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A Preliminary Investigation of Neuroantibody Levels, Prevalence, and Environmental Factors in Patients with Neurological Dysfunction

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A Preliminary Investigation of Neuroantibody Levels, Prevalence, and Environmental Factors in Patients with Neurological Dysfunction

by

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Submitted to the faculty of the School of Sciences and Engineering in partial fulfillment
of the Degree of Master of Public Health
September 2021

Abstract

Nervous system damage comprises a large proportion of diseases worldwide. It is also the most difficult to diagnose. Biomarkers for nervous system damage are needed in order to diagnose these diseases early enough to allow for intervention. In this study, we recruited patients from two Egyptian hospitals with MCI, ET and NMS and health references. Serum autoantibody levels against neural proteins and heavy metals were analyzed and compared. Immunoglobulin G (IgG) antibodies against neurofilament H (NFH), Glial Fibrillary Acidic Protein (GFAP), Myelin Basic Protein (MBP) and alpha synuclein (a-SYN) and Immunoglobulin M (IgM) antibody against neurofilament light (NFL) were among the most significant promising biomarkers of nervous system damage. Among the heavy metals, Zn and Pb were the most significant showing an association with neurotoxicity and had a positive correlation with the aforementioned autoantibodies. To conclude, this is a case-control study of 94 patients to determine general biomarkers of nervous system damage. This study gives us a new insight about the pathogenesis of these diseases and provides a starting point for more research in this area of biomarkers research in neurodegenerative diseases.

Acknowledgements

List of Abbreviations and Symbols

A-beta   Amyloid beta

A-SYN   Alpha Synuclein

AD   Alzheimer's disease

APC   Antigen Presenting Cells

As   Arsenic

Ba   Barium

BMI   Body Mass Index

Cd   Cadmium

CJD   Creutzfeldt Jakob Disease

CK2   Casein Kinase 2

CNS   Central nervous system

Co   Cobalt

Cr   Chromium

CSF   Cerebrospinal fluid

Cu   Copper

DALYs   Disability adjusted life years

DLB   Dementia with Lewy Bodies

DSM IV   Diagnostic and Statistical Manual of Mental Disorders, 4th Edition

ELISA   Enzyme-linked immunosorbent assay

ET   Essential Tremor

FAT   Fast Axonal Transport

Fe   Iron

FTLD	Frontotemporal lobar degeneration
GFAP	Glial fibrillary acidic protein
ICD-10	International Classification of Diseases, Tenth Revision
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile range
MAG	Myelin-associated-glycoprotein
MBP	Myelin basic protein
MCI	Mild Cognitive Impairment
Mn	Manganese
MS	Multiple sclerosis
NF	Neurofilament
NFH	Neurofilament Heavy Chain
NFL	Neurofilament Light Chain
NFM	Neurofilament Medium Chain
NMS	Neuroleptic Malignant Syndrome
NS	Nervous system
Pb	Lead
PD	Parkinson's disease
PSD	Post-stroke dementia
TBI	Traumatic Brain Injury
VaD	Vascular dementia

YLL   Years of life lost due to disease

YLD   Years of life lost due to disability

Zn   Zinc

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I. Introduction and Background

Access to the Central Nervous System (CNS) in a non-invasive fashion poses a challenge to its routine study and detection of disease. While methods like spinal tap or imaging techniques such as MRI and CT to inform ourselves of the brain's condition offer advantages, they are costly and require highly-trained personnel for interpretation. This limits their utility in many locations. In recent years, attempts have been made to develop and validate blood-based methods, and in some reports, urine. However, to date, there is no validated blood-based biomarker that reflects nervous system (NS) damage, regardless of etiology, or any that may be disease -specific. The current study seeks to answer the first of these questions: can NS damage be indicated by a blood (serum)-based biomarker, irrespective of cause. This is what we refer to as a first-tier (Tier I) approach. A recent review, discusses the development of an algorithm that factors in blood- and CSF-based biomarkers to predict dementia (El-Fawal, 2014).

I.   **Nervous System Cellular and Structural Protein Heterogeneity Guides Biomarker Discovery**

1. **Nervous System Heterogeneity**

The NS heterogeneous architecture of neurons and glial cells, with their cell-specific proteins, and those that may be associated with disease processes, provide guidance for NS insult signatures. Glia, which include astrocytes, microglia, oligodendrocytes and

ependymal cells, play an important role in the brain as they provide structural and functional stability for the neurons. Microglia, derived from monocytes, have a protective and inflammatory role, and may participate in adaptive immunity as antigen-presenting cells (APC). This may play a role in leveraging the immune system to detect NS insult, as will be discussed later. It should be noted, however, is that whether neurons, astrocytes, or the myelin elaborated by oligodendrocytes and Schwann Cells, these cellular substrates play a role in the disease process, whether as targets, or in some cases effectors of the disease process. Each of these cell types have cell-specific proteins that are lost, or increase, as a result of insult. Indeed, detection of these proteins in CSF, plasma and/or serum has figured prominently in the quest for biomarker development over the course of forty years, or more (El-Fawal et al., 1999).

2. The Quest for Biomarkers of Nervous System Insult

Neurodegenerative diseases are progressive and therefore require timely intervention at the earliest point of the detection in order to intervene in this progression when suitable therapeutic paradigms are developed (Beach, 2017). Relying on signs and symptoms in these conditions for diagnosis is of little benefit since symptoms and clinical signs manifest only after a large number of neurons have degenerated. Therefore, the pressing question is detection in absence of overt clinical manifestations. Sensitive biomarkers, with target specificity and sensitivity, for early detection of these conditions, preferably non-invasive and providing high throughput are required. Until now there is no fully validated blood-based biomarker for neurodegeneration, although current diagnosis based on imaging, genetic testing, in the case of familial ND, cognitive assessment and psychiatric evaluation

provide invaluable insights in biomarker evaluation (Lausted et al., 2014). For many ND, like Alzheimer's Dementia, ultimately autopsy provides the definitive diagnosis.

Research in recent decades has focused on the use of cerebrospinal fluid (CSF) for ND diagnosis (Lausted et al., 2014). Due to intimate relationship with the CNS, it is thought to reflect any change occurring there instantaneously (Blennow et al., 2010). Nevertheless, access to the CSF requires an invasive technique (lumbar puncture) and is not regularly or routinely done (Hampel et al., 2011; Lausted et al., 2014). Until now, non-specific markers of injury include CSF:serum albumin ratio, which is used as a standard biomarker for BBB function, with an increased ratio representing damage. This is seen in neuroborreliosis, Guillian-Barré syndrome and vascular dementia (Blennow et al., 2010). High levels of neurofilaments concentration in the CSF are found in vascular dementia, frontotemporal dementia and normal pressure hydrocephalus (Blennow et al., 2010). Of note, is that validated biomarkers in the CSF are specific to the molecular pathology of disease (e.g., amyloid beta and tau in AD) (Blennow, 2017).

The blood (and the subsequently derived serum and plasma) is a common medium among all organs, making it an ideal vehicle to ferry proteins and biomolecules. While this makes it harder to analyze less abundant proteins, it does provide a substrate for detection of NS-specific proteins detection (Lausted et al., 2014). For example, neurofilament light (NF-L), which is a neuron specific protein, was found to highly correlate between plasma and CSF concentrations. Autoantibodies against NF-L were measured with an even better sensitivity (Blennow, 2017). Furthermore, the blood allows repeated measures to monitor any change in the biomarker and disease progression, using a panel of quantifiable blood-borne biomarkers (Lausted et al., 2014).

However, although these protein/antigens, such as the neurofilament (NF) triplet are released into the CSF and blood, these proteins don't have a long half-life due to degradation in the periphery. Table 1 (adapted from (El-Fawal, 2014)), gives examples of the limitations of some commonly assayed or suggested protein biomarker targets.

In our studies, the premise is that neuronal and glial proteins lost due to degenerative processes (see Figure 1) stimulate the immune system to produce antibodies. While the neuronal and glial proteins may be short-lived in blood (see Table 1 and 2) immunoglobulins, M and G have a longer half-life and are produced/elevated under persistent insult or degeneration. This is also important for follow-up monitoring. In PD for example, autoantibodies against α-synuclein were found in the serum at a higher prevalence compared to controls (Henchcliffe et al., 2011).

Table 1. Serum/plasma half-life of some proteins.

	Half life	**Reference**
NFL	3 weeks to a month or two	(Barry et al., 2007; Hepner et al., 2019)
GFAP	10-17 hours	(El-Fawal, 2014)
MBP	4 hours in plasma, 12 minutes in serum	(El-Fawal, 2014)
a-SYN	16 hours	(Gupta & Dawson, 2010)

Neuron-specific enolase	48 hours	(Pelinka et al., 2004)
S100β	20-120 minutes	(Ghanem et al., 2001; Townend et al., 2006)

Reproduced from (El-Fawal, 2014).

3. Capitalizing on the Immune System to Indicate Nervous System Damage

Given the significant global burden of neurological disorders of diverse etiologies, inherited, trauma, or environmental (Feigin et al., 2019), there is a pressing need for the development of reliable biomarkers for NS damage. A biomarker, by definition, can be physiological, biochemical, genomic, or a combination. It should provide high-throughput rapid screening, identifying risk in the absence of symptoms, ideally early in the disease pathogenesis. Success stories have included the incorporation of HbA1C and blood cholesterol for early indication of type II diabetes and atherosclerosis.

In light of the limitations posed by NS access, and challenges of assaying NS-specific proteins, in absence of overt clinical signs and their short half-lives, investigators in the Neurotoxicology and Biomarkers Laboratory, have proposed capitalizing on the immune system's humoral response to indicate NS insult, as a first tier approached. This is premised on the fact that neuroantigens (autoantigens) are processed in the lymphoid tissue to generate autoantibodies (i.e., neuroantibodies or NAb), with a longer half-life, depending on immunoglobulin (Ig) class or isotype. Assay of autoantibodies has long been recognized as an effective tool in the study of autoimmunity and autoimmune disease. Figure 1, adapted from (El-Fawal, 2014), summarizes the concept.
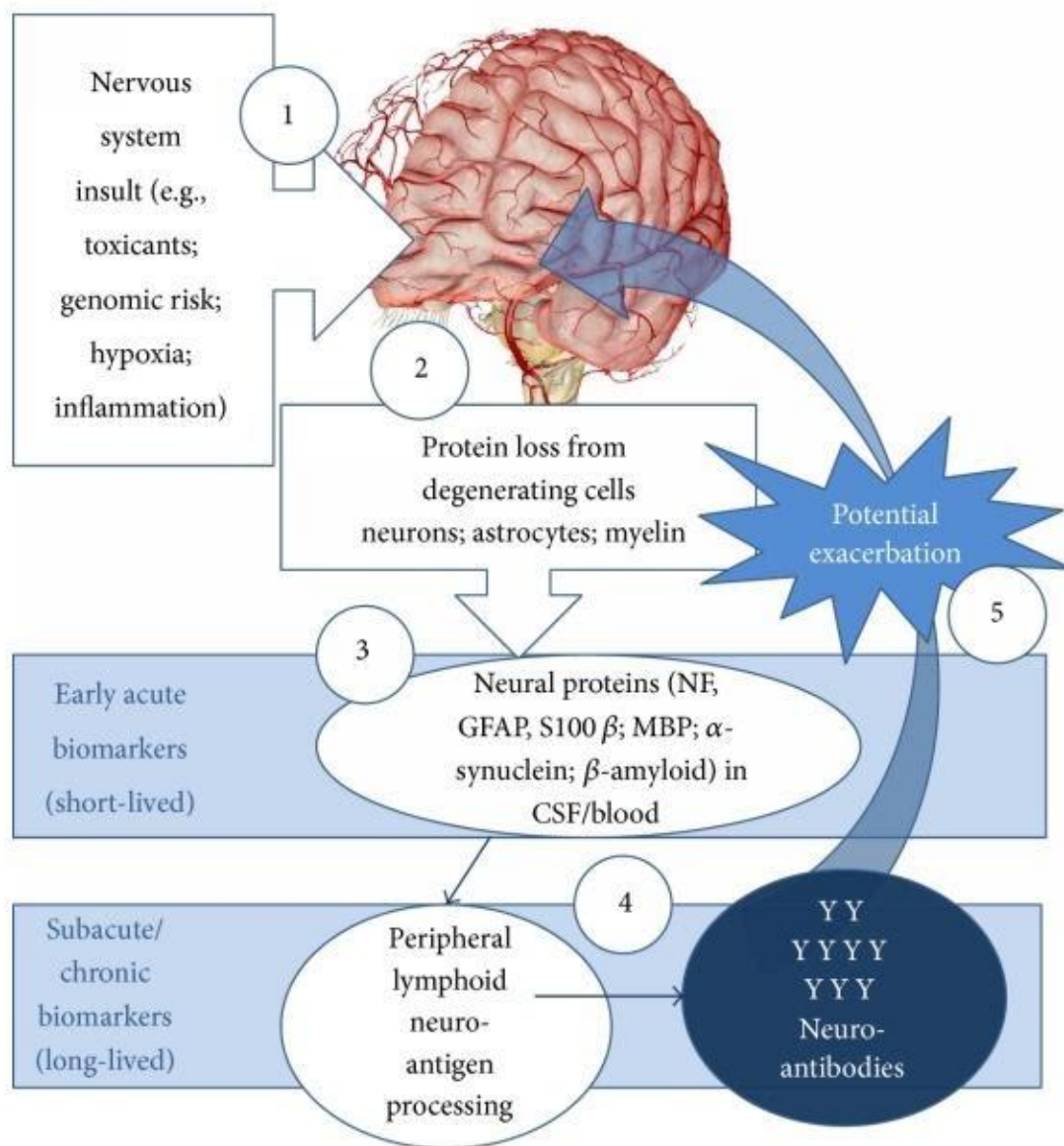
Figure 1. (1, 2) In the presence of toxicant-induced neurodegeneration, alterations in intracellular structural proteins and proteolysis, antigens (e.g., neuronal neurofilaments, α-synuclein, β-amyloid) are released into (3) the CSF and blood where (4) they are processed by antigen-presenting cells (APC) in the lymphoid tissue to induce humoral autoimmune responses. This autoimmune response, manifested in the form of autoantibodies, provides

an accessible biomarker of neurotoxic effects (El-Fawal, 2011). These antibodies may propagate neurodegenerative changes through complement activation and direct targeting of the neural and vascular architecture, particularly in the presence of increased vascular permeability. Modified from (El-Fawal, 2014).

The validation of such an approach would prove valuable for the early detection of *state* (i.e., the presence or absence of NS insult), and *stage* (i.e., severity and progression), and may provide a means of monitoring intervention (El-Fawal & McCain, 2008). It is important to note, that the Tier I approach does not answer the question of etiology, or identify specific neuronal substrates (i.e., cholinergic vs dopaminergic), but the absence or presence of insult. A Tier II approach, using cell-specific autoantigens, may be developed to identify unique neuronal populations that may be a source of autoantigens.

The present proof-of-concept research recruited patients with mild cognitive impairment (MCI), neuroleptic malignant syndrome (NMS), and essential tremor (ET) and analyzed their serum for neuro antibodies. It also determined IgM and IgG classes of these NAb.

4. IgM *vs* IgG: What do they suggest?

As is well established in adaptive humoral immunity, including vaccination, the primary challenge or exposure to antigen, with its processing by APCs, and the T-helper (CD4+) orchestrated cytokine release, B lymphocytes initially produce the pentameric IgM, with less affinity Fab, However, with the development of immunological memory, partially driven bey antigen concentration, plasma cells (the Ig-secreting B cells), are programed to switch classes from IgM to IgG. This occurs at the level of DNA programming. IgG has a longer half-life (23 days) than IgM (5 days) (Stavnezer, 1996). IgM is also characterized

by being a pentamer with multiple identical Fab epitopes, but possesses low affinity. Its continued generation, given its short half-life, is suggestive of chronic, or ongoing, insult, whereas IgG with its higher affinity and stability, is associated with immunological memory and the initial acute insult. It is the predominant circulating antibody.

II.   Neurodegeneration

As mentioned previously, 1 in 6 individuals suffers a neurological disorder, of diverse etiology and manifestations (Feigin et al., 2019). Sadly, the etiologies of most disorders, including neurodegenerative diseases (ND) and aside from direct trauma, are unknown, although gene-environmental interaction are suggested as strong determinants (Nelson et al., 2016). Neurons, the primary effectors of communication and body function, are the most vulnerable to insult because of their pronounced metabolic needs and the functional manifestations of their loss. In addition, their limited regenerative capacity and/or compensation depends on the location and severity of the insult. Interest in environmental factors such as heavy metals, pesticides and medications have drawn much interest in recent years as agents that may precipitate ND (Nelson et al., 2016).

As first described by Schaumburg and Spencer in 1976 (El-Fawal, 2011), neuropathologies may directly involve the neuronal cell body, irreversible neuronopathy, the axon, potentially reversible axonopathy, or myelin/myelinating cells, potentially reversible myelinopathy. However, in this Tier I approach, autoantigens common to all neurons, regardless of pathology, astrocytes and myelin, in addition to α-synuclein were selected. Their location, function and peripheral half-lives are summarized in Table 2.

Table 2. Capture Autoantigens used for Neuroantibody Detection in Tier I Studies.

Protein Antigen	Cellular Function
Neuronal	
Neurofilament Triplet (NF)	
NF-L	Neuronal intermediate filament (IF), in dendrites, soma and axon.
NF-M	Neuronal IF in dendrites, soma and axon.
NF-H	Neuronal IF, exclusive to the axon.
α-Synuclein	Multifunctional neuronal modulator; Association with PD and other ND.
Glial Fibrillary Acidic Protein (GFAP)	IF of astrocytes. A hallmark of astrogliosis and scar formation.
Myelin Basic Protein (MBP)	Myelin Compaction Protein of CNS and PNS (30% of myelin)

The present research tested the use of NAb detection against common neural cell protein substrates to indicate NS insult in subjects with diverse neurological conditions. Furthermore, the possible involvement or association of known neurotoxic environmental agents, heavy metals, as well as the influence of age and sex were determined. The neurological conditions included mild cognitive impairment (MCI), essential tremor (ET), and neuroleptic malignant syndrome (NMS). It is noteworthy that some of these conditions are reported to involve neuroinflammatory events and/or immune activation. The

prevalence and incidence, globally, and regional, whether in Egypt or the Middle East-North Africa (MENA) region, when available, are summarized in Table 3.

Table 3. The Global and Regional Burden of Dementia, Essential Tremor and Neuroleptic Malignant Syndrome.

