent research has found that TBBPA is not entirely metabolized upon exposure and that TBBPA can accumulate in human fluids over time (Jakobsson *et al*., 2002).

Not only was TBBPA found to accumulate in human fluids, but it was also later shown to build up in the adipose tissues of both animals and humans (Johnson-Restrepo, Adams, & Kannon, 2008). In a French human biomonitoring study of random women volunteers and their newborns, 44% of TBBPA was found in analyzed breast milk samples and 30% in both maternal and cord serum samples (Cariou, 2008). In a Japanese human mother-infant study, TBBPA was measured in maternal blood, maternal milk, cord blood, and umbilical cords. Researchers detected levels of TBBPA and concluded that the chemical could pass through the blood-placenta barrier and lead to perinatal exposure (Kawashiro, 2008). Recently, in a prospective study of 304 mothers and their children, flame retardants were measured in breast milk at three months' post-partum and again in 36 months: more than 70% of the subjects had detectable levels (Adgent, 2016). It appears that TBBPA is not as readily metabolized as had been first theorized in 1979, and it may contribute to the rising epidemic of chemical-induced neuroinflammation and neurological autoimmunity. This study was conducted to further investigate the potential theory that chemicals such as TBBPA may play a role in the worldwide epidemic of neurological autoimmunity.

A quantitative cross-sectional correlation research study was conducted to assess the relationships between human subjects with TBBPA bound to albumin antibodies with myelin basic protein (MBP) antibodies, myelin oligodendrocytic glycoprotein (MOG) antibodies, aquaporin-4 antibodies, alpha-synuclein antibodies, and S100B antibodies. These antibodies represent the target sites for neuroinflammatory and neurological diseases such as multiple

sclerosis, demyelinating diseases, PD, NMO, neuromyelitis optica spectrum disorder (NMOSD), and biomarkers for the breakdown of the blood-brain barrier (BBB). The goal of this study was to investigate whether there are any associations and risks between chemical, immune reactivity to TBBPA, found in flame-retardants, in human subjects and biomarkers used to diagnose inflammatory and autoimmune diseases of the nervous system. Non-identifiable, non-disease blood samples from 96 blood donors were for enzyme-linked immunosorbent assay (ELISA) analysis in a clinical laboratory. The ELISA methodology evaluated optical density measurements for antibodies to TBBPA bound to human albumin, myelin oligodendrocytic glycoprotein (MOG), myelin basic protein (MBP) alpha-synuclein, aquaporin-4 (AQP4), and S100B. Three separate immunoglobulin assays, which included IgG, IgA, and IgM, were tested for each antibody.

Statistical analyses were conducted to determine the correlation between the following variables:

(1) correlation between TBBPA bound to albumin antibodies and MBP antibodies

(2) correlation between TBBPA bound to albumin antibodies and MOG antibodies

(3) correlation between TBBPA bound to albumin antibodies and alpha-synuclein antibodies

(4) correlation between TBBPA bound to albumin antibodies and AQP4 antibodies

(5) correlation analysis will be performed between TBPA bound to albumin antibodies and S100B antibodies.

The presence of statistically significant correlative relationships was conducted with Pearson's r, Kendall's tau, and Spearman's rho, which are parametric and non-parametric association measures for IgG, IgA, and IgM independently. A Bonferroni correction was conducted with the

p-value to avoid a false discovery rate when testing for multiple comparisons. The adjusted alpha for statistical significance was set to 0.01.

Risk ratios were calculated by converting each of the optical density continuous variables into binary variables. One standard deviation above the mean for each continuous variable was classified as a significant binary value of one. Any value below one standard deviation of the mean was classified as a non-significant binary value of zero. Risk ratio analysis was conducted, labeling the TBBPA antibodies as exposure variables and the neurological target protein antibodies as the disease outcome variable for each of the 15 relationships.

The results of the study found a positive linear relationship that is statistically significant (p-value <0.0001) between TBBPA bound to human protein antibodies and myelin basic protein, myelin-associated glycoprotein, aquaporin, and alpha-synuclein with all three forms of immunoglobulins, such as IgA, IgG, and IgM. The degree of correlation ranges from moderate to significant correlation. The highest degree of association is with IgM. These correlations coefficients ranged from 0.85–0.92. Risk ratio analysis found a significant risk for the development of neurological antibodies with subjects that exhibited antibodies to TBBPA bound to human albumin. These risks ranged from a two-fold to a ten-fold increase risk even when using the lowest tail of the 95% confidence interval. In summary, the results of the investigation found that a significant linear association and risk in human subjects who exhibit antibodies to TBBPA with antibodies to a diverse list of neurological autoimmune target protein sites.