Disease or Disorder (or risk factor)	Global Burden (Prevalence or Incidence)	Egypt and/or MENA Burden	Reference
Dementia	Incidence: 4.6 million new cases annually. Prevalence: 43.8 million people have dementia today.		(Ferri et al., 2005; Nichols et al., 2019; Who, 2008)
• AD	Prevalence: 588.8/100,000		
• VaD			(Global Burden Of Disease, 2017)
			(Who, 2008)

	Prevalence: 500-600 per 100,000 (varies according to population)		
• Ischemic stroke (CVD)			
Essential Tremors	Prevalence: 0.4-0.9% across all ages, 4.6–6.3% above age 60–65, and up to 21.7% above age 95.		(Ong et al., 2019; Sepúlveda Soto & Fasano, 2020)
NMS	Incidence: 0.4-1.4%		(S. Lee et al., 1989)

1. Dementia and Mild Cognitive Impairment (MCI)

The literature review regarding biomarkers will use the example of Alzheimer's Disease as in the context of biomarker research it has received the most attention, compared to vascular dementia which is also a form of dementia. Dementia is a syndrome, or a collection of symptoms, that has many potential causes. Dementia does not start suddenly; rather, it develops over years ("2017 Alzheimer's Disease Facts and Figures," 2017; Gauthier et al., 2006). Research shows that 50% of patients with dementia have MCI five years prior to dementia diagnosis. MCI is diagnosed when there is greater than expected decline in cognitive function at a specific age. It is distinguished from dementia in that it does not interfere with the daily activities and function of the patient (Gauthier et al., 2006). Some people with MCI may stay stable or recover.

The most common cause of dementia is Alzheimer's Disease or Alzheimer's Dementia (AD), followed by stroke ("2017 Alzheimer's Disease Facts and Figures," 2017). AD is usually preceded by MCI. It is at the MCI stage or even before it, when intervention would be most useful for AD patients (Blennow, 2017). Figure (2) below summarizes the MCI to AD/dementia continuum ("2020 Alzheimer's Disease Facts and Figures," 2020). Dementia is characterized by the gradual deterioration in memory, language, and cognitive skills, and eventually leading to loss of basic functions such as walking and swallowing and, ultimately death ("2017 Alzheimer's Disease Facts and Figures," 2017). Amyloid-beta accumulation intracellularly is the hallmark of AD and amnestic MCI. The deposition of amyloid-beta in MCI patients is thought to start in the neocortex and progress to involvement all the cortical areas such as the entorhinal cortex and subiculum, and eventually the basal ganglia, thalamus, hypothalamus, the midbrain, and medulla oblongata and finally the pons and cerebellum. It is assumed that some factors such as nuclear factor of activated T cells 3(NFAT3), which are prevalent in MCI patients, play a role in the amyloid-beta neurodegeneration early on in the development of dementia (Mufson et al., 2012). The cholinergic basal forebrain is also implicated in the pathogenesis of MCI, as well as AD, where the neurodegeneration of cholinergic neurons takes place early in the pathogenesis (Mufson et al., 2012). Intracellular soluble amyloid-beta is seen early in the AD pathogenesis and has been shown to cause neurodegeneration in the cerebral cortex, hippocampus, and amygdala. Some hypotheses claim that amyloid beta activates casein kinase 2 (CK2) and impedes fast axonal transport (FAT), which is essential for the neurons' survival, thereby leading to neurodegeneration. FAT is the machinery by which axons carry proteins and essential metabolites from and to the cell body (Pigino et al., 2009). Neuronal

loss was observed in the entorhinal cortex layer II in MCI patients, supporting the early

onset of neurodegeneration in these patients and later leading to dementia (Mufson et al.,

2012). Hippocampal synaptic plasticity, which is also important in the memory circuit, also

tends to diminish in MCI patients (Mufson et al., 2012).

Stage	Description
Preclinical AD	No signs or symptoms. Abnormal levels of beta-amyloid as shown on PET scans and in CSF, and decreased metabolism of glucose.
Mild cognitive impairment (MCI)	Mild symptoms that do not interfere with daily living 38% of individuals with MCI develop dementia after 5 years.
Mild AD	Symptoms interfering with a few daily activities.
Moderate AD	Symptoms interfering with many daily activities.
Severe AD	Symptoms interfering with most daily activities.

Figure 2. MCI-AD Continuum.

1.1 Types of dementia: Suspected Alzheimer's Dementia

Alzheimer's dementia is the most common cause of dementia. Specifically, 60-80% of dementia cases are due to AD. It is a progressive disease where neurons degenerate and die, due to amyloid beta fibrils accumulation outside, and tau proteins aggregation inside the neurons. Clinical signs and symptoms show after the neurons have already started to degenerate or have degenerated. That further underscores the imperative of early diagnosis ("2017 Alzheimer's Disease Facts and Figures," 2017). Other types of dementia include Dementia with Lewy bodies (DLB), vascular dementia (VaD), mixed dementia, Frontotemporal lobar degeneration (FTLD), Parkinson's disease (PD), normal pressure hydrocephalus and Creutzfeldt Jakob disease. ("2017 Alzheimer's Disease Facts and Figures," 2017).

VaD is caused by a stroke or bleeding in the brain. While it can coexist with AD, initial symptoms more likely include impaired planning, organizing, and decision-making rather than memory loss. Nearly half of the stroke survivors develop post-stroke dementia (PSD) approximately 25 years after experiencing their stroke. The prevalence of PSD in stroke survivors is 30% (Leys et al., 2005). There is no functional neuroimaging such as single-photon emission CT, PET, functional MRI, or spectroscopy capable of identifying predictors of PSD in the majority of patients with stroke ("2017 Alzheimer's Disease Facts and Figures," 2017; Leys et al., 2005). Stroke survivors also suffer cognitive decline which is characteristically rapid. In addition, PSD is associated with some specific comorbidities namely, age, hypertension, diabetes mellitus, cardiac dysfunction, recurrent stroke or low educational level (Leys et al., 2005; Pendlebury & Rothwell, 2009).

DLB's early symptoms include visual hallucinations and sleep disturbance, and parkinsonian movement features. It can also coexist with AD and VaD. Lewy bodies are aggregates of alpha-synuclein proteins, which are also seen in PD. But when Lewy bodies accumulate in the cortex of the brain, dementia results. The difference in both conditions is the onset of motor symptoms. In PD, motor symptoms show first, while in DLB psychiatric symptoms prevail. DLB comprises nearly 20% of all dementia cases. DLB comprises nearly 20% of all dementia cases ("2017 Alzheimer's Disease Facts and Figures," 2017; McKeith, 2007). Mixed dementia, as deduced from its name, is a collection of hallmarks from more than one dementia type ("2017 Alzheimer's Disease Facts and Figures," 2017).

1.1a Epidemiology of Alzheimer's Dementia

Dementia affects 40-50 million people worldwide. The global burden of disease statistics showed that dementia was the fifth leading cause of death worldwide in 2016, following ischemic heart disease, chronic obstructive pulmonary disease, intracerebral hemorrhage, and ischemic stroke. It is also the second leading cause of death in people over 70 years, secondary to ischemic heart disease. Taking into consideration that 28.8 million disability adjusted life years (DALYs) were lost due to dementia in 2016, this disease has an adverse economic impact. To illustrate, 818 billion dollars accounted for dementia medical costs in 2015, a figure that was estimated to reach a trillion in 2018 (Nichols et al., 2019; Vijayan & Reddy, 2016). Dementia also tends to be sex-biased, targeting women more than men and resulting in higher female mortality rates as well (Nichols et al., 2019). With a long prodromal phase of 20-30 years, dementia provides ample time for a myriad of factors to

act as candidates affecting the pathology of the disease, for example heavy metals as suggested by some and discussed in the current study. It should also be pointed out that dementia is a product of genes, environment, and lifestyle. Accordingly, there are specific risk factors linked to dementia, including high BMI, smoking, high fasting plasma glucose, and high intake of sugar-sweetened beverages (Nichols et al., 2019). At an older age, a steeper rate of increase was seen in the YLL (years of life lost due to disease) rates than the YLD (years of life lost due to disability). The prevalence of dementia increases with age, particularly between the ages of 50-80 a substantial increase is seen (Nichols et al., 2019). The diagnostic criteria for dementia include DSM-IV, ICD-10 and NINCDS-ARADA. This is in accord with established dementia evidencing neuropathology. Therefore, new drugs that are considered disease-modifying can never live up to their full potential, particularly since optimum efficiency in treating the disease relies on early pathogenesis, specifically in the amyloid beta aggregation phase. This argues for the vital role of early diagnostic biomarkers for dementia, which can decrease the prevalence by 9 million cases by 2050 if the onset was delayed by 1 year (Hampel et al., 2010).

1.1b Alzheimer's Dementia in Egypt

A systematic review by Elshahidi showed that studies published until 2016 showed that the prevalence of dementia in Egypt ranged from 2.01% to 5.07%. It was also notable that dementia was more common among illiterate groups (Elshahidi et al., 2017).

A door-to-door study in Al-Kharga District in Egypt showed that dementia prevalence increased with age, just like the rest of the world. In the population aged 80 and above, the prevalence of dementia reached 18.48 % while the prevalence in the population aged 50

and above was 2.26%. The most common type of dementia was found to be AD followed by vascular dementia (El Tallawy et al., 2012).

Another door-to-door study in Qena governorate in Egypt showed that the prevalence of dementia in people aged 60 and above was 1.72%, while the highest prevalence was among the age of 85 and above, which was 5%. It was also shown that living in industrialized areas and being illiterate are risk factors for dementia. In general, Qena shows lower prevalence of dementia compared to Egypt in general (Khedr et al., 2015). In Al-Quseir City, the prevalence of dementia was 2.01% and 3.83% in people aged 50 and above and 60 and above, respectively. AD was also the most common type among the population and lack of education was a risk factor for dementia (El Tallawy et al., 2013).

The prevalence of dementia in Egypt is similar to the rest of the world, and it increases with age. More research and epidemiological studies are needed to assess the magnitude of this public health issue, as in the previous 20 years not many studies have been found concerning this issue.

1.1c. Heavy Metals in Dementia

Chronic exposure to high- or low-level Pb results in CNS damage, cognitive impairment and neurological symptoms (Geiger & Cooper, 2010). Exposure to Pb was linked to accelerated cognitive decline and dementia (Bakulski et al., 2020). The neurotoxicity of Pb is exhibited by its excitotoxic effect on neurons resulting from the overactivation by calcium (Bakulski et al., 2020). Neuroinflammation and epigenetic changes were also noted due to Pb exposure (Bakulski et al., 2020). Amyloid-beta and tau protein, the hallmarks of AD, were found to be elevated in rats' brains when exposed to Pb. Memory

and learning impairment are also features of chronic Pb exposure (Bakulski et al., 2020). Higher Pb concentrations in bone were associated with lower MMSE scores in an epidemiological study (Bakulski et al., 2020).

Cadmium is a known neurotoxicant (both at high and at low-persistent levels), its effects include neuroinflammation and apoptosis in neurons (Bakulski et al., 2020). Specifically in AD, it is known to promote amyloid-beta aggregation and tau protein production (Bakulski et al., 2020). In addition, Cd promotes neurodegeneration by activating MAPK, protein kinase B, and mTOR pathways. Due to its high affinity to sulfhydryl groups, it binds to antioxidant molecules such as glutathione, inhibiting it, promoting oxidative stress (Bakulski et al., 2020). Postmortem studies showed elevated Cd levels in AD brains compared to controls. Blood Cd levels were also higher in AD patients compared to controls (Bakulski et al., 2020; Huat et al., 2019). Whole blood Cd level was also linked to lower cognitive function (Bakulski et al., 2020).

Mn is an essential trace element in our body (Bakulski et al., 2020). Nevertheless, long-term exposure to high levels of Mn is pathologic. It mainly affects the brain, seen as slowed visual reaction time, psychological disturbances and eye-hand coordination (Geiger & Cooper, 2010). Mn was also found to accumulate in dopamine-rich areas in the brain, which explains the side effect (tremors) seen in Manganism (Geiger & Cooper, 2010). Mn displays its neurotoxic effects by mechanisms including oxidative stress, accumulation of metabolites, apoptosis, and autophagy dysregulation. In AD, Mn increases amyloid-beta

levels by promoting its accumulation (Bakulski et al., 2020; Huat et al., 2019). Abnormal Mn levels were found in AD patients as well (Bakulski et al., 2020).

Cu is an important element in our bodies for redox reactions, it is especially essential for brain function (Huat et al., 2019). Genetic conditions such as Menkes disease results in cerebral and cerebellar neurodegeneration due to lack of Cu transport to the brain (Huat et al., 2019). On the other hand, Wilson's disease results in the deposition Cu in the brain and liver leading to neuropsychological deficits among other side effects (Huat et al., 2019). Cu concentration must therefore be tightly regulated (Huat et al., 2019). Postmortem studies revealed that Cu was present in the insoluble neuritic plaques in AD patients. Whole blood levels of Cu were also elevated in AD compared to controls (Huat et al., 2019). The role of Cu in AD seems to involve the production of amyloid-beta, and promoting its aggregation (Huat et al., 2019). It also promotes neuroinflammation by activating microglia by forming a complex with amyloid-beta (Huat et al., 2019).

Zn is an essential element in our body, it is found at the highest concentrations in the brain, as a structural or catalytic component (Huat et al., 2019). Concentrations of Zn above 300 nM were seen to aggregate amyloid beta. It was also associated with tau hyperphosphorylation (Huat et al., 2019). Oxidative stress is also another mechanism by which Zn contributes to AD pathology (Huat et al., 2019). Zn toxicity was seen to damage nerve endings in the nose (Geiger & Cooper, 2010).

Al is a known neurotoxin, causing memory deficits, cerebral impairment and loss of concentration (Huat et al., 2019). It exerts its toxic effects by looking for a negatively charged ooxygen donor such as the DNA, RNA or ATP affecting gene expression and energy metabolism. It also promotes the aggregation of cytoskeletal proteins such as the neurofilaments and MAPs due to their hyperphosphorylated states resulting in apoptosis and neurodegeneration (Huat et al., 2019). In AD, Al was found to promote the production and aggregation, inhibit the degradation as well as interact with tau protein (Huat et al., 2019). In addition Al affects the cholinergic system which plays a role in the pathogenesis of AD (Huat et al., 2019).

1.1d. Neuroantibody Precedent in Dementia and Alzheimer's Disease

Autoantibodies are found in the sera of human beings. Their level, type and specificity depends on different factors including sex, age, and chronic conditions (Wu & Li, 2016). In neurodegenerative conditions such as AD, the environment and lifestyle might also affect this. Autoantibodies against GFAP and microglia were found in high levels AD patients (Wu & Li, 2016). NAb against GFAP were found to be higher in AD patients compared to dementia and aged healthy controls. Antibodies against microglia were found at high concentration in the CSF of AD patients (Wu & Li, 2016). Autoantibodies can also play a pathogenic role in the pathogenesis of diseases, because they were rarely found in the brains of healthy individuals compared with AD patients (Wu & Li, 2016). Future immunotherapies can be developed in response to this latter hypothesis, which might halt the progression of this dreadful disease. Autoantibodies can also confer a protective effect. This is all still unknown and warrants for more vigorous research (Wu & Li, 2016). A new class of antibodies called functional antibodies which bind to receptors have been studied in vascular dementia. Their aim is to prevent the overstimulation of certain receptors which are related to disease, in order to prevent apoptosis/neurodegeneration. This is one of the therapeutic modalities that we might use in order to prevent/treat AD in the future (Wallukat et al., 2018).

1.2 Types of Dementia: Stroke and Vascular Dementia

Stroke is the second most common cause of dementia. The risk of stroke increases after the age of 55 (Vijayan & Reddy, 2016). Stroke can be either hemorrhagic or ischemic, 85% is ischemic. Ischemic stroke is the most common cause of vascular dementia. In fact, 70% of

dementia after stroke is vascular dementia (Raj N. Kalaria et al., 2016). About 20-25% of stroke patients develop dementia within a few months (3-12) after the incident (Barba et al., 2000; R. N. Kalaria & Ballard, 2001; Mijajlović et al., 2017; Pendlebury & Rothwell, 2009). 50% of stroke survivors develop delayed dementia over 5 years (R. N. Kalaria & Ballard, 2001). Elderly with an ischemic stroke medical history has a 5 fold risk of developing dementia compared to the elderly without ischemic stroke history (Tatemichi et al., 1994). Dementia does not have a single cause but it shows through the interplay of cerebrovascular disease, including infarct and hemorrhage (Savva & Stephan, 2010). The chance of developing dementia after first-ever stroke is lower than assumed. Long term cognitive impairment is predicted by the infarct size and prediabetic status (Johnen et al., 2020). Although AD represents the majority of dementia cases, it cannot be neglected that mixed dementia is present in many AD patients. The most common mixed dementia is Vascular dementia and AD. 43% of VaD patients present with A-beta deposits and neurofibrillary tangles. During autopsy, 87% of VaD patients had either AD alone (58%) or AD with cerebrovascular disease (42%). 40% of patients who have pathological features of VaD have concurrent AD pathology (Qian et al., 2012). Dementia after stroke can be seen on neuroimaging showing the following: silent brain infarcts, white matter changes, lacunar infarcts, and medial temporal lobe atrophy (Raj N. Kalaria et al., 2016). In a study by Honig et al, 1766 patients in New York were followed up for 5 years. The risk of AD development was measured and was found to increase with a history of stroke and diabetes, together and independently. The history of stroke combined with other vascular risk factors was found to be the most possible combination to lead to AD pathology (Honig et al., 2003). A retrospective study by Huang used data from Taiwan National Health Insurance

bank and. 1487 stroke, and 1402 non-stroke patients developed dementia after 5 years and the findings showed a 6.09 greater risk of dementia after stroke compared to controls (Huang et al., 2015). A systematic review measuring the excess risk of dementia in the population showed that strokes double the risk of dementia in the elderly. The increased risk did not correlate with any cardiovascular or demographic risk factors, but a higher risk was seen in patients with an APOE 4 allele. Reaching the age of 85 conferred both groups (with and without stroke) the same risk of dementia (Savva & Stephan, 2010). Early-onset dementia occurs within six months of the stroke incident. Delayed onset dementia occurs most commonly from 1-8 years post-stroke (Mok et al., 2016). A longitudinal cohort study by Mok, recruited 1007 patients where 88 (8.7%) patients developed early-onset dementia (less than 6 months), while 40 (4.4%) developed delayed onset dementia (Mok et al., 2016). A study by Yang, compared clinical findings and imaging findings in patients with and without incident dementia after stroke/TIA. Incident dementia would occur at 3-6 months after stroke/TIA. The findings showed that diabetes mellitus history, age, white matter changes and medial temporal lobe atrophy were associated with incident dementia (Yang et al., 2015).

Prevention is key to dealing with strokes and dementias. Prevention of strokes can subsequently prevent more than a third of dementias, with strokes being risk factors for dementia. This is not impossible, knowing that 90% of strokes and 35% of dementias are preventable (Hachinski et al., 2019).