There are several limitations of this study. The data analysis in this study used correlational analytic procedures. Correlation is not causative, and this indicates that there is no distinct understanding if immune reactivity to TBBPA is causative of neurological autoimmunity. Overzealous immune responsiveness may occur due to loss of overall

immunological tolerance, leading to both neurological and chemical immune reactivity. Correlative statistical does not permit to statistically account for confounding and effect modification factors in statistical data analysis. Additionally, the conversion of the data from a continuous variable to a binary variable to determine risk ratios significantly reduced power in the statistical analysis of this study. Although the effect size of the risk ratio was considerable and statistically significant, the confidence intervals for the risk ratios were extremely wide.

The results of this study provide scientific, legislative, and clinical recommendations. Furthermore, study designs that provide greater causal inference regarding the role of TBBPA should be investigated. Although clinical trials provide the greatest evidence for causal relationships, they are ethically not permissible in toxicology studies with humans. However, other study designs, such as prospective studies and case-control studies, can provide greater evidence for the potential toxic role that TBBPA may play in autoimmune disease, and thus, should be considered.

The outcomes of this study suggest that measuring antibodies to TBBPA bound to human albumin may provide a different perspective regarding biomonitoring studies, which are used to evaluate the safety profile of TBBPA in humans and animals. These safety concerns regarding the use of TBBPA in modern society provide further evidence of legislative safety concerns recommendations of mandatory use of fire retardants. Despite a gradual shift to remove mandatory guidelines, there is an ongoing need to publish research that identifies safety concerns for flame retardants. Lastly, the findings of this study may impact the clinical management of patients suffering from neuroinflammatory and neuroautoimmune diseases that have been found with specific tissue antibodies used in this study (MOG, MBP, S100B, AQP4, and alpha-synuclein). Although there is not enough evidence from this study to suggest that these

chemicals play a direct causative role in the development of the neurological autoimmune diseases, this study does provide evidence that individuals who have antibodies to the target proteins found in this study are strongly associated with immunological reactivity to TBBPA. Whether TBBPA is causative or not, the co-occurrence of this immunological sensitivity does have a potential clinical consideration for the avoidance of toxic chemicals. Reducing exposure to TBBPA in the household by avoiding furniture, foam mattresses, and upholstery sprayed with TBBPA may reduce the exposure of an environmental immune trigger that has been found to be associated with neurological autoimmune target proteins investigated in this study. In summary, the outcomes of this research provide scientific, legislative, and clinical recommendations regarding the impact of TBBPA and human health risk.

Appendix A: Laboratory Permission Letter

December 26, 2016

Brianna Kent, PhD
Associate Chair, Department of Health Care Sciences
Director of PhD in Health Science Program
Health Professions Division College of Health Care Sciences
Terry Building 1216
3200 South University Drive
Fort Lauderdale, FL 33328

Re: Permission to Conduct Research for PhD Dissertation

Dear Dr. Kent,

I, Aristo Vojdani, PhD, Director of Immunosciences Laboratory, 822 South Robertson Boulevard #312, Los Angeles, California 90035, formally approve Datis Kharrazian, D.H.Sc. to conduct research with ELISA methodology to evaluate the association between TBBPA and neurological autoimmunity for his PhD research dissertation at Nova Southeastern University.

Sincerely Yours,

Aristo Vojdani, Ph.D.
Director of Immunoscienes Laboratory

Appendix B: Power Calculation

```
. power onecorrelation 0.3 0.6

Performing iteration ...

Estimated sample size for a one-sample correlation test
Fisher's z test
Ho: r = r0   versus   Ha: r != r0

Study parameters:

        alpha =    0.0500
        power =    0.8000
        delta =    0.3000
           r0 =    0.3000
           ra =    0.6000

Estimated sample size:

          N =        57


. power onecorrelation 0.3 0.7

Performing iteration ...

Estimated sample size for a one-sample correlation test
Fisher's z test
Ho: r = r0   versus   Ha: r != r0

Study parameters:

        alpha =    0.0500
        power =    0.8000
        delta =    0.4000
           r0 =    0.3000
           ra =    0.7000

Estimated sample size:

          N =        29



. power onecorrelation 0.3 0.8

Performing iteration ...

Estimated sample size for a one-sample correlation test
Fisher's z test
Ho: r = r0   versus   Ha: r != r0

Study parameters:

        alpha =    0.0500
        power =    0.8000
        delta =    0.5000
           r0 =    0.3000
           ra =    0.8000

Estimated sample size:

          N =        16
```