In the context of the present study, it is important to note a precedent for the use of NAb detection. The presence of CNS antigens have been reported in the cervical lymph nodes 3 days post-stroke, with their levels corresponding to brain damage (Planas et al., 2012). Specific IgG antibodies were found in the CSF of 17.9% ischemic stroke patients in a retrospective study compared with 2.5% of controls (Prüss et al., 2012). Whether these antibodies contribute to the subsequent damage to the brain leading to cognitive decline is still unknown. A study in mice showed that MBP antibody titer was the only predictor of post-stroke cognitive decline (Becker et al., 2016). Anti MBP antibody titers were elevated in ischemic stroke patients 30 days after stroke. They were also associated with worse outcome (Shibata et al., 2012). Astrogliosis usually occurs in the tissue adjacent to where stroke or hemorrhage occurred in the brain. This is proven by the presence of antibodies against GFAP (Magaki et al., 2018).

2. Essential Tremor

Essential tremor (ET) is one of the most common neurological disorders (Louis, 2011). It is a specifically characterized pattern of kinetic tremor that is progressive over time (Louis, 2011, 2014). It can occur at any age and mainly affects the hands and/or head causing an action tremor (Louis, 2014; Louis et al., 2003). Some patients may experience the involvement of parts of the brain such as the cerebellum, basal ganglia and suffer from cognitive deficits (Louis et al., 2003). It is distinct from the age related enhanced physiologic tremor. Prevalence, nevertheless, increases with age; 20.5% in people who are in their 60's and 70's present with ET while prevalence in the age group above 40 is 4% (Louis, 2011, 2014; Louis et al., 2003). Both genetic and environmental factors contribute to the disease occurrence, which is found in familial or sporadic form (Louis, 2014; Louis

et al., 2003). Some of the loci which have been linked to ET are 2p22-25, 3q13, and 6p23 (Louis, 2014; Ong et al., 2019; Sepúlveda Soto & Fasano, 2020). Other genes found to increase the risk of developing ET are: LINGO1, SLC1A1/EAAT2, SORT1, SCN4A, NOS3, KCN52 (Sepúlveda Soto & Fasano, 2020).

Gene-environment interaction seems to play a significant role in the pathogenesis of ET. A case-control study showed that family history combined with agricultural work and frosted glass exposure were significantly associated with ET (Schaumburg & Spencer, 1976). Age at onset of ET was also lower in individuals who had a family history, who were exposed to iron-manganese alloys, or alcohol (Schaumburg & Spencer, 1976). Lead (Pb) is also a common toxicant which exposes individuals to the development of action tremors upon exposure (Louis, 2014; Louis et al., 2003). Pb toxicity destroys cerebellar Purkinje cells, and chronic Pb exposure leads to lower cognitive ability and intellectual performance (Louis et al., 2003). Lifestyle factors such as dietary factors including caffeine, meat, B-carboline alkaloids, alcohol or smoking have all been investigated as risk factors for ET, but the results are inconclusive, some may be protective, while others may be toxic (Ong et al., 2019).

2.1 Pathogenesis

The involvement of different brain structures in ET has been studied, but is not completely understood until now. The cerebellum seems to play a major part in the pathogenesis, as patients present with problems in tandem gait and balance. In addition, more than half of ET patients present with intention/cerebellar tremor of the hands and action tremor. The

intention tremor usually spreads to the head in 10% of the cases. Unilateral cerebellar stroke has been described to stop ipsilateral arm tremor in ET patients. Neuroimaging studies have supported this finding, where there's evidence of cerebellar hemispheric dysfunction in ET shown on MRI and PET scans (Louis, 2011). Autopsies comparing ET patients with controls have shown that the most common structural pathology was in relation to cerebellar involvement, which was present in more than 75% of the patients with ET. This included cerebellar degeneration, 6-fold swelling increase in Purkinje cell axon (which represents response to injury) also called torpedoes, a decrease in 40% of the number of Purkinje cells, Purkinje heterotopias, and dendrite swelling. This presents the first type of ET called "cerebellar ET". The second type of ET, called "Lewy body variant ET" involves Lewy bodies in the locus ceruleus without the presence of torpedoes in these brains. The Lewy bodies distribution is nevertheless different from PD, DLB and the normal aging pattern of distribution (Louis, 2011). ET patients are likely to develop PD late in life, and mild cognitive impairment was also known to be part of ET pathogenesis (Louis, 2011).

3. Neuroleptic Malignant Syndrome (NMS)

NMS is a rare syndrome in the form of adverse drug reaction which is potentially fatal. Neuroleptics are antipsychotic drugs (both typical and atypical) that are mainly the cause of this syndrome (Pileggi & Cook, 2016). The main trigger for this reaction is dopamine (D2) receptor blockade (Berman, 2011). The common manifestations of this syndrome include hyperthermia, muscle rigidity presented as part of extrapyramidal symptoms, mental status changes and autonomic dysfunction (Berman, 2011). NMS laboratory findings include an increase in creatinine phosphokinase due to rhabdomyolysis, iron

deficiency and metabolic acidosis. CSF and imaging studies are usually normal (Berman, 2011). The most common typical antipsychotics which can cause NMS include: haloperidol, fluphenazine, chlorpromazine, trifluoperazine, and prochlorperazine. Atypical neuroleptics can also cause NMS but have a lower risk. These include risperidone, clozapine, quetiapine, olanzapine, aripiprazole, and ziprasidone (Berman, 2011). Medications other than which are dopamine antagonists have also shown potential risk of inducing NMS. These medications include metoclopramide, promethazine, tetrabenzine, droperidol, diatrizoate, and amoxapine (Berman, 2011). Abrupt cessation or lowering doses of dopamine agonists such as levodopa in PD may have the same effect (Pileggi & Cook, 2016). General dysregulation in the dopamine pathway is generally a common factor in the NMS pathogenesis (Pileggi & Cook, 2016). Neuroleptic drugs themselves can cause adverse motor effect (extrapyramidal symptoms) such as parkinsonism, tremors, tardive dyskinesia and dystonia (Ward & Citrome, 2018).

3.1 Pathogenesis

There are two theories proposed to explain the pathogenesis of NMS, one is thought to be related to dysregulation in the central neuroregulatory mechanisms induced by the antipsychotics and the second is abnormal reaction of predisposed skeletal muscle. The effect on muscles is hypothesized to be due to calcium dysregulation and uptake by muscles (Adnet et al., 2000). Neurologic sequelae have been described in case studies where patients presented with brain damage after NMS attacks. The symptoms the patients presented with were suggestive of cerebellar and basal ganglia damage. These included nystagmus, ataxia, hypotonia, dysmetria, titubation, scanning speech, and pendular jerks

for cerebellar damage and abnormal involuntary movements such as choreoathetosis, dystonia, and myoclonus for basal ganglia damage (Chakrabarti et al., 2001; Lyons & Cohen, 2013). Generalized brain atrophy, especially the cerebellum, was also present on MRI imaging (Chakrabarti et al., 2001). Survival rate of NMS have increased over the years because of early detection and treatment of the syndrome. The survivors nevertheless may suffer the consequences, which might include neurologic sequelae (Chakrabarti et al., 2001). The author of this thesis suggests that NMS might confer an increased risk of PD. Extrapyramidal symptoms are common in NMS and they are very similar to PD symptoms.

Although not generally accepted, case studies suggest that there may be primary or secondary neuropathology in NMS. For example neurogenic degeneration has been proposed as a pathologic mechanism, because of increased protein levels found in CSF, resulting in persistent neurologic sequelae seen in NMS (Fukushima et al., 2020). Another case study reported an association with demyelinating disorders without the use of dopamine depleting drugs. This was reflected in the CSF of this patient which had increased MBP levels (Delgado et al., 2016). Most studies propose a central hypodopaminergic state that results from antagonism of dopamine-D2 receptor (specifically the basal ganglia) by antipsychotic medications as the cause of NMS (Anglin et al., 2010; Khanzode, 2017; Lyons & Cohen, 2013). Another suggestion is the sympathoadrenal hyperactivity as the cause (Anglin et al., 2010). In addition, NAb against neurotransmitter receptors have been reported. Indeed, (Anglin et al., 2010) has proposed a neuroimmunologic hypothesis for the development of NMS. It suggests that individuals with previous NMS have autoantibodies against neurotransmitter receptors, due to

antipsychotic medications interacting with endogenous proteins in the brain (Anglin et al., 2010).

4. Heavy Metals as Environmental Etiological Factors

Heavy metals are found naturally in the Earth's crust from which they are mined for industrial use. However, due to their wide use in numerous industries, aside from mining, there is occupational exposures, as well as being released into the environment through upstream industrial and agricultural processes, which contribute to chronic exposure of communities. These exposures have been shown to be associated with the development of cognitive disorders in children and nervous system disorders in the adults (Surendran & El-Fawal, 2008). Some heavy metals may disrupt the BBB and gain access to vulnerable primary neuronal targets, or secondarily by affecting glial function (El-Fawal et al., 1999). The patients in the current study were recruited from the Governorate of Mansoura, Sohag and the Delta area. Published papers indicated wide-spread environmental heavy metal exposures in the same regions our subjects were recruited from. Cadmium levels in pregnant women from Mansoura were far above the safe limit with an average of 3.28 mcg/dL which is less than the number in our study and average Cu level was 50.32 mcg/dL. Both of which are far from the safe levels (Motawei & Gouda, 2016). Drinking groundwater was assessed using 54 samples from different districts in that same governorate to measure some heavy metals levels including Mn, Pb, Cd, Zn, Cu and Co. Only two samples were not suitable for drinking due to increased Cd levels (Mandour & Azab, 2011b). Another study with the same aim showed increased Al, Pb and Cd (Mandour & Azab, 2011a). A study by (Mortada et al., 2002) measured blood Cd levels in healthy individuals in the Mansoura governorate and found it to be within the safe limits (2

mcg/dL). Blood Pb levels on the other hand were lower in the Mansoura population (124.3 mcg/L) compared to Cairo (180 mcg/L). This might be due to increased traffic in Cairo as mentioned by the researcher (Mortada et al., 2002). A more recent study by (Elwakil et al., 2017) in the Delta area (Mansoura) showed an elevated level of Pb, Cd, and As in the blood of cancer patients, which were corresponding to the increase in the levels of these heavy metals in the soil, edible plants and water samples tested in this study. Control groups in this study had 0.237 ppm blood level of Pb, 0.006 ppm blood level of Cd, and 0.019 ppm blood level of As. All of which exceeded the safe levels of 0.015, 0.005, and 0.005 ppm respectively for Pb, Cd and As (Elwakil et al., 2017). Living in Mansoura, close by the sea, allows for higher intake of seafood. Heavy metals such as As, Cd and Pb are known to deposit in some kinds of fish. This was assessed in a study by (Abd-Elghany et al., 2020) which found increased levels of these metals in shrimps and crabs. This concentration was significantly reduced after cooking (boiling or grilling), but this doesn't eliminate the health risk completely and might confer to an increased risk of environmental exposure to the population living in this area (Abd-Elghany et al., 2020). Since the neurotoxicity of most heavy metals is well established, these findings are summarized in Table 4.

Tables 4. Heavy Metals Exposures and Toxicity Summary.

Heavy Metal	Point Sources/Uses	Range or Cutoff	Toxicity
Aluminum (Al)	Cosmetics, drugs, food packaging, utensils, drinking	Serum levels in healthy	Dysfunction in motor movement,

	water, or construction and electrical industry (Bojanić et al., 2020).	individuals range from 1 to 3 µg/L ("Toxicological Profile for Aluminum," 2002).	locomotion and balance (Fernandes et al., 2021), memory and learning deficits (Silva et al., 2013), impaired cognition (Maya et al., 2016).
Arsenic (As)	Cosmetics, agriculture, paints, fungicides, insecticides, pesticides, herbicides, wood preservatives, cotton desiccants (Ratnaike, 2003), contaminated groundwater (Bjørklund et al., 2017).	Whole blood levels below 1 mcg/L (ATSDR, 2007).	Neurological problems such as peripheral neuropathy, tremor, hand-eye coordination, grip strength and finger tapping deficits (Rodríguez et al., 2003), cognitive impairment and peripheral

			neuropathy (Bjørklund et al., 2017).
Cadmium (Cd)	Tobacco consumption or contaminated substances (Branca et al., 2018).	Whole blood levels range from 0.1 to 4 mcg/L (Nordberg, 2009).	Cancer and organ system toxicity such as skeletal, urinary, reproductive, cardiovascular, central and peripheral nervous, and respiratory systems (Rahimzadeh et al., 2017).
Copper (Cu)	Windblown dust, volcanic eruptions, found in air due to suspended soils, combustion sources, and manufacturing or processing of copper-containing materials or mine tailings (ATSDR, 2004a).	Serum levels <120 mcg/g in females and <109 in males mcg/g (ATSDR, 2004b).	Liver and kidney damage, anemia, immunotoxicity, developmental toxicity, nausea, vomiting, and abdominal pain

			(Dorsey et al., 2004).
Chromium (Cr)	Ferrochrome production, ore refining, chemical and refractory processing, cement-producing plants, automobile brake lining and catalytic converters for automobiles, leather tanneries, contaminated water and food, and chrome pigments (Geiger & Cooper, 2010).	Serum levels range from 0.1 to 0.16 mcg/L (ATSDR, 2012).	Respiratory tract effects (shortness of breath, coughing, wheezing, ulcerations), gastrointestinal (vomiting, nausea, hemorrhage), liver, immune and kidney systems dysfunction (Geiger & Cooper, 2010).
Lead (Pb)	Batteries, paint pigments and industry (Bjørklund et al., 2017).	Whole blood levels range from 0.15 to 1.5 μmol/L (30-300 mcg/L) (National Center	Convulsions, ataxia and coma, anxiety, fatigue, cognitive dysfunction, depression (Bjørklund et al.,

		for Health Statistics, 2012).	2017), neurological deficits, manual dexterity, and psychomotor speed in adults (Louis et al., 2003).
Manganese (Mn)	Contaminated water or food (ATSDR, 2000).	Whole blood levels range from 4 to 15 µg/L (ATSDR, 2000). Serum levels range from 0.4 to 0.85 µg/L (ATSDR, 2000).	Neurotoxicity (Huat et al., 2019), tremors, difficulty walking, and facial muscle spasms (ATSDR, 2000).
Zinc (Zn)	Mining, metallurgic operations, releases to soil, smelter slags, wastes, mine tailings, coal and bottom fly ash, and the use of commercial products such as	Lower limit of normal fasting plasma Zn is 10.7 mmol/L	Nausea, vomiting, epigastric pain, lethargy, fatigue, Cu deficiency/ impaired immune

	fertilizers and wood preservatives (Roney et al., 2007).	(Nordberg et al., 2014).	function (Fosmire, 1990), neurotoxicity (Huat et al., 2019).

Arsenic (As). As was used previously in cosmetics and agriculture to protect crops from pests. As is used to manufacture paints, fungicides, insecticides, pesticides, herbicides, wood preservatives, and cotton desiccants in industry (Ratnaike, 2003). As exposure can be through diet inhalation or dermal contact (Manthari et al., 2018). In chronic exposure to As, its accumulation can occur in the brain (Ratnaike, 2003). Chronic environmental exposure to As inhalation due to working in industry can cause neurological problems such as peripheral neuropathy, tremor, hand-eye coordination, grip strength and finger tapping deficits (Rodríguez et al., 2003). Contaminated groundwater is another source of As intoxication which also led to cognitive impairment and peripheral neuropathy in the community who drank this water (Bjørklund et al., 2017). Associations between As exposure and neurodegenerative diseases have been shown in clinical studies (Bjørklund et al., 2017).

Pathology of As Exposures. Chronic As exposure is known to cause neurotoxicity. Some of the effects include concentration and learning difficulties, delirium and encephalopathy. As also affects astrocytes by reducing the expression of signaling proteins and it could also damage axons and white matter which later lead to degeneration (Abou-Donia et al., 2013). Previous studies have shown that As causes permanent neuronal damage (Manthari et al.,

2018). As can cross the BBB, specifically targeting tight junctions, and cause oxidative stress resulting in neuronal death (Manthari et al., 2018).

Lead (Pb). Lead is widely used in batteries and paint pigments, usually in older houses. It's chemical properties make it very useful in the industry as it has a low melting point and high vapor temperature. Blood lead levels below 250 µg/L were shown to have an effect on cognitive function in kids, so the new level set by the US is 150 µg/L (Bjørklund et al., 2017). Signs of Pb toxicity in acute cases include convulsions, ataxia and coma, while chronic intoxication can present as anxiety, fatigue, cognitive dysfunction or depression (Bjørklund et al., 2017). Associations between Pb exposure and neurodegenerative diseases have been shown in clinical studies (Bjørklund et al., 2017). Even low levels of lead below 10 ug/dL have been associated with neurological deficits, manual dexterity, and psychomotor speed in adults (Louis et al., 2003). *Pb pathogenesis*. Lead plays a role in the immune system by stimulating B-lymphocytes. In vitro it enhanced the production of antibodies. It can also stimulate T-lymphocytes to produce IL-2 which in turn increase the responsiveness of B-lymphocytes. Some studies even suggest that low levels of lead are capable of stimulating immunoglobulin production (El-Fawal et al., 1999).

Pb and Cd are both toxic heavy metals found in the environment and when they enter the human body, they stay there for a long time. There has been literature about their toxic effects on the human body separately, but only few assesses the combined toxicity. A study tested this effect on rats, which showed that a combination of Pb and Cd exposure affects learning and memory. Cd decreased the density of dendritic spines which is an important

post synaptic structure. This also affects synaptic plasticity on the long-term. Both Cd and Pb combined, affected learning and memory through altering the spine density. Cd on its own was not causal of neural dysfunction (Zhou et al., 2020).

Cadmium (Cd). Cd is one of the ubiquitous heavy metals found in the environment and is regarded as one of the most important occupational and environmental pollutants. It is mainly man made and enters our body through tobacco consumption or contaminated substances. It usually accumulates in the body because of its long half-life. Cd cannot penetrate the BBB of adults. Older studies demonstrated damage in the BBB as a mechanism of Cd in the pathogenesis of neurodegenerative diseases (Branca et al., 2018).

Zinc (Zn). Zn is an important transition metal in the human body. Fluctuation in its concentration in the plasma affects mainly the CNS and the immune system. It is neuroprotective and acts as a cofactor for over 300 enzymes including metalloproteinases (MMP). It regulates the immune system by affecting serum thymulin which is needed for the maturation of T helper cells. Both its deficiency and excess is implicated in AD. If Zn concentration is reduced intracellularly, destabilization of the microtubules occurs which further leads to tau release and neurofibrillary tangle formation. If Zn concentration increases intracellularly, it leads to A-beta formation and ROS generation (Mezzaroba et al., 2019) .