Appendix C: Pearson's Correlation Data IgA

```
. pwcorr TBBPA MBP MOG S100B Aquaporin4 αSynuclein , sig
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.6126 0.0000	1.0000				
MOG	0.6816 0.0000	0.3790 0.0002	1.0000			
S100B	0.6146 0.0000	0.2947 0.0039	0.8415 0.0000	1.0000		
Aquaporin4	0.6976 0.0000	0.4336 0.0000	0.6756 0.0000	0.6683 0.0000	1.0000	
αSynuclein	0.7225 0.0000	0.4647 0.0000	0.8354 0.0000	0.8191 0.0000	0.6739 0.0000	1.0000

Appendix D: Spearman's Correlation Data IgA

```
. spearman TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(rho p)
(obs=94)
```

Key
rho
Sig. level

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.3283	1.0000				
	0.0012					
MOG	0.7125	0.2220	1.0000			
	0.0000	0.0315				
S100B	0.6254	0.2244	0.8220	1.0000		
	0.0000	0.0297	0.0000			
Aquaporin4	0.6661	0.1636	0.6121	0.5207	1.0000	
	0.0000	0.1152	0.0000	0.0000		
αSynuclein	0.7203	0.2610	0.8249	0.8072	0.5973	1.0000
	0.0000	0.0111	0.0000	0.0000	0.0000	

Appendix E: Kendall's Correlation Data IgA

```
. ktau TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(taub p)
(obs=94)
```

```
┌─────────────────┐
│ Key             │
├─────────────────┤
│ tau_b           │
│ Sig. level      │
└─────────────────┘
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	0.9979					
MBP	0.2315	1.0000				
	0.0033					
MOG	0.5418	0.1596	0.9998			
	0.0000	0.0431				
S100B	0.4604	0.1674	0.6279	0.9991		
	0.0000	0.0350	0.0000			
Aquaporin4	0.4930	0.1183	0.4376	0.3761	0.9989	
	0.0000	0.1339	0.0000	0.0000		
αSynuclein	0.5314	0.1950	0.6459	0.6241	0.4341	0.9998
	0.0000	0.0138	0.0000	0.0000	0.0000	

Appendix F: Pearson's Correlation Data IgG

```
. pwcorr TBBPA MBP MOG S100B Aquaporin4 αSynuclein , sig

             |   TBBPA      MBP      MOG     S100B  Aquapo~4  αSynuc~n
-------------+------------------------------------------------------------
       TBBPA |   1.0000
             |
             |
         MBP |   0.8076   1.0000
             |   0.0000
             |
         MOG |   0.5042   0.4234   1.0000
             |   0.0000   0.0000
             |
       S100B |   0.3470   0.3339   0.3984   1.0000
             |   0.0006   0.0010   0.0001
             |
  Aquaporin4 |   0.8308   0.8411   0.6783   0.4302   1.0000
             |   0.0000   0.0000   0.0000   0.0000
             |
  αSynuclein |   0.7617   0.7027   0.6693   0.4369   0.8394   1.0000
             |   0.0000   0.0000   0.0000   0.0000   0.0000
```

Appendix G: Spearman's Correlation Data IgG

```
. spearman TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(rho p)
(obs=94)
```

Key
rho
Sig. level

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.7460 0.0000	1.0000				
MOG	0.5499 0.0000	0.5960 0.0000	1.0000			
S100B	0.5333 0.0000	0.6063 0.0000	0.6260 0.0000	1.0000		
Aquaporin4	0.7704 0.0000	0.8219 0.0000	0.7132 0.0000	0.5902 0.0000	1.0000	
αSynuclein	0.6277 0.0000	0.6645 0.0000	0.7052 0.0000	0.7201 0.0000	0.8002 0.0000	1.0000