Copper (Cu). Cu is a vital element for many enzymes including dopamine β hydroxylase and monoamine oxidase which play a role in neurotransmitter synthesis. In brief, Cu is

hypothesized to play a role in AD because Cu in excess can cause accumulation of A-beta extracellularly. Intracellularly Cu can stimulate tau hyperphosphorylation and lead to the formation of intracellular tangles. Both of which are the hallmarks of AD. However, Cu deficiency is also problematic for the brain. This is obvious in genetic mutations causing loss of function of Cu transporters which present as neurologic manifestations in patients (Mezzaroba et al., 2019). In PD, Cu increases oxidative stress leading to Lewy body formation. Serum Cu concentration in PD patients was nevertheless not altered compared to controls in some studies (Mezzaroba et al., 2019). A meta-analysis in China showed higher serum Cu concentration in AD patients compared to references (D. D. Li et al., 2017; Z. X. Wang et al., 2015). Zn and Cu were both found in the rim of senile plaques of autopsied brains of AD patients in higher amounts compared to controls (Lovell et al., 1998).

Manganese (Mn). Mn is an essential element for development and cellular homeostasis. It is also important for the normal immune response. Mn is also a cofactor to many enzymes involved in neurotransmitter synthesis or glial and neuronal function. In excess, Mn can be toxic to the CNS. Manganism is the condition with symptoms similar to PD and results from overexposure to Mn. These extrapyramidal and cognitive symptoms surface from a neurodegeneration process. These arise due to the effect of Mn on the basal ganglia. The cerebellum is also one of the area affected by Mn toxicity. In AD, Mn plays a role in senile plaques formation by affecting the superoxide dismutase (SOD) scavenger system. Astrocytes are also a target for Mn, as are other glial cells (Mezzaroba et al., 2019). Iron-

Manganese alloy exposure was associated with an older age of ET onset in one study (Ong et al., 2019).

Aluminium (Al). Humans are exposed to Al through cosmetics, drugs, food packaging, utensils or drinking water. It is mainly used in the construction and electrical industry (Bojanić et al., 2020). Al in the peripheral blood is transported by the transferrin complex and it crosses the BBB by endocytosis. In cases of reduced renal or liver function, higher concentrations of Al can be found in the blood due to reduced filtration or lack of detoxification processes, respectively (Fernandes et al., 2021). Al was found in significantly higher levels in patients with neurodegenerative diseases compared to controls (Bojanić et al., 2020). In many diseases Al plays a role in the pathogenesis depending on where accumulation occurs. In AD for example, Al accumulation was found to be in the encephalon (Fernandes et al., 2021). A-beta was formed in the hippocampus of animals exposed to Al as were neurofibrillary tangles (Bojanić et al., 2020). Al also targets the substantia nigra pars compacta, affecting motor function, locomotion and balance (Fernandes et al., 2021). Al is also associated with parkinsonism, dementia and ALS (Bojanić et al., 2020). Al intoxicated rats showed a decrease in the number of astrocytes and neuronal loss in the hippocampus resulting in memory and learning deficits (Silva et al., 2013). Al is a known neurotoxin which exerts its effect in multiple ways. Some of the proposed mechanisms include oxidative stress, cell mediated toxicity, apoptosis, inflammatory events in the brain, glutamate toxicity, effects on calcium homeostasis, gene expression and Al induced Neurofibrillary tangle (NFT) formation (Maya et al., 2016). Most notably, Al acts as a cholinotoxic agent that affects cholinergic and noradrenergic

neurotransmission. It induces dysfunction the cholinergic system which is important for memory and cognition (Maya et al., 2016). Al binds to phosphorylated amino acids leading to the aggregation of cytoskeletal proteins, neurofilaments and microtubule associated proteins. This leads to death of neurons and glial cells (Maya et al., 2016).

III.   Hypothesis and Specific Aims

In light of the above, and the imperative for the development and continuous validation of biomarkers, the two-phased hypothesis tested was:

Neuroantibodies (NAb) have a higher frequency of prevalence and titer levels in subjects with diagnosed neurological conditions, compared to a reference group with no overt neurological involvement. Levels of NAb are modified by intrinsic risk factors, age and sex, and extrinsic risk factors, environmental exposure, as indicated by biomarkers of exposure (i.e., blood heavy metal levels) given that these are potential risk factors for neurodegenerative changes.

Specific Aims

Specific Aim 1. Determine the prevalence and levels of NAb against neuron-specific (NF-L, NF-M and NF-H) and glia-specific (GFAP and MBP) antigens in subjects with diagnosed NMS, MCI/Stroke and Essential Tremors, compared to a reference group with no overt neurological involvement;

Specific Aim 2. Determine the statistical association between whole blood levels of known neurotoxic metals and levels of NAb titers. Delineate the dose-response relationship based on stratification;

Specific Aim 3. Delineate the association between subject sex and age, regardless of diagnosis, as determinants of NAb prevalence and titer levels.

III. Experimental Design and Methods

1. Ethical Considerations

This study gained the approval of the International Review Board at the American University in Cairo and from the ethical committees at each respective hospital. An informed consent was obtained from all participants in the study.

2. Patient Recruitment

A total of 96 subjects were recruited by clinicians at Delta University Hospital, Mansoura University Hospital, and Sohag University Hospital. Demographic (Table 6) and medical history collection and examination were conducted by a team of neurologists as follows: MMSE examination (ElKholy et al., 2019; Folstein et al., 1975), NMS diagnostic criteria (Five & Online, 2013), and movement disorder society criteria for tremor (Deuschl et al., 1998). The DSM-5 and MERCK manual for neurological diseases were used by the clinicals for diagnosis. This criteria is summarized in Appendix 1. Whole blood samples were obtained from the reference group (n=30), MCI (n=18), NMS (n=30) and ET (n=18) patients at time of examination and serum separated according to standard Clinical

Pathology Laboratory protocols at those hospitals, or on receipt on ice, at the Neurotoxicology and Biomarker Laboratory. They were all initially stored at -20°C until arrival to our lab, then stored in the -80° c until day of NAb or heavy metal analysis. In the case of NMS, the history of neuroleptics taken was collected, and is summarized, with their classification and association with NMS, in the table 5 below.

Table 5. Neuroleptics taken by patients diagnosed with NMS.

Antipsychotic Medication	Classification	Duration (range in years)	Association with NMS Y=yes; N= no
Haloperidol	Typical		Y
Chlorpromazine	Typical		Y
Trifluoperazine	Typical		Y
Risperidone	Atypical		Y, but less than the typical agents
Olanzapine	Atypical		Y, but less than the typical agents
Aripiprazole	Atypical		Y, but less than the typical agents
Quetiapine	Atypical		Y, but less than the typical agents
Clozapine	Atypical		Y, but less than the typical agents

3. Autoantibodies Measurement

The concentration of antibodies against 6 different antigens was measured using the method described by (El-Fawal et al., 1996). Purified human proteins NFL, NFM, NFH, GFAP and a-synuclein (American Research Products, Belmont, MA) and MBP (Sigma-Aldrich, St. Louis , MO) were set using 10 mM (pH 7.4) Tris buffer saline at a concentration of 2.5 ug/ml and at 25 ug/ml for MBP. 100 microliter of the purified protein was injected in each well of the 96-well microtiter plate overnight at 4 degrees Celsius. After 24 hours, we washed the microtiter plate three times with tris and triton X 0.05%. Then, non-specific binding sites were blocked using blotto (tris and skimmed milk). Dilutions of the patients serum samples were prepared by adding blotto. 100 microliters of the 1:100 diluted samples were added to each well in the microtiter plate. Then they were incubated for 2 hours. Half an hour before incubation was over, IgG antibodies were prepared, by mixing with blotto and at the 2 hour timepoint, the samples were discarded. Subsequently washing was carried out using tris and tween before adding 100 microliter of the antibody solution. After 30 minutes incubation, washing with blotto and tween 3 times and with tris 2 times was done. Alkaline phosphatase substrate was added after that. plates were read at 405 nm in a plate reader after 30 minutes and 40 minutes of adding the substrate.

4. Heavy Metals Measurement

Sampling. Whole blood was obtained from MCI and ET patients, and serum from the NMS and reference group. Serum was obtained by centrifugation of the whole blood samples. Disinfection with 70% isopropyl alcohol was carried out before disposable stainless steel needles were used for blood sampling. Samples collected were stored at −80℃ until they were used in the test. The test was only performed after the samples were thawed at room temperature.

Reagents. Calibration standards were prepared using Agilent Technologies ICP-OES Wavelength Calibration Solution 5mg/L Al, As, Ba, Cd, Co, Cr, Cu, Mn, Pb, Se, Zn in 5% HNO3/tr. Concentrated, 68% (v/v) nitric acid and ultrapure deionized water were used for all sample preparation and analysis procedures.

Whole blood sample preparation. The whole blood of patients and controls were kept at −80℃. Right before the analysis, they were thawed at room temperature. The protocol for whole blood digestion was adapted from Lee et al. (J. Y. Lee et al., 2012) To analyze heavy metals with ICP-AES, 1 ml of whole blood was mixed with 2 ml of nitric acid and 0.2 ml of hydrogen peroxide and heated for 2 hours at a temperature of 110 degrees, and for 1 hour at 250 degrees. Then the samples which had a volume of less than 7 ml were diluted with ionized water until they reached 7 ml in order to be analyzed by the ICP-AES. This was all conducted in the hood.

Serum sample preparation. The protocol for serum digestion was adapted from Lim et al. (Lim et al., 2019) In brief, 200 µL were then transferred to acid washed digestion vessels and 3 ml nitric acid was added to each vial. Digestion was performed on a hotplate using 180℃ for 30 minutes or until clear. After cooling to room temperature, the solutions were diluted to a final volume of 10 mL with deionized water. Sample analysis was performed

by Agilent Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Working standards were prepared by serial dilution of standard stock solutions with 2% nitric acid.

5. Statistical analysis

Initially data was tested for normal distribution. Given the sample size/diagnosis, non-parametric statistics were used, where median levels of NAb titers or heavy metal levels were compared either using Mann-Whitney U-test or Kruskal-Wallis test. Spearman Correlation was used to determine association between titers and determinants of heavy metals, sex and age. For these determinants, stratifications into tertiles was based on interquartile ranges (IQR) and graphically represented as boxplots.

The odds ratio (OR) and the relative risk/risk ratio (RR) are measures of association. They compare between exposure of two groups and the health outcome. Generally, an RR or OR above 1 indicate an increased risk/odds of a specific outcome among the exposed compared to the unexposed. RR compares the risk of the event (finding antibodies) in the group of interest (MCI, NMS and ET groups) with the risk of finding antibodies among the reference group. While OR represents the probability that an event will occur (antibody presence) in the presence of certain exposure (disease groups i.e. nervous system damage) compared to the probability that the event will occur (antibody presence) in the absence of this exposure (reference group with no nervous system damage). In this study, we examined the risk/odds of detecting autoantibodies against neural proteins in those diagnosed with ET, NMS or MCI, compared to the references.

OR and RR were determined according to the following formulae:

Relative Risk (RR), the ratio of the probability of NAb detection occurring in the disease group versus the probability of NAb detection occurring in the non-disease group, was calculated using MedCalc Software Ltd. Relative risk calculator. [https://www.medcalc.org/calc/relative_risk.php (Version 20.010; accessed August 26, The relative risk (RR), its standard error and 95% confidence interval are calculated according to Altman, 1991].

The relative risk or risk ratio is given by

$$RR = \frac{a/(a+b)}{c/(c+d)}$$; where a= number positive for NAb in disease group; b= number negative for NAb in disease group; c= number with positive NAb in reference group; and d= number with negative for NAb in refence group.

with the standard error of the log relative risk being

$$SE\{\ln(RR)\} = \sqrt{\frac{1}{a} + \frac{1}{c} - \frac{1}{a+b} - \frac{1}{c+d}}$$

and 95% confidence interval

$$95\% \text{ CI} = \exp\left(\ln(RR) - 1.96 \times SE\{\ln(RR)\}\right) \quad \text{to} \quad \exp\left(\ln(RR) + 1.96 \times SE\{\ln(RR)\}\right)$$

Odds Ratio (OR), the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure was calculated using MedCalc Software Ltd. Odds ratio calculator. https://www.medcalc.org/calc/odds_ratio.php (Version 20.010; accessed August 26, 2021)

$$OR = \frac{a/b}{c/d}$$

$$= \frac{a \times d}{b \times c}$$; where a= number positive for NAb in disease group; b= number negative for NAb in disease group; c= number with positive NAb in reference group; and d= number with negative for NAb in refence group.

with the standard error of the log odds ratio being

$$SE\{\ln(OR)\} = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}$$

and 95% confidence interval

$$95\% \ CI = \exp\left(\ln(OR) - 1.96 \times SE\{\ln(OR)\}\right) \quad to \quad \exp\left(\ln(OR) + 1.96 \times SE\{\ln(OR)\}\right)$$

Values of $p \leq 0.05$ were considered statistically significant.

IV.   Results

Only statistically significant results (p value below or equal to 0.05) will be mentioned.

1. Patient Demographics and Clinical History

Out of the 96 subjects, nineteen patients were clinically diagnosed with MCI and presented with a history of stroke. Thirty patients were clinically diagnosed with NMS and nineteen patients were diagnosed with ET. Twenty-eight reference individuals were chosen after no signs of neurological deficits were found on the neurological examination.

Table 6 summarizes the demographics of the sample population studied. As expected from the clinical diagnosis, those with neurological disorders were likely to be older (52.25) than the reference group (36.7).   Although not significant overall (p=1.26). There were significant age differences between age of those in the different diagnoses. The sex distribution was similar in all groups except the ET population, which had a very low

percentage of females compared to males. We did not have data about the smoking status of the ET and MCI populations, but we did for the NMS and reference groups. The reference group had a slightly higher smoker percentage than the NMS group.

Table 6. Demographics for the whole study group. ET, essential tremor; SD, standard deviation; MCI, mild cognitive impairment; NMS, neuroleptic malignant syndrome.

	n	Average age (SD) [Range]	Sex (%F)	Smokers	Duration of Diagnosis years (SD)
Reference	28	36.7 (9.7) [17-53]	46.40%	57.10%	NA
		NMS>Reference (p=0.0001) MCI>Reference (p<0.00001)			
Essential Tremor	19	44.7 (21.7) [10-83]	11.10%	NA	11.4 (11.9)
		MCI > ET (p=0.00018)			
NMS	30	46.6 (8.5) [33-68]	43.30%	46.70%	4.6 (1.7)
		MCI>NMS (p<0.00001)			

MCI/Stroke	19	69 (6.7) [51-79]	38.90%	NA	NA
SUM	96				

Of the 30 NMS patients 17 had bipolar disease, 8 had schizophrenia and 5 had schizoaffective disorder. Table 7 presents the data of medication distribution among the NMS population. Nearly half of the NMS population were on both atypical and typical antipsychotics. 60% of them were on Olanzapine and nearly half were on either risperidone or trifluoperazine or both. From the other medications, 60% of the patients were on biperiden, which is an anticholinergic drug used in the treatment of PD and neuroleptic-induced extrapyramidal symptoms (Kostelnik et al., 2017). Other medication the patients were on were Lithium and benztropine.

Table 7. Distribution of medications among the NMS group.

	Condition	Antipsychotics	Other medications		Distribution of medications among the whole NMS population
	Bipolar (56.7%)	58.8% Risperidone or Aripiprazole	70.6% Valproate	General	46% Both
		52.9% Olanzapine	47.1% Benztropine		40% SGA

NMS					
		41.2% Trifluoperazine	29.4% Lithium		13.3% FGA
		35.3% Clozapine	41.2% Biperiden		
		11.8% Chlorpromazine		Specific antipsychotics	60% Olanzapine
		11.8% Quetiapine			46.7% Risperidone
	Schizophrenia (26.7%)	75% Trifluoperazine	75% Lithium		46.7% Trifluoperazine
		50% Olanzapine	75% Biperiden		20% Clozapine
		25% Chlorpromazine	50% Valproate		16.7% Chlorpromazine
		12.5% Risperidone			
	Schizoaffective (16.7%)	60% Olanzapine and Risperidone	100% Biperiden	Other medications	60% Biperiden
					36.7% Lithium
		20% Chlorpromazine and Olanzapine and Risperidone	80% Valproate		33.3% Benztropine
		20% Trifluoperazine and Olanzapine and Aripiprazole			13.3% Valproate

SGA, Second Generation Antipsychotic. FGA, First Generation Antipsychotic. NMS, Neuroleptic Malignant Syndrome.

Table 8 summarizes the MCI population description. Memantine is a medication which acts on the NMDA receptors, thus reducing the neurotoxicity associated with glutamate overload. Donepezil and rivastigmine are both acetylcholinesterase inhibitors which improve cognition, behavior or functional capacity. Nevertheless, none of them affected the progression rate in AD. (D. D. Li et al., 2019) 16/18 of the patients in the MCI group have suffered some kind of hematoma leading to hemiparesis on one side of the body, most of which were left. As for the comorbidities, HTN and DM were the most common in all the MCI patients, three presented with IHD, one with atrial fibrillation, and two with bronchial asthma. Nearly all of the MCI patients suffered previous strokes. The prevalence of DM and HTN in these patients are well associated with post-stroke dementia. (Leys et al., 2005; Pendlebury & Rothwell, 2009) Some of the MCI population had lacunar infarcts which were seen on neuroimaging in patients with dementia after stroke. (Raj N. Kalaria et al., 2016)

Table 8. MCI population description.

	MMSE average (SD)	Drugs	History	Comorbidities
MCI	18.3 (3.4)	77.8% Memantine	72.2% Left side hemiparesis	61.1% HTN
		22.2% Donepezil	11.1% Right side hemiparesis	44.4% DM
		16.7% Rivastigmine		22.2% IHD or AF
				11.1% Bronchial asthma
				5.6% Pneumonia

				5.6% Liver cirrhosis
				5.6% Benign prostatic hyperplasia

Further information about the ET group was lacking.

2. Sensitivity and specificity of ELISA for NAb.

    Table 9 and Figures 3 A-F show the ROC values and graphs which represent the sensitivity and specificity of the ELISA technique for NAb measurement. Noticeable is that the specificity is high for most of the IgM antibodies, except for GFAP and a-SYN. While the IgG showed high sensitivity and specificity for all antigens except NFM. The assay seems to be sensitive and specific overall, so we find the data to be highly reliable

Table 9. ROC values representing the sensitivity and specificity of ELISA for NAb (determined using MedCalc software).

	NF-L IgM	NF-L IgG	NF-M IgM	NF-M IgG	NF-H IgM	NF-H IgG	GFAP IgM	GFAP IgG	MBP IgM	MBP IgG	SYN IgM	SYN IgG
Area under the ROC curve (AUC)	0.803	0.73	0.538	0.583	0.934	0.757	0.698	0.856	0.65	0.766	0.588	0.804
Significance level P (Area=0.5)	<0.0001	0.0001	0.5352	0.1544	<0.0001	<0.0001	0.0003	<0.0001	0.0082	<0.0001	0.0359	<0.0001
Sensitivity	67.65	76.47	26.47	26.47	88.24	82.35	91.18	79.41	54.41	79.41	97.06	75
Specificity	92.86	78.57	96.43	100	85.71	64.29	46.43	85.71	78.57	78.57	21.43	82.14

Figure 3. ROC graphs representing the sensitivity and specificity of ELISA for NAb (determined using MedCalc software).