Appendix H: Kendall's Correlation Data IgG

```
. ktau TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(taub p)
(obs=94)
```

```
┌─────────────┐
│ Key         │
├─────────────┤
│  tau_b      │
│  Sig. level │
└─────────────┘
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.5754	1.0000				
	0.0000					
MOG	0.3875	0.4285	1.0000			
	0.0000	0.0000				
S100B	0.3873	0.4365	0.4634	1.0000		
	0.0000	0.0000	0.0000			
Aquaporin4	0.5915	0.6438	0.5474	0.4290	1.0000	
	0.0000	0.0000	0.0000	0.0000		
αSynuclein	0.4656	0.4891	0.5187	0.5431	0.6217	1.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	

Appendix I: Pearson's Correlation Data IgM

```
. pwcorr TBBPA MBP MOG S100B Aquaporin4 αSynuclein , sig
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.8746	1.0000				
	0.0000					
MOG	0.8821	0.7495	1.0000			
	0.0000	0.0000				
S100B	0.9182	0.8402	0.8723	1.0000		
	0.0000	0.0000	0.0000			
Aquaporin4	0.8847	0.7802	0.9084	0.8700	1.0000	
	0.0000	0.0000	0.0000	0.0000		
αSynuclein	0.8468	0.7351	0.8929	0.8544	0.9623	1.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	

Appendix J: Spearman's Correlation Data IgM

```
. spearman TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(rho p)
(obs=94)
```

```
┌─────────────┐
│ Key         │
├─────────────┤
│  rho        │
│  Sig. level │
└─────────────┘
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.8430	1.0000				
	0.0000					
MOG	0.8610	0.7603	1.0000			
	0.0000	0.0000				
S100B	0.8242	0.7718	0.8732	1.0000		
	0.0000	0.0000	0.0000			
Aquaporin4	0.8445	0.8016	0.8699	0.8561	1.0000	
	0.0000	0.0000	0.0000	0.0000		
αSynuclein	0.8468	0.7941	0.8745	0.8647	0.9736	1.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	

Appendix K: Kendall's Correlation Data IgM

```
. ktau TBBPA MBP MOG S100B Aquaporin4 αSynuclein, stats(taub p)
(obs=94)
```

```
┌─────────────┐
│ Key         │
├─────────────┤
│  tau_b      │
│  Sig. level │
└─────────────┘
```

	TBBPA	MBP	MOG	S100B	Aquapo~4	αSynuc~n
TBBPA	1.0000					
MBP	0.6658	1.0000				
	0.0000					
MOG	0.6768	0.5704	1.0000			
	0.0000	0.0000				
S100B	0.6509	0.5958	0.6915	1.0000		
	0.0000	0.0000	0.0000			
Aquaporin4	0.6649	0.6176	0.7009	0.6956	1.0000	
	0.0000	0.0000	0.0000	0.0000		
αSynuclein	0.6638	0.6220	0.7044	0.6991	0.8642	1.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	

Appendix L: Risk Ratio TBBPA and MBP IgA

```
.  cs MBPcat TBBPAcat
```

	TBBPAcat Exposed	Unexposed	Total
Cases	7	3	10
Noncases	7	77	84
Total	14	80	94
Risk	.5	.0375	.106383

	Point estimate	[95% Conf. Interval]	
Risk difference	.4625	.1973008	.7276992
Risk ratio	13.33333	3.906828	45.50438
Attr. frac. ex.	.925	.7440379	.9780241
Attr. frac. pop	.6475		

```
                  chi2(1) =     26.81  Pr>chi2 = 0.0000
```

Appendix M: Risk Ratio TBBPA and MOG IgA

```
.  cs MOGcat TBBPAcat
```

	TBBPAcat Exposed	Unexposed	Total
Cases	10	4	14
Noncases	4	76	80
Total	14	80	94
Risk	.7142857	.05	.1489362

	Point estimate	[95% Conf. Interval]	
Risk difference	.6642857	.4228757	.9056958
Risk ratio	14.28571	5.197975	39.26175
Attr. frac. ex.	.93	.8076174	.9745299
Attr. frac. pop	.6642857		

```
                          chi2(1) =    41.48  Pr>chi2 = 0.0000
```

Appendix N: Risk Ratio TBBPA and S100B IgA

. cs S100Bcat TBBPAcat

	TBBPAcat		Total
	Exposed	Unexposed	
Cases	11	3	14
Noncases	3	77	80
Total	14	80	94
Risk	.7857143	.0375	.1489362

	Point estimate	[95% Conf. Interval]	
Risk difference	.7482143	.5292817	.9671468
Risk ratio	20.95238	6.678406	65.73459
Attr. frac. ex.	.9522727	.8502637	.9847873
Attr. frac. pop	.7482143		

chi2(1) = 52.62 Pr>chi2 = 0.0000

Appendix O: Risk Ratio TBBPA and Aquaporin IgA

```
.  cs Aquaporin4cat TBBPAcat

                   │ TBBPAcat
                   │  Exposed    Unexposed  │    Total
───────────────────┼────────────────────────┼──────────────
            Cases  │      9           5     │     14
         Noncases  │      5          75     │     80
───────────────────┼────────────────────────┼──────────────
            Total  │     14          80     │     94
                   │
             Risk  │ .6428571      .0625     │  .1489362
                   │
                   │  Point estimate        │ [95% Conf. Interval]
───────────────────┼────────────────────────┼──────────────
  Risk difference  │      .5803571          │  .3238201     .8368942
       Risk ratio  │      10.28571          │  4.041295     26.17872
  Attr. frac. ex.  │      .9027778          │  .7525546      .961801
  Attr. frac. pop  │      .5803571          │
───────────────────┴────────────────────────┴──────────────
                       chi2(1) =     31.66   Pr>chi2 = 0.0000
```

Appendix P: Risk Ratio TBBPA and Alpha-Synuclein IgA

```
. cs αSynucleincat TBBPAcat
```

	TBBPAcat		
	Exposed	Unexposed	Total
Cases	13	4	17
Noncases	1	76	77
Total	14	80	94
Risk	.9285714	.05	.1808511

	Point estimate	[95% Conf. Interval]	
Risk difference	.8785714	.7354624	1.02168
Risk ratio	18.57143	7.067243	48.80234
Attr. frac. ex.	.9461538	.8585021	.9795092
Attr. frac. pop	.7235294		

chi2(1) =   62.08   Pr>chi2 = 0.0000

Appendix Q: Risk Ratio TBBPA and MBP IgG

```
. cs MBPcat TBBPAcat
```

	TBBPAcat		
	Exposed	Unexposed	Total
Cases	9	5	14
Noncases	2	78	80
Total	11	83	94
Risk	.8181818	.060241	.1489362

	Point estimate	[95% Conf. Interval]	
Risk difference	.7579409	.524337	.9915447
Risk ratio	13.58182	5.554011	33.21307
Attr. frac. ex.	.9263722	.81995	.9698914
Attr. frac. pop	.595525		

chi2(1) =   44.02   Pr>chi2 = 0.0000

Appendix R: Risk Ratio TBBPA and MOG IgG

```
. cs MOGcat TBBPAcat
```

	TBBPAcat Exposed	Unexposed	Total
Cases	5	7	12
Noncases	6	76	82
Total	11	83	94
Risk	.4545455	.0843373	.1276596

	Point estimate	[95% Conf. Interval]	
Risk difference	.3702081	.0699441	.6704722
Risk ratio	5.38961	2.063681	14.07577
Attr. frac. ex.	.8144578	.5154289	.9289559
Attr. frac. pop	.3393574		

chi2(1) =   11.95   Pr>chi2 = 0.0005

Appendix S: Risk Ratio TBBPA and S100B IgG

`. cs S100Bcat TBBPAcat`

	TBBPAcat Exposed	Unexposed	Total
Cases	5	5	10
Noncases	6	78	84
Total	11	83	94
Risk	.4545455	.060241	.106383

	Point estimate	[95% Conf. Interval]	
Risk difference	.3943045	.0956333	.6929757
Risk ratio	7.545455	2.592786	21.95857
Attr. frac. ex.	.8674699	.6143145	.9544597
Attr. frac. pop	.4337349		

chi2(1) =   15.88 Pr>chi2 = 0.0001

Appendix T: Risk Ratio TBBPA and Aquaporin IgG