A. Anti-NF-L

B. Anti-NF-M

C. Anti-NF-H

Test_5

Test_6

D. Anti-GFAP

Test_7

Sensitivity: 91.2
Specificity: 46.4
Criterion: ≤1.536

AUC = 0.698
P < 0.001

Test_8

Sensitivity: 79.4
Specificity: 85.7
Criterion: >21.2

AUC = 0.856
P < 0.001

E. Anti-MBP

Test_9

Sensitivity: 54.4
Specificity: 78.6
Criterion: ≤16.03

AUC = 0.650
P = 0.008

Test_10

Sensitivity: 79.4
Specificity: 78.6
Criterion: >77.35

AUC = 0.766
P < 0.001
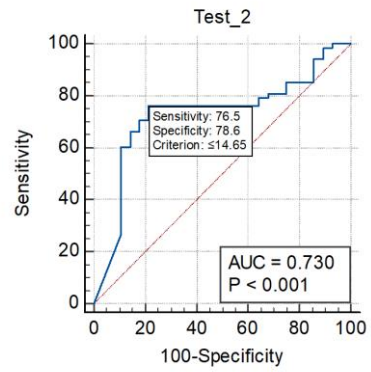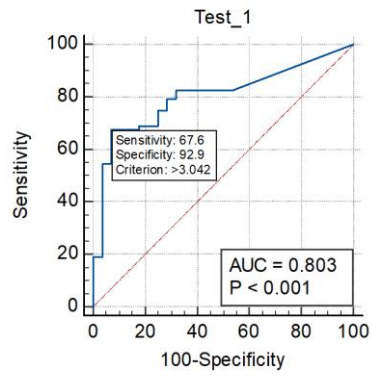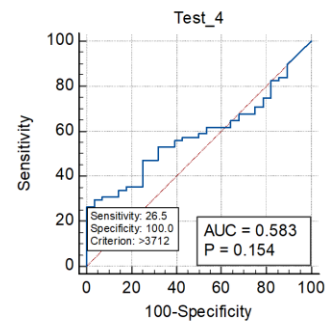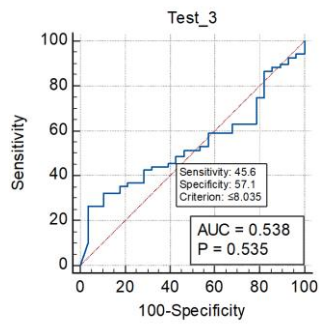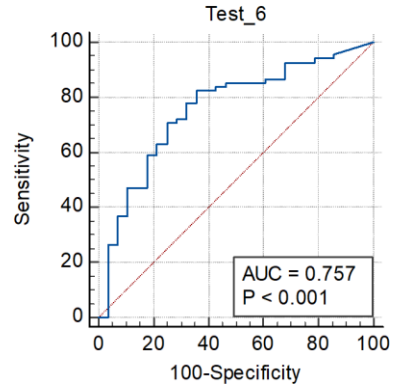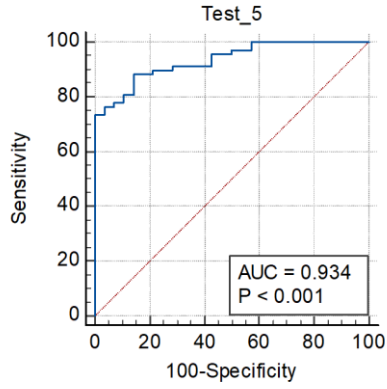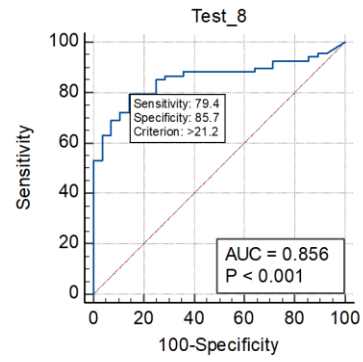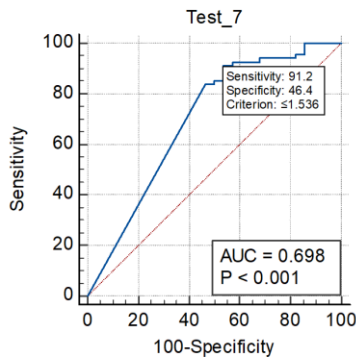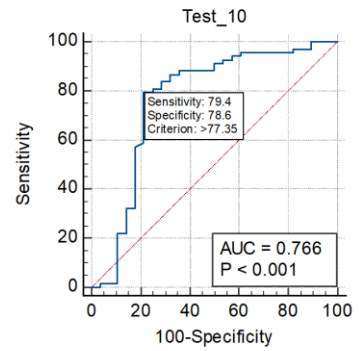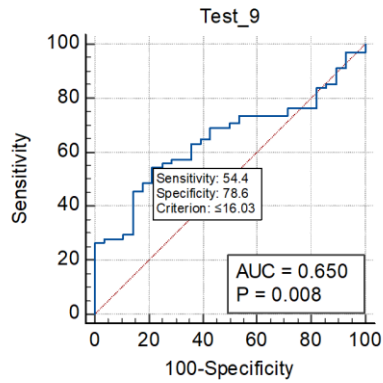
F. Anti-α-SYN
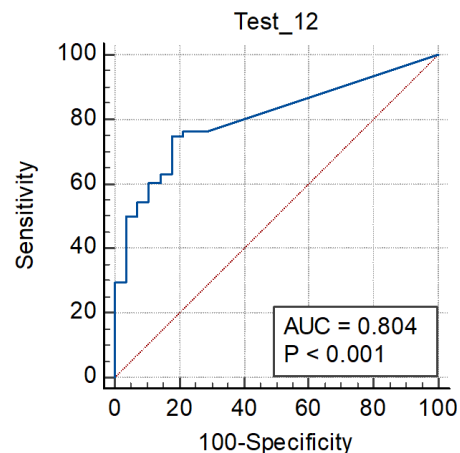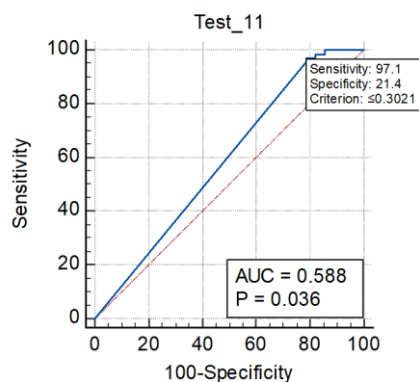
*ROC= Receiver Operating Characteristic is the plot of the true positive rate (Sensitivity) in function of the false positive rate (100-Specificity) for different cut-off points of a parameter. The AUC nearing 1 (or >0.5) indicates sensitivity (fraction of true positives to all with disease) and specificity (fraction of true negatives to all without disease) of the test. Florkowski CM. Sensitivity, specificity, receiver-operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. Clin Biochem Rev. 2008;29 Suppl 1(Suppl 1):S83-S87.

3. Prevalence of Serum Neuroantibodies

Table 10 shows the prevalence of the NAb and how they compare to the reference groups for both IgM and IgG class. Figure 4 represents the segregation of the statistically significant prevalence for six (6) of the twelve (12) NAb detected.

Table 10. Prevalence of NAb in Reference Group and Patients with Neurological Disease Diagnoses.

									Frequency (Prevalence)												
Neuroantibody	NFL IgM	p=	NFL IgG	p=	NFM IgM	p=	NFM IgG	NFH IgM	p=	NFH IgG	p=	GFAP IgM	p=	GFAP IgG	MBP IgM	MBP IgG	a-Syn IgM	p=	a-Syn IgG	p=
Reference (n=28)	0.39		0.11		0.96		0.89	1.00		0.14		0.54		0.93	1.00	0.89	0.21		0.29	
NMS (n=30)	0.67	0.03*	0.63	0.0001*	1.00	ns	0.90	0.50	0.0001*	0.90	0.0001	0.33	ns*	0.97	1.00	1.00	0.07	ns*	0.50	ns*
Stroke/Dementia (n=19)	1.00	0.0001* 0.006**	0.84	0.0001*	0.95	ns ns	0.89	0.05	0.0001* 0.001**	1.00	0.0001	0.00	0.0001* 0.005**	1.00	0.95	1.00	0.00	0.0001* ns**	1.00	0.0001* 0.0003**
Essential Tremors (n=19)	0.89	0.0007* 0.09** ns***	0.79	0.0001*	0.68	0.009* 0.004** 0.01***	0.89	0.11	0.0001* 0.005** ns***	1.00	0.0001	0.11	0.0001* 0.08** ns***	1.00	1.00	1.00	0.05	0.0001* ns** ns***	0.95	0.0001* 0.001** ns***

*=compared to Reference group; ** compared to NMS; ***compared to Dementia; ns=not significant
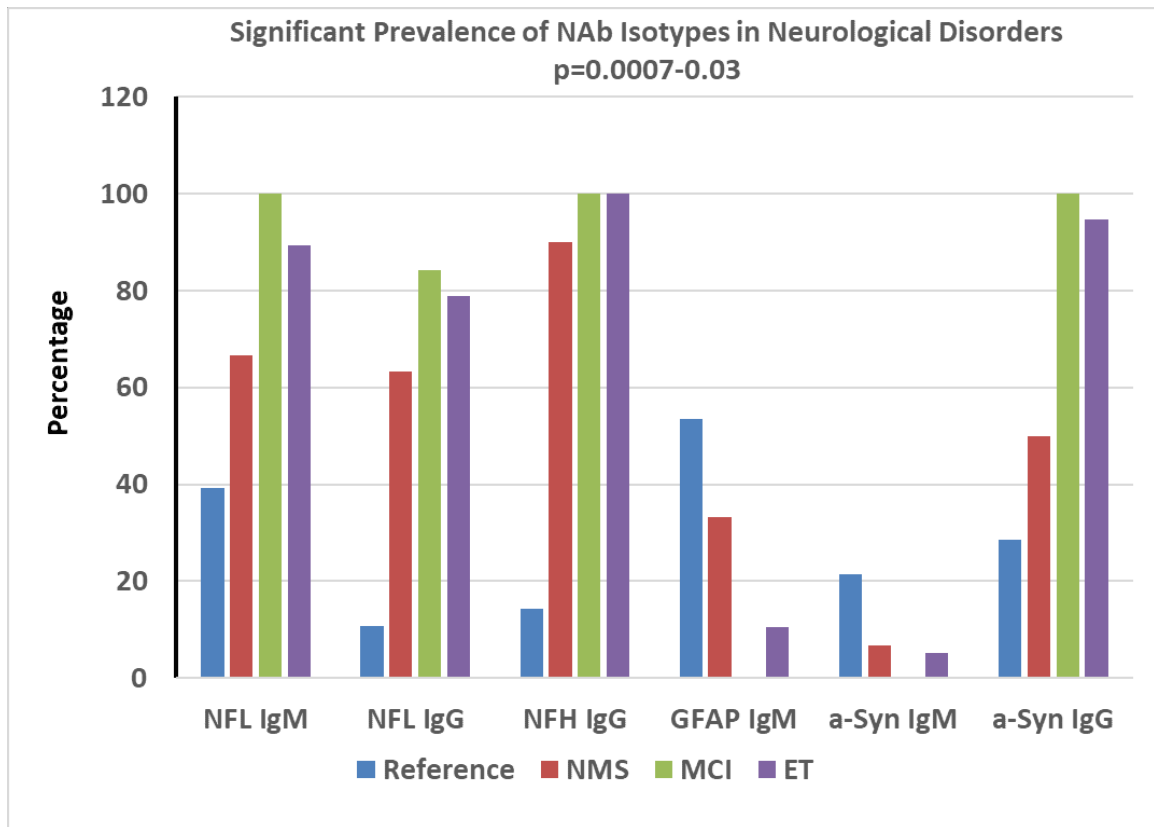
Figure 4. Statistically Significant Prevalence for Detected Neuroantibodies.

Relative Risk and Odds Ratio

Four (4) of the 12 NAb assayed, were highly associated with neurological disease diagnosis. Furthermore, they were statistically significant, in the case of MCI and ET. The choice of NF-L, IgM and IgG, NF-H IgG and α-SYN IgG, was based on their significantly high prevalence (Table 8 and 10 and Figure 4).

Table 11. Odds Ratio and Relative Risk.

	OR	p value	95% Confidence Interval	RR	P value	95% Confidence Interval
NMS						
NF-L IgM	1.8214	ns	0.7146 to 4.6425	1.697	0.04	1.0034 to 2.8699
NF-L IgG	2.4351	0.04	1.4929 to 21.4186	5.9111	0.001	1.9610 to17.8183
NF-H IgG	8.5714	0.001	2.3218 to 31.6430	7.2	0.0001	2.8566 to 18.1472
a-Syn IgG	1.4286	ns	0.6139 to 3.3245	1.75	ns	0.8810 to 3.4762
MCI/stroke						
NF-L IgM	23	0.03	1.3584 to 422.1849	2.7273	0.0001	1.7040 to 4.3649
NF-L IgG	5.6548	0.01	1.4929 to 21.4186	7.8596	0.0002	2.6509 to 23.3032
NF-H IgG	33.5263	0.01	2.2906 to 146.5430	7	0.0001	2.8253 to 17.3434
a-Syn IgG	28.0526	0.02	1.6002 to 491.7962	3.5	0.0001	1.9486 to 6.2866
Essential Tremor						
NF-L IgM	5.7679	0.02	1.1919 to 27.9121	2.2775	0.0009	1.4014 to 3.7014
NF-L IgG	4.2411	0.01	1.2703 to 14.1598	7.3684	0.0003	2.4670 to 22.0077
NF- H IgG	33.5263	0.01	2.2906 to 146.5430	7.25	0.0001	2.9186 to 18.0097
a-Syn IgG	13.5714	0.01	1.6766 to 109.8543	3.3158	0.0001	3.8285 6.0127

4. Comparison of Nab Titers Based on Diagnosis

Figures 5A-K present the levels of the IgG and IgM antibodies against the different neural antigens in the three different disease groups and the reference group.

Figure 5A-K. Box plots (median $\pm$ IQR) for NAb titer comparison between reference and groups with neurological disease diagnosis. Non-parametric statistics indicate statistically significant difference between groups and reference, and between each other for 11 of the 12 NAb assayed, as indicated by the p values displayed.

A, B=Anti-NF-L, IgM and IgG; **C, D**=Anti-NF-M, IgM and IgG; **E, F**=Anti-NF-H, IgM and IgG; **G, H**=Anti-GFAP, IgM and IgG; **I, J**=Anti-MBP, IgM and IgG; **K**=Anti-α-SYN, IgG, respectively.

E

Ant-NF-H IgM ng/ml

0.000001
0.000001
0.000001
0.0001
0.01

Reference NMS Dementia Tremors

F

Ant-NF-H IgG ng/ml

0.00002
0.00002
0.05

Reference NMS Dementia Tremors

G

Ant-GFAP IgM ng/ml

0.006
0.00001
0.03 0.05

Reference NMS Dementia Tremors

H

Anti-GFAP IgG ng/ml

0.000001
0.000001
0.001
0.004

Reference NMS Dementia Tremors
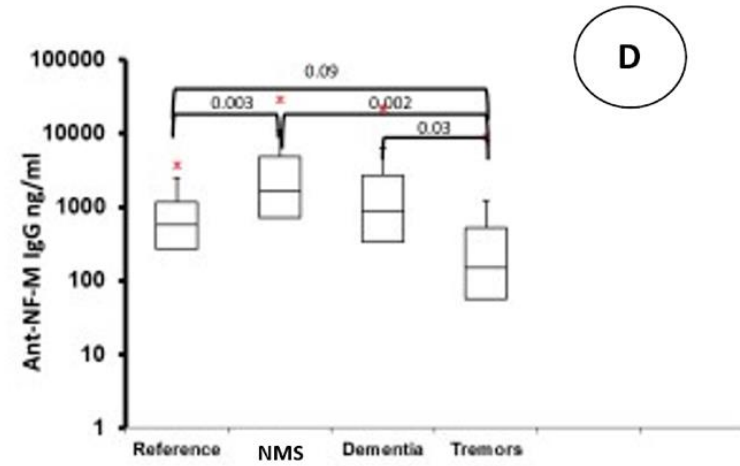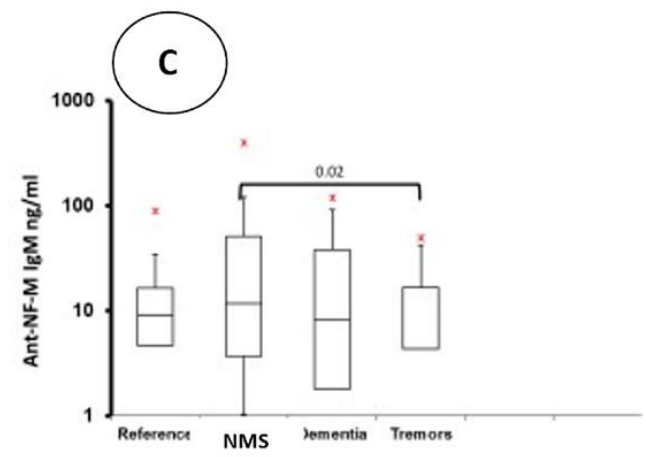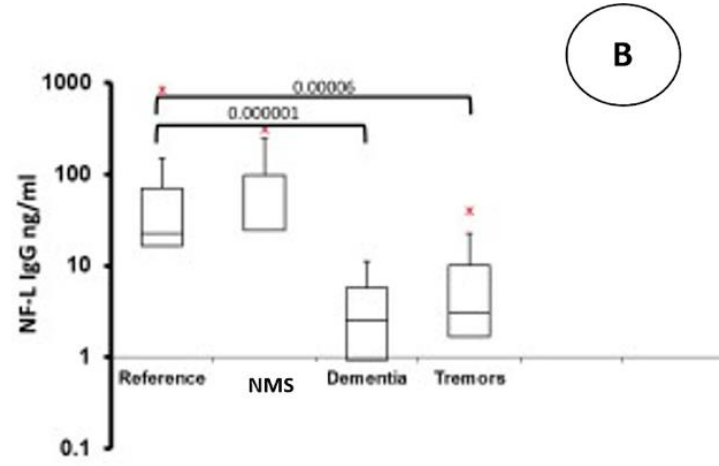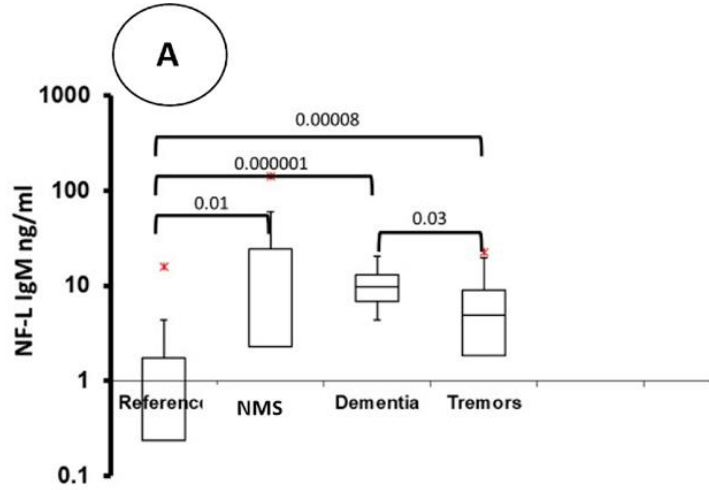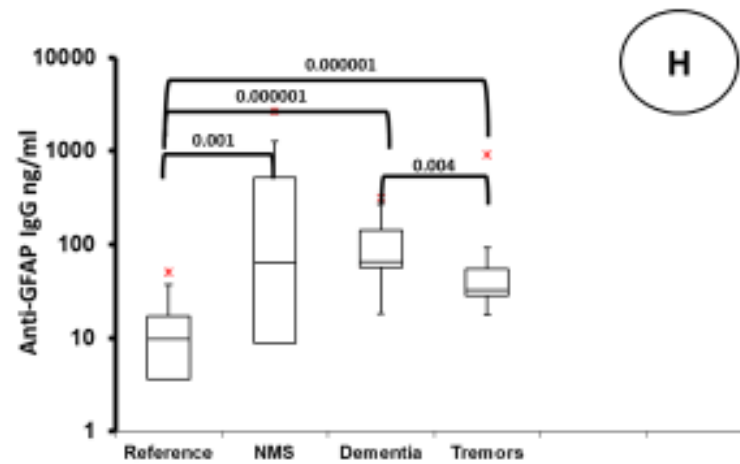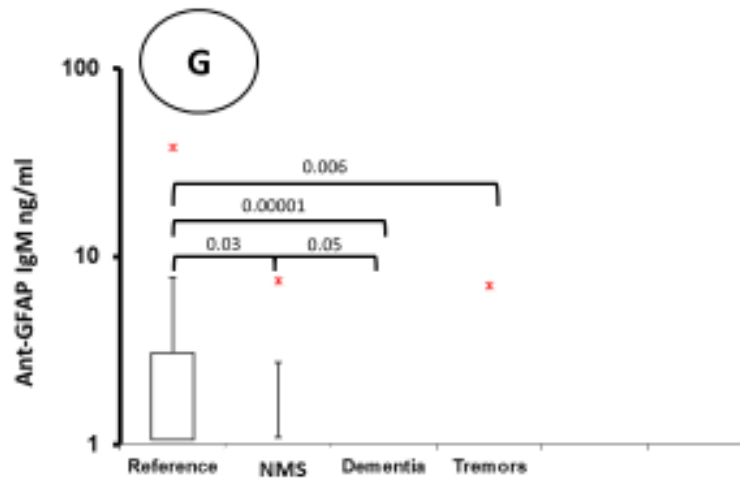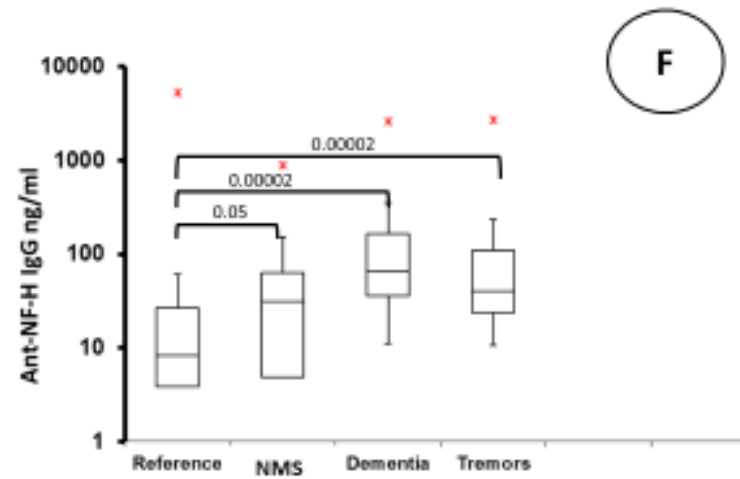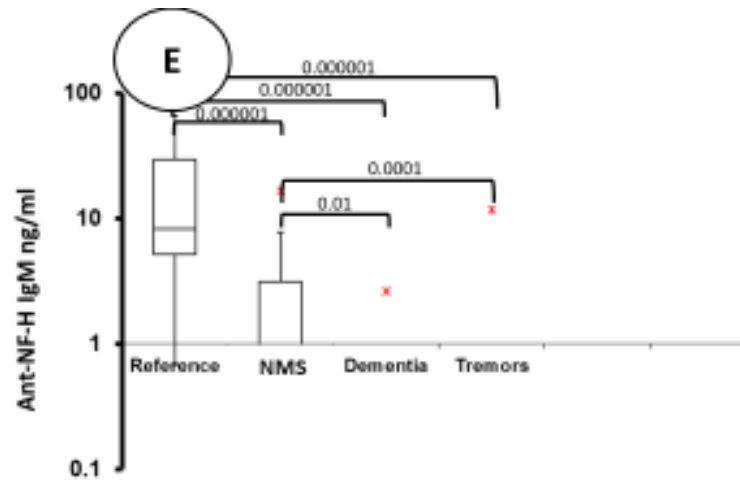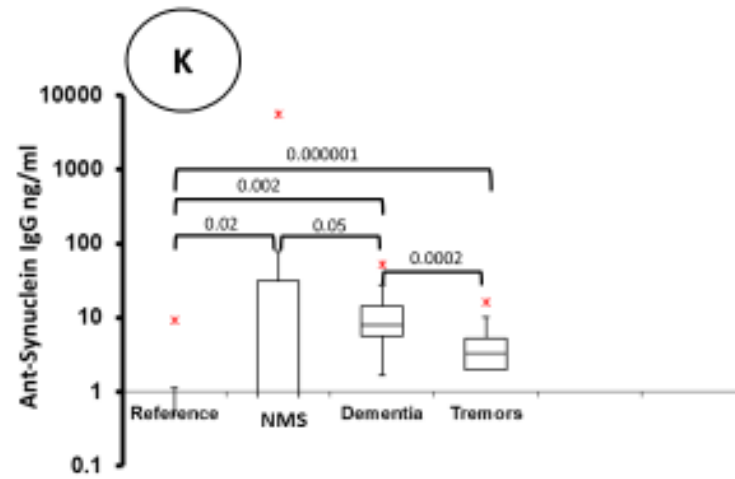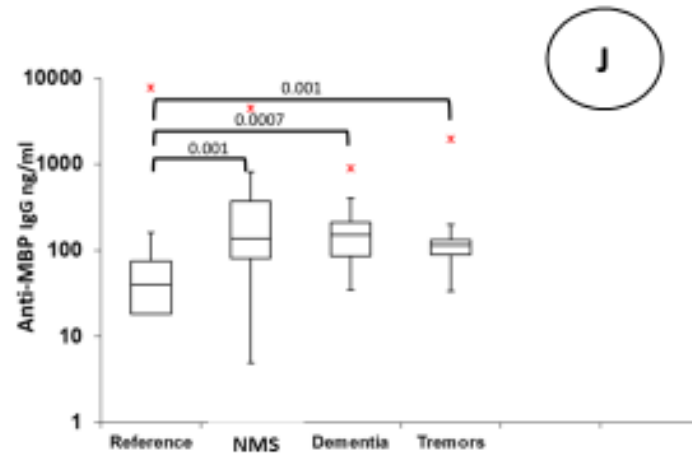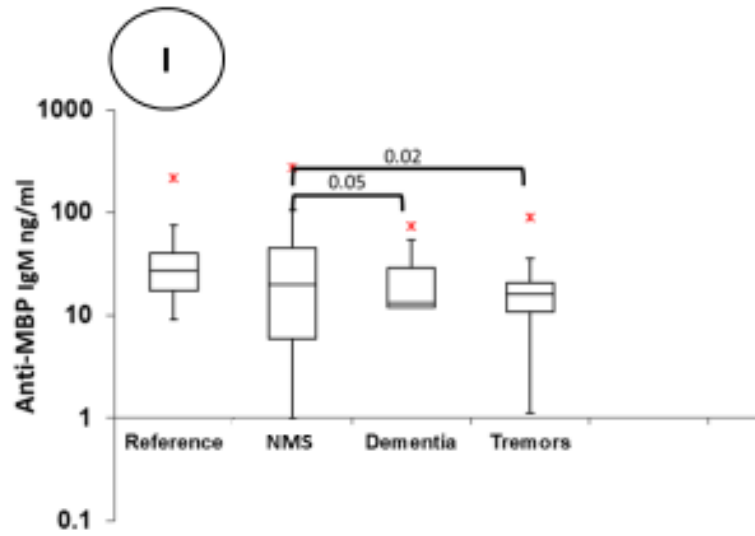
5. Age as a determinant of NAb Titers

Table 12 shows the spearman correlation for age of all subjects and NAb levels. The association is further clarified for IgM and IgG against the different autoantigens by tertile stratification in Figures 6A-F.

Table 12. Spearman correlation coefficients and statistically significant direct and inverse associations between age of all individual assayed for NAb and age.

		AGE
NF-L IgM	R	0.35000339
	p	0.0004
NF-L IgG	R	-0.3146731
	p	0.001
NF-H IgM	R	-0.5077845
	p	0.00000001
NF-H IgG	R	0.24735839
	p	0.01
GFAP IgM	R	-0.3350695
	p	0.0008
GFAP IgG	R	0.32802249
	p	0.001
MBP IgM	R	-0.1003454
	p	ns
MBP IgG	R	0.23705291
	p	0.02
a-Syn IgM	R	-0.1894823
	p	0.06
a-Syn IgG	R	0.44290499
	p	0.0000001

Figure 6A-F. Age tertile stratification of NAb levels show significant, as indicated by the p values, age-dependent titer differences. For Anti-NF-L, IgM, Anti-NF-H, GFAP, MBP and α-SYN, IgG, the data suggests ongoing circulating autoantigen and degenerative changes that are age-dependent. For all of Anti-NF-H, GFAP, MBP and α-SYN, IgM, the antibody associated with primary challenge, they appear to be in decline, as IgG, the antibody associated with memory and the historical record increases.
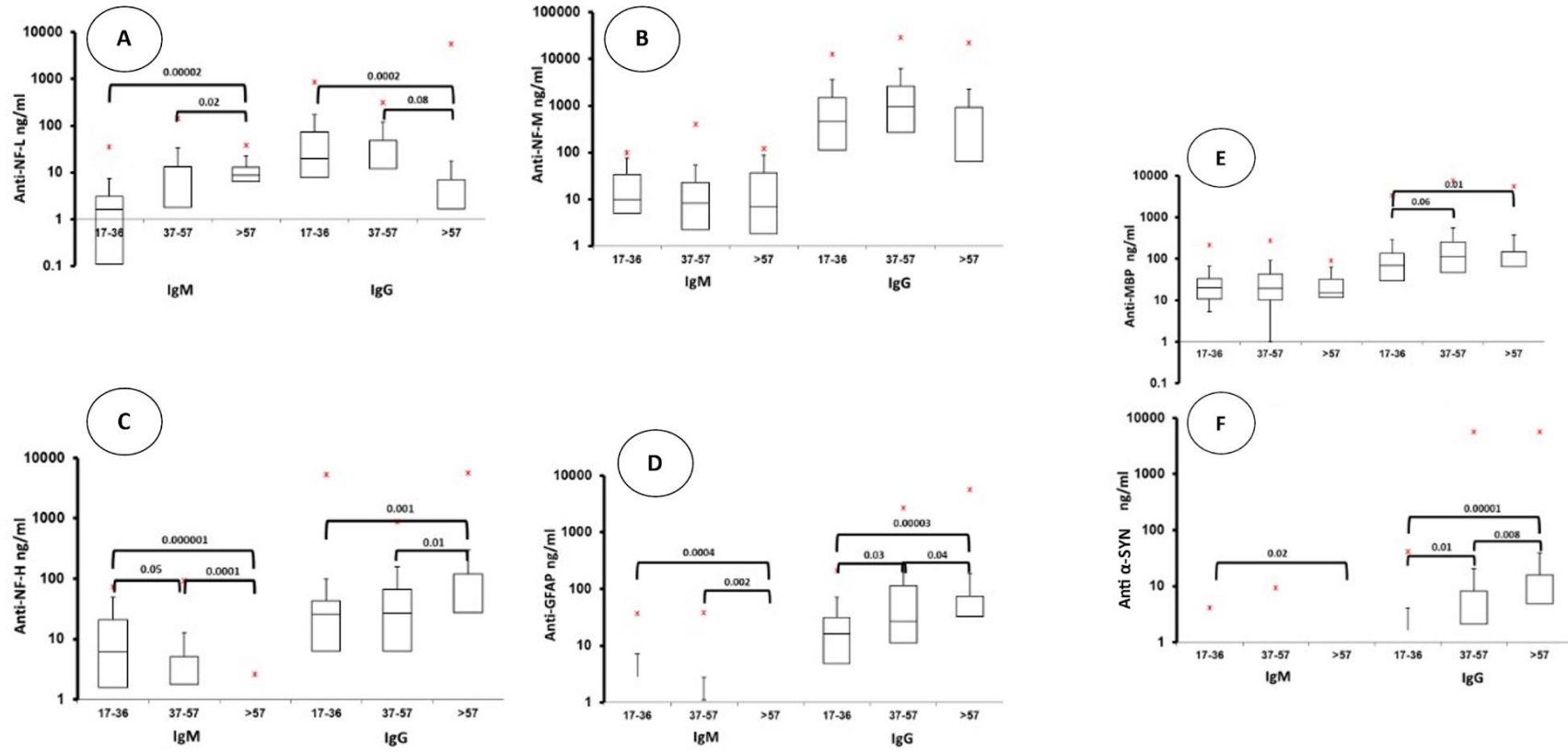
6. Sex as a Determinant of Nab Titers

Figure 7A-D illustrates the sex differences in NAb titer levels, while E indicates the differences in prevalence according to sex.
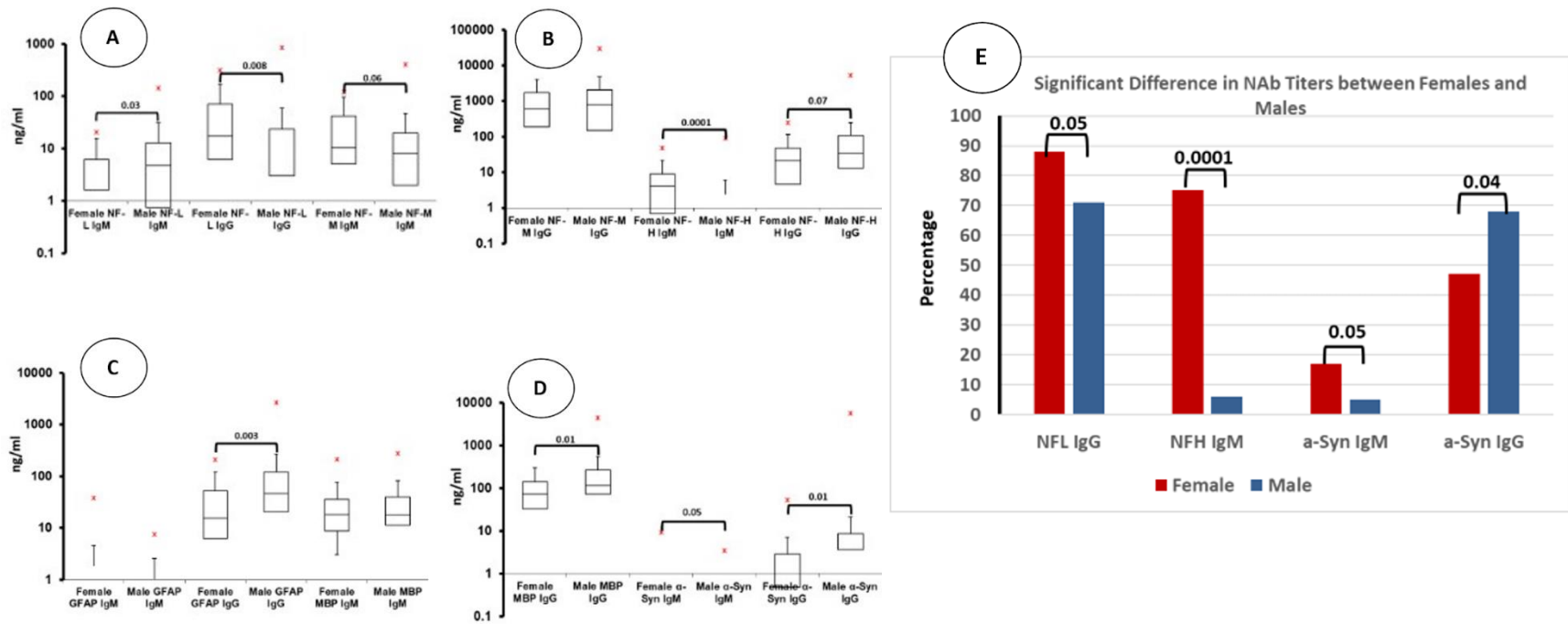
Figure 7A-D. Comparison of median titer levels and IQR between female (n=36) and male (n=60) participants, regardless of diagnosis.

As autoimmunity is more prevalent in females, this comparison was to ascertain whether this held true for NAb detection and, therefore,

clinical progression. Overall, NAb of the IgG isotype appeared to predominate in males, suggesting that they were likely to be more affected. **6E** demonstrates the statistically different prevalence for 4 or the 12 NAb assayed.

7. Heavy Metals as Determinants of Nab Titers

Heavy metals levels in the serum were all summarized in Table 13.

Table 13. Median heavy metal levels (µg/dl) in all subjects and comparison between subjects with neurological diseases diagnosis and reference.