```
. cs Aquaporin4cat TBBPAcat

                 | TBBPAcat
                 | Exposed    Unexposed |      Total
-----------------+----------------------+-------------
           Cases |       8            1 |          9
        Noncases |       3           82 |         85
-----------------+----------------------+-------------
           Total |      11           83 |         94
                 |                      |
            Risk | .7272727    .0120482 |  .0957447

                 |    Point estimate    |  [95% Conf. Interval]
-----------------+----------------------+-------------------------
 Risk difference |       .7152245       |   .4509929     .9794562
      Risk ratio |       60.36364       |   8.322304     437.8317
  Attr. frac. ex.|       .9834337       |    .879841      .997716
  Attr. frac. pop|       .8741633       |
-----------------+----------------------+-------------------------
                         chi2(1) =    57.39   Pr>chi2 = 0.0000
```

Appendix U: Risk Ratio TBBPA and Alpha-Synuclein IgG

```
. cs TBBPAcat αSynucleincat
```

	αSynucleincat Exposed	Unexposed	Total
Cases	8	3	11
Noncases	4	79	83
Total	12	82	94
Risk	.6666667	.0365854	.1170213

	Point estimate	[95% Conf. Interval]	
Risk difference	.6300813	.3602863	.8998763
Risk ratio	18.22222	5.596219	59.3346
Attr. frac. ex.	.945122	.8213079	.9831464
Attr. frac. pop	.6873614		

chi2(1) = 40.22 Pr>chi2 = 0.0000

Appendix V: Risk Ratio TBBPA and MBP IgM

```
. cs MBPcat TBBPAcat
```

	TBBPAcat		
	Exposed	Unexposed	Total
Cases	8	3	11
Noncases	2	81	83
Total	10	84	94
Risk	.8	.0357143	.1170213

	Point estimate	[95% Conf. Interval]	
Risk difference	.7642857	.5132115	1.01536
Risk ratio	22.4	7.067182	70.9986
Attr. frac. ex.	.9553571	.8585009	.9859152
Attr. frac. pop	.6948052		

chi2(1) =   50.52 Pr>chi2 = 0.0000

Appendix W: Risk Ratio TBBPA and MOG IgM

```
. cs MOGcat TBBPAcat
```

	TBBPAcat Exposed	Unexposed	Total
Cases	8	4	12
Noncases	2	80	82
Total	10	84	94
Risk	.8	.047619	.1276596

	Point estimate	[95% Conf. Interval]	
Risk difference	.752381	.5003148	1.004447
Risk ratio	16.8	6.147579	45.91076
Attr. frac. ex.	.9404762	.8373343	.9782186
Attr. frac. pop	.6269841		

chi2(1) =   45.42   Pr>chi2 = 0.0000

Appendix X: Risk Ratio TBBPA and S100B IgM

```
. cs TBBPAcat S100Bcat
```

	S100Bcat Exposed	Unexposed	Total
Cases	9	1	10
Noncases	1	83	84
Total	10	84	94
Risk	.9	.0119048	.106383

	Point estimate	[95% Conf. Interval]	
Risk difference	.8880952	.7007157	1.075475
Risk ratio	75.6	10.65757	536.2725
Attr. frac. ex.	.9867725	.90617	.9981353
Attr. frac. pop	.8880952		

```
                        chi2(1) =    74.14  Pr>chi2 = 0.0000
```

Appendix Y: Risk Ratio TBBPA and Aquaporin IgM

```
. cs Aquaporin4cat TBBPAcat
```

	TBBPAcat		
	Exposed	Unexposed	Total
Cases	9	1	10
Noncases	1	83	84
Total	10	84	94
Risk	.9	.0119048	.106383

	Point estimate	[95% Conf. Interval]	
Risk difference	.8880952	.7007157	1.075475
Risk ratio	75.6	10.65757	536.2725
Attr. frac. ex.	.9867725	.90617	.9981353
Attr. frac. pop	.8880952		

chi2(1) =   74.14 Pr>chi2 = 0.0000

Appendix Z: Risk Ratio TBBPA and Alpha-Synuclein IgM

```
. cs αSynucleincat TBBPAcat
```

	TBBPAcat Exposed	Unexposed	Total
Cases	9	1	10
Noncases	1	83	84
Total	10	84	94
Risk	.9	.0119048	.106383

	Point estimate	[95% Conf. Interval]	
Risk difference	.8880952	.7007157	1.075475
Risk ratio	75.6	10.65757	536.2725
Attr. frac. ex.	.9867725	.90617	.9981353
Attr. frac. pop	.8880952		

```
                         chi2(1) =    74.14  Pr>chi2 = 0.0000
```

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