		Zn		Cd		Ba		Cu		As		Co		Pb		Cr		Al		Mn	
		Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)	Median	(IQR)
									Serum Metal (µg/dL)													
Reference (n=28)		16.9	76.05	23.4	4.2	2.2	2.05	30	7.75	20.2	6.8	10.4	3.35	13.9	9.9	44	5.25	161.8	25.45	2.6	0.4
NMS (n=30)		84.9	35.65	16.9	3.4	1.8	1.75	23	18.05	12.7	4	8.8	8.2	14.5	26.35	40.2	14.45	129.6	43.55	2.2	1.65
vs Reference	p		0.0001		0.00003		0.08		0.01		0.0001		0.08		ns		0.007		0.00000001		0.02
vs Dementia	p		0.02		0.00008				ns		ns		ns		ns		ns		0.0000001		0.008
vs Tremors	p		0.03		0.00009		0.003		ns		0.00001		ns		ns		ns		0.0000001		0.01
Dementia (n=19)		101.6	30.7	8.4	10.5	0.8	0.9	21	4.1	10.4	11.6	9.6	2.3	22.2	12.8	42	1.1	184.6	24.8	2.6	0.7
vs Reference	p		0.00001		0.000001		0.00003		0.00005		0.001		ns		0.01		ns		0.001		ns
vs Psychosis	p		0.02		0.00008		0.005		ns		ns		ns		ns		ns		0.0000001		0.008
vs Tremors	p		ns		0.000002		ns		0.00004		0.000003		ns		ns		ns		0.001		ns
Tremors (n=19)		103.2	30.8	21.4	3.8	0.8	0.3	27.2	3.7	25.2	2.9	10.4	1.6	23.8	10.7	41.6	2.1	171	9.6	2.6	0.4
vs Reference	p		0.00001		ns		0.000001		0.05		0.000003		ns		0.001		0.001		ns		ns
vs Psychosis	p		0.03		0.00009		0.003		ns		0.00001		ns		ns		ns		0.0000001		0.01
vs Dementia	p		ns		0.000002		ns		0.00004		0.000003		ns		ns		ns		0.001		ns

Table 14. Spearman correlation and significance figures for direct and inverse associations between heavy metals and NAb Isotypes.

		Zn	Cd	Ba	Cu	As	Co	Pb	Cr	Al	Mn
NF-L IgM	R	0.3668224	-0.2794216	-0.439763	-0.1896	0	-0.02773	0.51176528	-0.26598	0.028057	0.061518
	p	0.0002	0.005	0.0000001	0.06	ns	ns	0.00000001	0.008	ns	ns
NF-L IgG	R	-0.305477	0.23302577	0.3684042	0.180863	0.01	-0.03887	-0.4427306	0.377085	-0.04949	0.004326
	p	0.0002	0.02	0.0002	0.07	ns	ns	0.000001	0.0001	ns	ns
NF-M IgM	R	-0.119494	0.0595938	0.0702372	0.044601	0.04452	-0.04794	0.02951274	-0.01923	-0.05764	0.030909
	p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NF-M IgG	R	0.0790425	-0.2872304	-0.067057	-0.28059	-0.27568	-0.20831	-0.0604031	-0.18947	-0.32402	-0.25066
	p	ns	0.004	ns	0.05	0.006	0.04	ns	0.06	0.001	0.01
NF-H IgM	R	-0.553456	0.49796521	0.4938267	0.364638	0.088606	0.042339	-0.4126334	0.358303	-0.00697	0.00519
	p	0.0000001	0.00000001	0.000001	0.0002	ns	ns	0.00002	0.0003	ns	ns
NF-H IgG	R	0.4399783	-0.2229897	-0.41614	-0.07776	0.011004	-0.02372	0.38571218	-0.1509	0.010868	0.080521
	p	0.0000001	0.03	0.00002	ns	ns	ns	0.0001	ns	ns	ns
GFAP IgM	R	-0.327385	0.41026806	0.2712799	0.376618	0.199175	0.218762	-0.1009648	0.190963	0.011094	0.177405
	p	0.001	0.00003	0.007	0.0001	0.05	0.03	ns	0.06	ns	0.08
GFAP IgG	R	0.4173589	-0.3902172	-0.472105	-0.24415	-0.11453	-0.04007	0.42358219	-0.31333	-0.1035	0.007656
	p	0.00002	0.00008	0.000001	0.01	ns	ns	0.00001	0.001	ns	ns
MBP IgM	R	-0.09638	0.15320855	-0.069999	0.028961	0.174166	0.026723	0.19134549	-0.18234	-0.10399	0.013198
	p	ns	ns	ns	ns	0.08	ns	0.06	0.07	ns	ns
MBP IgG	R	0.3239387	-0.3750114	-0.153404	-0.28889	-0.06845	-0.10713	0.36999219	-0.39079	-0.1542	-0.09619
	p	0.001	0.0001	ns	0.004	ns	ns	0.002	0.00008	ns	ns
a-Syn IgM	R	-0.223361	0.37068847	0.2960122	0.328498	0.228714	0.232155	-0.0464522	0.100426	0.183605	0.232006
	p	0.03	0.0002	0.003	0.001	0.02	0.02	ns	ns	0.07	0.02
a-Syn IgG	R	0.3844418	-0.3489185	-0.382368	-0.18153	-0.03221	0.024943	0.48543949	-0.36457	0.084056	0.232006
	p	0.0001	0.0005	0.0001	0.07	ns	ns	0.0000001	0.0002	ns	0.02

Figure 8A-D. Distribution and significant difference between NAb titer isotypes based on stratification of heavy metal levels showing the internal dose-NAb response correlated in Table 12.

A. For lead, levels of NAb titers, whether IgM or IgG, appear to follow a dose-response pattern which may correlate with severity of toxicity.

A. SERUM Pb: TERTILES
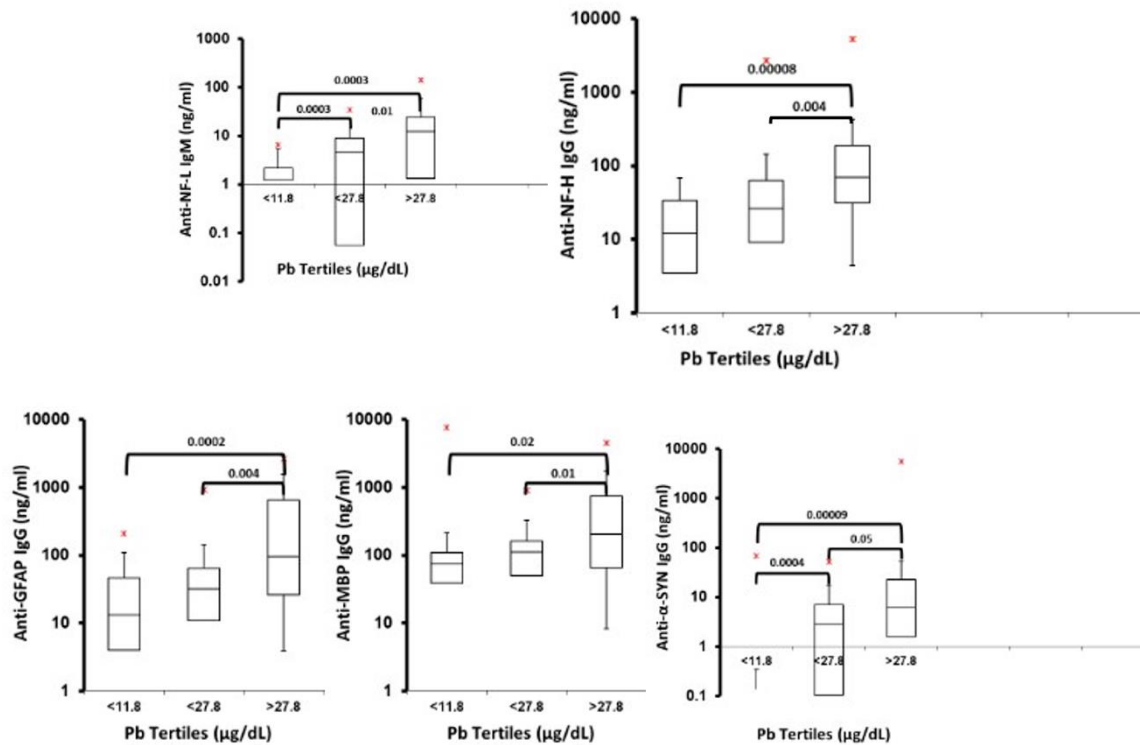
B. For Zinc, the dose-response, in the form of NAb, appears to be consistent, suggesting that increased neurodegenerative response with levels of internal heavy metal dose.

B. SERUM Zn: TERTILES
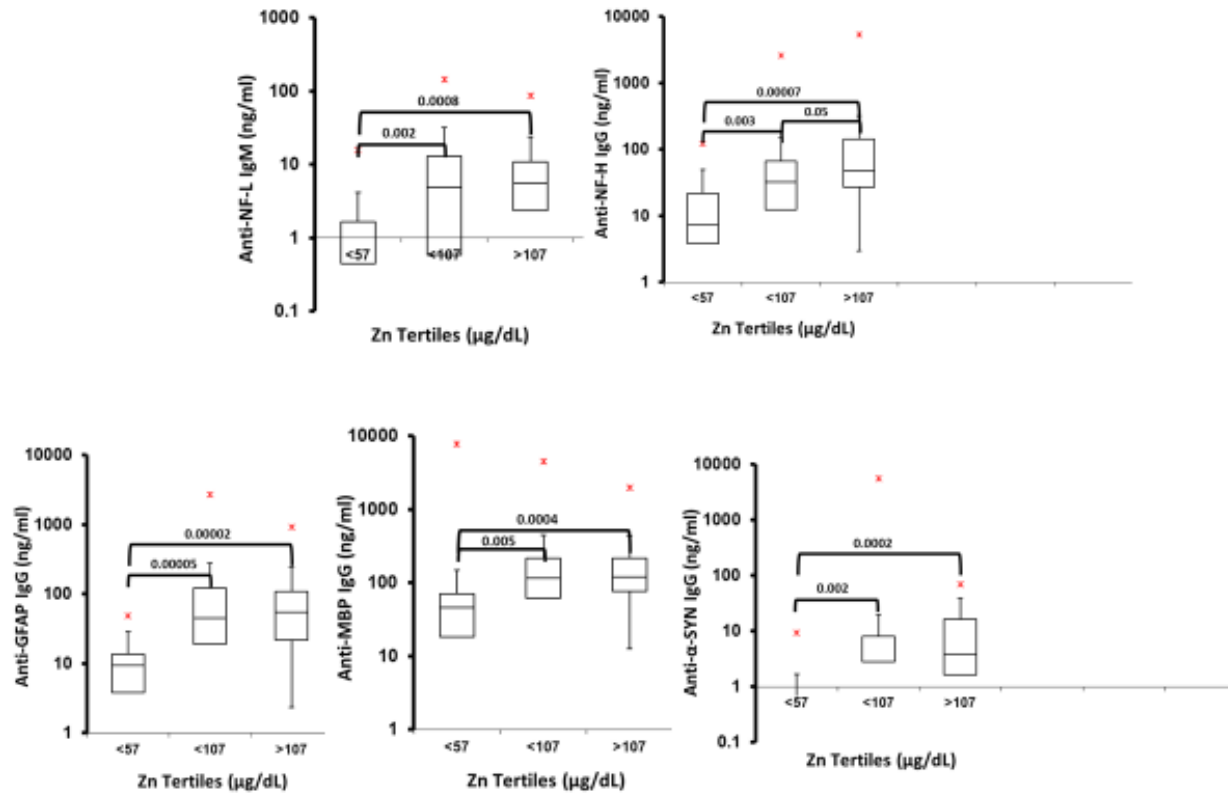
C. For Cd, a dose-response relationship was also noted.

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C. SERUM Cd: TERTILES
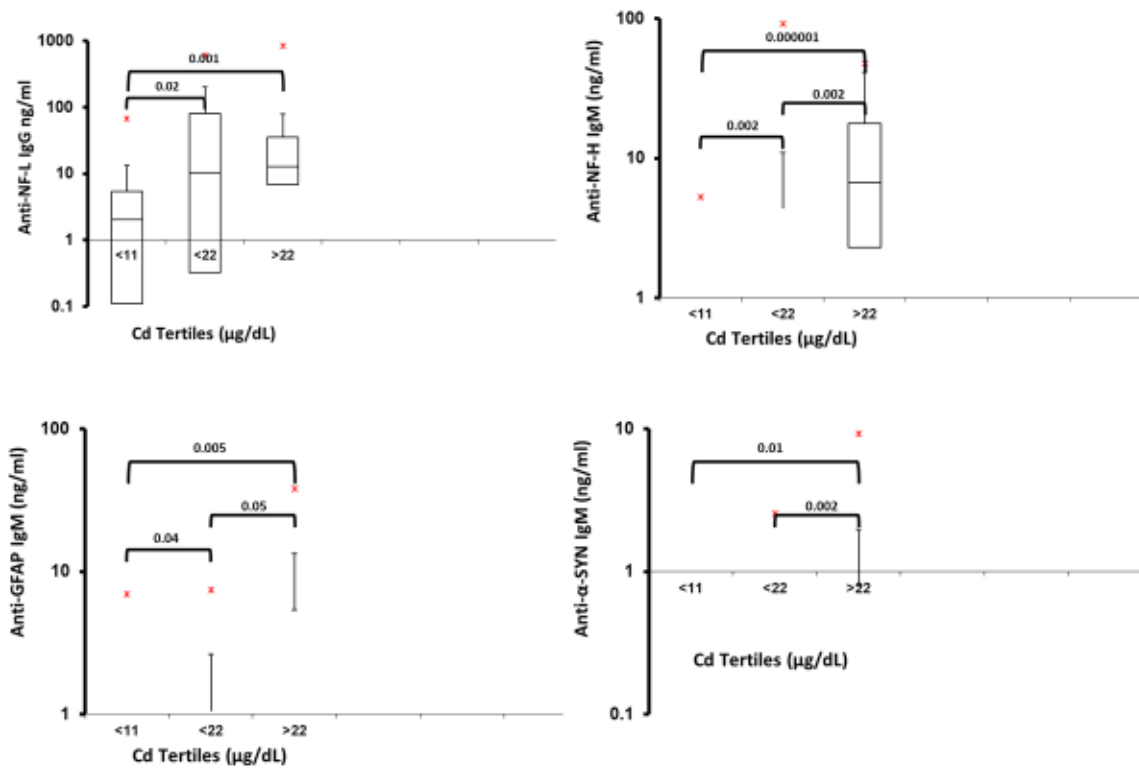
D. and for Copper.

D. SERUM Cu: TERTILES
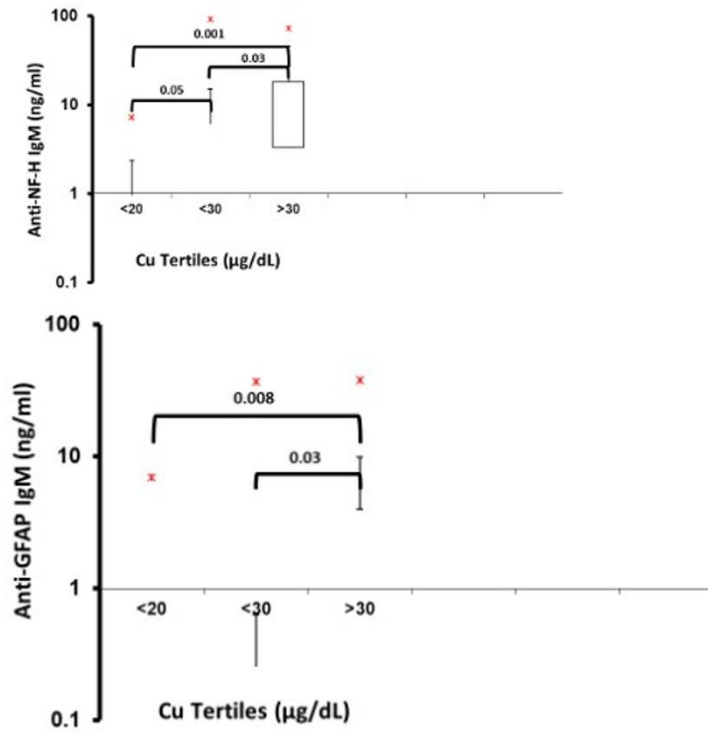
Sex also appeared to be a significant determinant of some heavy metal levels, suggesting that the exposure did differ between males and females. Figure 8A-D show the heavy metals levels according to the sex.

Figure 9A-D. Differences in median heavy metal levels between females (n=36) and males (n=60). This was only significantly higher in females for Cr and Al, while for Zn and Pb, levels were significantly higher in males, possibly reflecting their industrial occupations.
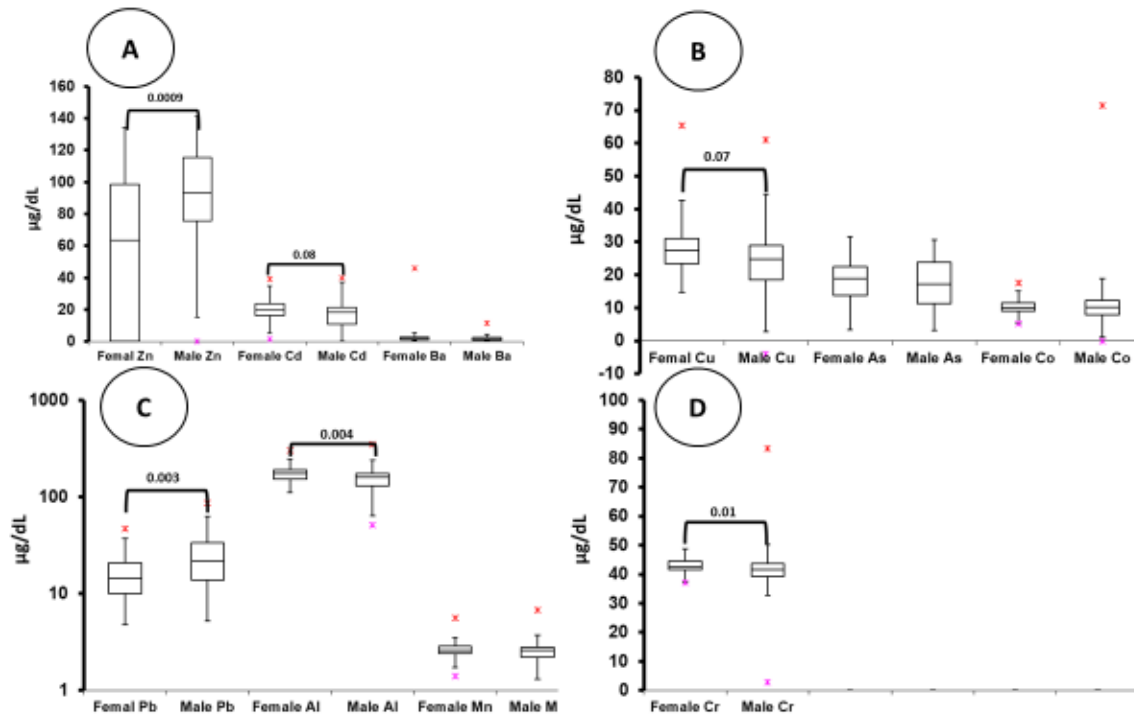
V. Discussion

Objective determination of NS insult due to various etiological factors, including familial, sporadic and environmentally-induced, disorders pose unique challenges. This is due, to a great extent, the inaccessibility of the CNS. More often, than not, overt signs and symptoms evident at the time of clinical exam become the basis of diagnosis. Unfortunately, by the time these develop, significant neuronal loss has occurred, thereby narrowing the window for effective intervention and/or disease mitigation. In this context, the discovery and validation of biomarkers for NS damage is an imperative. In this study, we have chosen to blindly analyze the serum of patients with NS damage, as identified by collaborating clinicians, for antibodies against neural proteins, what we refer to as neuroantibodies. Antibodies are produced by the B-lymphocytes in response to proteins that are considered xenobiotic in origin, including intracellular sequestered proteins. This has been reported in the case of neurodegeneration, and environmentally-induced neurotoxicity (El-Fawal, 2011). Capitalizing on the immune system to indicate NS insult, IgM antibodies indicate a preliminary or chronic response. With the development of immunological memory, immunoglobulin class switching occurs, and the longer-lived IgG response, also indicative of acute injury are generated (El-Fawal, 2014). During the neurodegeneration process, proteins are translocated to the lymphoid tissue (e.g., cervical lymph nodes) and autoantibodies against these proteins are generated (Koehler et al., 2013). Whether these NAb are pathogenic, and may lead to further pathology remains to be elucidated (El-Fawal 2014; Salama et al., 2018).

The purpose of the present study is to address the challenge of developing a marker that indicates nervous system insult, irrespective of etiology, in what is referred to as a Tier I study. With validation, in various neurological disorders, it should be fine-tuned to allow for differential diagnosis, in what is known as Tier II studies. This may be coupled with genomic studies and studies of intervention.

1. General markers of NS damage: Neuroantibodies and Relation to Disease

With the exception of anti-NF-M, IgM and IgG, anti-NF-J, IgM, anti-GFAP, IgG, and anti-MBP, IgM and IgG, all other NAb were more prevalent in those diagnosed with neurological disorders of NMS, dementia and essential tremors. However, it should be pointed out that since the NF proteins exist in a triplet, NAb against one is likely to be indicative of neuronal damage. Differences in prevalence and/or titers may reflect the differences in biochemical immunogenicity (El-Fawal, 2014). Nevertheless, a high prevalence of these NAb in the reference population prompted the investigation of environmental exposures in all, as the regions of recruitment are industrialization centers. Thus, the determination of heavy metal levels described below.

 Prevalence in terms of frequency of occurrence in itself is not sufficient, thus relative risk, the ratio of the probability of NAb detection occurring in the disease group versus the probability of NAb detection occurring in the reference group, and the odds ratio, the odds that an outcome will occur given a particular exposure (diagnosis), compared to the odds of the outcome occurring in the absence of that exposure (diagnosis) was also determined (Table 11). In general, four NAb appeared to be closely associated with diagnosis: anti-NF-L, IgM and IgG, anti-NF-H, IgG, and anti-α-synuclein IgG. Furthermore, the observation that the higher prevalence were found for anti-NF-L, anti-NF-H, and anti-α-synuclein, IgG isotype, would suggest that neuronal involvement has

persisted for some time. It should be noted that some pre-clinical and clinical studies suggest the presence of a "natural repertoire" of autoantibodies may exist against diverse self- and foreign antigens (Simell et al., 2008).

2. Titer Levels and Diagnoses

In general, and as summarized in Figure 5 (A-K), median levels of IgG titers against most autoantigens tended to be significantly higher in subjects diagnosed with neurological disorders compared to the reference population, even for the NAb not found to be significantly different in prevalence. On the other hand, median IgM levels, when present, tended to be higher in the reference and NMS populations.

Within the different neurological diagnoses, median levels of IgG tended to be higher in the stroke/dementia group compared to NMS and essential tremors. This is not surprising, given that an organic basis, if present, for these manifestations, if present, have yet to be defined, with reports in the literature being equivocal. For example, Louis (2011) implicates the cerebellar Purkinje neurons because of the reported ataxia, intention tremors of the hands. This is supported by neuroimaging studies using MRI and PET scans (Louis, 2011). In contrast, an organic basis for NMS has yet to be defined, although as mentioned earlier, both neurogenic and myogenic hypotheses have been put forth (Fukushima et al., 2020; Delgado et al., 2016). Interestingly, and what may be relevant to the present study, is the proposed neuroimmunologic hypothesis for the development of NMS. It suggests that individuals with previous NMS have autoantibodies against neurotransmitter receptors, due to antipsychotic medications interacting with endogenous proteins in the brain. In short, the generation of neoantigens to which the individual mounts and immune response (Anglin et al., 2010).

3. Specific Neuroantibodies

Anti-NFH IgG appears to be the most consistent antibody to reflect NS damage regardless of etiology. It is noteworthy that the NF-H intermediate filaments is exclusively axonal/synaptic, although found with NF-L and NF-M. As the highest molecular weight (200 kD) protein, it is likely to have more immunogenic epitopes (antigenic determinants). In the present study, prevalence results show that 90% of NMS patients and a 100% of MCI and ET patients have anti-NF-H IgG antibodies in comparison with <15% of references (Table 11). Median titers of this antibody were also higher in the neurological diagnoses groups compared to the references, but it could not differentiate between the disease groups (Figure 4F). Furthermore, relative risk (Table 9) strongly supports that NMS, MCI and ET were determinants of anti-NFH IgG antibodies being present. All the disease groups have significantly high OR for anti-NF-H IgG. Anti-NF-H titers were higher among higher age groups (Figure 6C), and although the correlation coefficient was low than 0.25, it was highly significant ($p \leq 0.01$) (Table 13). This further supports the aforementioned findings, as our MCI patient group is older compared to references and the other neurological diagnoses. So, the higher anti-NFH IgG levels are most probably not due to age, but to NS damage. No significant differences were found in the levels of this antibody between sexes (figure 7B). Moreover, serum heavy metals such as Pb and Zn affect the level of anti-NF-H IgG, showing a highly significant correlation (<0.0001; Table 14) and a consistent dose-response on metal dose-stratification (Figures 8A and B). This is relevant in light of the known neurotoxicity of heavy metals and their potential contribution to sporadic neurodegenerative diseases.

NF-H is one of the neurofilament triplet proteins, intermediate filaments, exclusive to the axon. Its elaboration during neurogenesis signals stabilization of the synapse. So, antibodies against this protein are indicative of neuronal/axonal damage (El-Fawal & McCain, 2008; Mondello & Hayes,

2015) which is very common in stroke patients, especially those who develop hemiparesis (Liu et al., 2021). Stroke patients present with early Wallerian degeneration in parts of the brain and axonal degeneration in the stroke affected side (Liu et al., 2021). This is further supported by another study where serum phosphorylated NF-H levels increased in ischemic stroke patients 1-3 weeks after the incidence compared to controls (Nielsen et al., 2020). Since information about the disease duration, it is assumed that these patients had the stroke weeks to years earlier, as serum IgM against NF-H were found to be low with negligible prevalence (5%). To date, there have been no studies about axonopathies in neuroleptic malignant syndrome patients.

To our knowledge, this is the first study to suggest that NMS leads to injury in the brain, with ensuing neurodegeneration. The results in our study strongly suggest neuronal insult in NMS patients, presented by high levels of anti-NF-L IgG and anti-NF-H IgG as well as their complementary anti-NF-H IgM and anti-NF-L IgM. NF phosphorylation and the number of NF in the ET cerebellum has been shown to be different than in controls, especially NF-H (Louis et al., 2012). This is in concordance with our results where IgG antibodies against NF-H were the highest in concentration in the ET population. NF-H levels are usually elevated by 14 days in the serum of patients with traumatic brain injury (TBI) correlating with the severity of disease on CT scan (Glushakova et al., 2018; Mondello & Hayes, 2015). A study by Bartos demonstrated a lower level of IgG antibodies against NF-H in the serum of AD patients compared to controls (Bartos et al., 2018). This is contradictory to our study where our MCI population presented with higher IgG antibodies against NF-H. However, it should be noted that in the presence of further insult and antigen release, circulating antibodies may be bound to antigen, thus not detectable in an indirect

ELISA. In the present study, even the NMS and the ET patients had a higher IgG antibody concentration against NF-H compared to references.

In summary, the present study suggests that detection of anti-NFH IgG is worth pursuing as a potential general biomarker for NS damage regardless of etiology.

Anti-NF-L IgG and IgM were NAbs found to be significantly different in all disease groups. Anti-NF-L IgM was found in more than 66% of patients in the disease groups compared to 39% of the references (Table 11). Antibodies were shown by the RR to be present due to diseases (NMS, ET and MCI) but the OR was only significant in the MCI and ET group. The levels of anti-NFL IgM were significantly higher in the disease groups compared to the references (Figure 5A). Anti-NF-L IgM levels also increased with age (Figure 6A) with a high correlation coefficient (Table 12). It was also significantly higher among males (Figure 7A), which may reflect the higher level of industrial exposures to neurotoxic heavy metals. Anti-NF-L IgG was prevalent in >60% of NMS, ET and MCI patients compared with 11% of the references (Table 11). RR showed that all disease groups were associated with this antibody being present, and the OR confirmed the low probability that this was due to chance (Table 11). Interestingly, the level of this antibody was nevertheless only significant in the MCI and ET groups, where the levels were much lower compared to the reference and the NMS groups (Figure 5B). However, this may also be reflective of the small sample size (n=18) in these groups, as well as the presence of outliers.

With age, anti-NFL IgG levels also decreased, and the negative correlation coefficient also supports this (figure 6A, Table 13). This is well correlating with our results because our MCI group, which had the lowest anti-NF-L titers, is older in age. However, it should be noted that IgM levels against NF-L were higher in this group compared to IgG. The environmental factors in our

study show significant correlations with anti-NF-L IgG, especially Cd, Ba, Cr, Pb and Zn (Table 14). Correlation coefficients with Pb and Zn were negative. Correlation coefficients with Cd, Ba and Cr were positive with this antibody. Serum Cr levels were only significantly lower in the NMS at ET groups (Table 14). Serum Cd was significantly lower in the NMS and MCI group (Table 14). Serum Ba level was significantly lower in all disease groups (Table 14).

Reconciliation of these IgM vs IgG results is feasible when we note that across MCI and ET groups IgM levels against NF-L were higher than IgG levels against the same antigen. This was highly significant for the MCI group ($p<0.00002$). It would appear that a chronic state of progression is occurring in these two sample populations. Furthermore, in the presence of progressing degeneration, it is expected that antibodies associated with memory (i.e., IgG) would be bound to newly released antigen. A recent review by Prüss, (2021) discusses the potential binding of autoantibodies in neurodegenerative disease progression. The logic is that autoantibodies bound to antigen (and/or cellular structures), and given that humoral immune responses are not indefinite, will not be available for detection in a laboratory (El-Fawal, 2014).

NF-L is also a protein unique to the neuron, it is part of the neurofilament triplet (NFL, NFM and NFH) (Mondello & Hayes, 2015). Found in dendrites, the soma and axon, antibodies against these neurofilaments are therefore indicators of neuronal insult (El-Fawal & McCain, 2008). Depending on the severity of the insult, it may progress to myelin degeneration and neuronopathy or myelinopathy which includes myelinating cells (oligodendrocytes) (El-Fawal, 2011; Mason et al., 2013). The present results for the MCI and ET group, where IgM antibodies against NF-L were found in higher levels compared to references, and OR as well as RR confirmed the association (figure 5A-B and table 12). The NMS group also showed higher anti-NFL IgM levels compared to references. IgG and IgM antibodies against NF-L were present in a small percentage of the

reference group, which had higher levels of IgG against NFL and lower levels of IgM against NF-L compared to the MCI and ET group, suggesting some past acute injury or pre-disease state. NF-L is usually released into the interstitial fluid to the CSF and then the systemic circulation. So, reference subjects can sometimes present with a low titer of autoantibodies as seen in our study as well (Table 11) (Mason et al., 2013; Osuna et al., 2014). As a clinical investigation, a true "control" does not exist, and presence of a "natural repertoire" must always be considered. But in case of AD, dementia and MCI, the level of NF-L increases markedly. NF-L was therefore associated with cognitive decline and disease related structural damage in other studies. Compared to controls, reports indicate that in AD and MCI patients had higher NF-L protein in both CSF and plasma (Andersson et al., 2020; Mattsson et al., 2017; Olsson et al., 2019; Yuan et al., 2017; Zetterberg et al., 2016). This should result in higher levels of antibodies against NFL. Another study showed elevated NF-L in the CSF of patients with neurodegenerative diseases (Sillman et al., 2018). NFL when found in the CSF is a sign of axonal injury and neurodegeneration following brain injury such as stroke, subarachnoid hemorrhage and traumatic brain injury (TBI) (Glushakova et al., 2018). In a study on MCI/AD patients, plasma NF-L was also found to be correlated with high CSF levels of NF-L. In all diagnostic groups CSF NF-L had the strongest correlations, and correlated with plasma NFL (Mattsson et al., 2017). This is mentioned to demonstrate that NFL concentration in the plasma is reflective of the CSF and therefore the brain. So, autoantibodies formed in response may prove reliable. NF-L concentrations are obviously not specific as it is found in the plasma of AD, supranuclear palsy, and multiple sclerosis, frontotemporal dementia and human immunodeficiency virus with brain engagement patients (Mattsson et al., 2017). The neurofilament triplet is not abundant in the same proportion. From low to high molecular weight they are found in a ratio of 4:2:1 or 6:2:1 (NFL:NFM:NFH) (El-Fawal & O'Callaghan, 2008).

Bigger proteins can also carry more epitopes leading to stronger immunogenicity (El-Fawal & O'Callaghan, 2008) The phosphorylation state of these neurofilaments also affect the autoantibody titers (El-Fawal & McCain, 2008). Our findings support this. Anti-NF-M IgG and IgM titers are higher than anti-NF-H IgG and IgM titers.

Anti-α-SYN IgG prevalence data were significant for MCI and ET groups only, where more than 94% of these patients had antibodies against a-SYN compared to 29% of references (Table 11). This is further supported by RR (Table 12), which show that anti-α-SYN IgG antibody was likely associated with MCI and ET disease presence but not the NMS. The high probability of this being a disease-associated outcome was confirmed by OR (Table 12). It would be useful in confirming this observation in these and other chronic neurodegenerative disease conditions. That being said, titer levels of anti- α-SYN IgG antibodies we find that their levels are significantly elevated in all disease groups compared to the references (Figure 4K). These levels also correlated with age (Figure 6F, Table 13). This is consistent with the pathology associated with aging, such as dementia of varying etiologies, with Parkinson's Disease and ALS. α-SYN had the highest correlation coefficient (r=0.55; p<0.0000001) with age. Our MCI group possesses an older population compared to the others, which might be the reason it had the highest levels of anti-α-SYN IgG. Males also had higher levels of anti-α-SYN (Figure 7D), as reflected in our ET group. Serum levels of Zn, Mn and Pb correlated with anti-a-SYN IgG levels, while Cd Cr and Ba correlated negatively (Table 14).

a-SYN is naturally found as an unfolded protein in the cytosol of brain cells under physiologic conditions. Some of its functions include augmentation of transmitter release, by affecting synaptic transmission, plasticity and neuroprotection. In PD, α-SYN is converted to an insoluble aggregated

form found in Lewy bodies intracellularly (Smith et al., 2012; Yanamandra et al., 2011). Once thought an exclusive hallmark of PD, α-SYN aggregation plays a role in the pathogenesis of many other diseases as well, such as multiple system atrophy (MSA), DLB, and PDD (Koehler et al., 2013). Damage to the neurons in the substantia nigra releases α-SYN into the extracellular space. Both α-SYN and its aggregated form cross the BBB, and they were found in the CSF and serum of PD patients (Yanamandra et al., 2011). Antibodies against α-SYN can influence their half-life in body fluids and brain, and prevent their accumulation (Koehler et al., 2013). Studies about α-SYN IgG antibodies in PD patients were mixed, some showed increased or decreased α-SYN in PD patients sera, and others showed no change at all (Scott et al., 2018). In another study, mild PD patients presented with higher α-SYN antibodies in both serum and CSF compared to controls and moderate PD patients (Horvath et al., 2017). Another study by (Wang et al., 2020) showed a negative correlation between serum antibodies against α-SYN in PD patients with dementia. These levels also correlated with PD severity, MMSE score and duration of disease. It is possible that antibodies against α-SYN of the IgM class might confer a protective effect to the brains of ET patients by attracting these aggregates from the brain and clearing them in the circulation (Koehler et al., 2013).

Anti-MBP IgG were highest in concentration in both the MCI and the ET groups, and the second highest in the NMS group (Figure 5J) compared to the reference group. It also increased with age (Figure 6E) with low, but significant correlation coefficient (r=0.23; p<0.02) (Table 13). It was also higher in males (figure 7C). Furthermore, Serum Zn and Pb levels correlated positively with this antibody, while Cd, Cr and Cu correlate inversely. This corresponds well with our previous findings with the other antibodies.

The odds ratio for this antibody was not significant, and it was prevalent in all our study subjects (Table 12).

MBP is an essential compaction protein of myelin, in both CNS and PNS. Due to attendant white matter loss in brains of AD patients, its reduction has been associated with the increase in A-beta 42 deposition (Papuc & Rejdak, 2020). As studies of multiple sclerosis have shown, MBP is released into the blood stream and lymphoid tissue which would correlate with elevated levels of IgG antibodies against MBP in all three disease groups. Myelin degeneration is the result of severe axonopathy or myelinopathies which is common in trauma, stroke (Liu et al., 2021) and ET (Louis, 2011) patients and seems possible in NMS patients. Stroke survivors showed elevated anti-MBP titers at 30 days after stroke. This also correlated with worse outcome (Shibata et al., 2012). Antibodies against MBP were also a predictor with cognitive decline in stroke patients (Becker et al., 2016).

Anti-GFAP IgG. The highest level of anti-GFAP IgG was found in the MCI group, followed by the NMS and then the ET group (Figure 5H). Levels of anti-GFAP IgG increase with age (figure 6D), with a correlation coefficient of >0.3 (table 12). It was also higher in males (figure 7C). Serum Zn and Pb correlate positively with anti-GFAP IgG, while Cd, Ba and Cu correlate inversely (Table 14). It may be elevated as a sign current astrocytic scar formation due to neuronopathy which would occur early on in MCI, and might later progress to dementia,

In the literature, GFAP antibodies were found in the sera of patients with AD, VaD, Senile AD, and aging healthy controls; suggesting GFAP antibody titer as a general marker of brain pathology and aging (Mecocci et al., 1995). Our results also demonstrated a positive correlation of anti-GFAP IgG with age (p=0.001). Neurodegenerative diseases in general have shown an increase GFAP

levels in CSF (Abu-Rumeileh et al., 2019). GFAP is produced when astrocytes hypertrophy, which usually happens during astrocytic scar formation. The astrocytic scar usually fills in the areas where neurons or their axons are lost, and therefore higher levels of GFAP released into the serum are indicative of neuronopathy/neurodegeneration (Mason et al., 2013). This excess GFAP loss is believed to be associated with scar stabilization (El-Fawal and O'Callaghan, 2008) and secondary to neuronal loss as may be taking place in the disease conditions in the present study. IgG antibody levels were higher in the disease groups compared to the controls, and IgM levels were lower. This further supports GFAP as a biomarker for acute NS damage. More studies are needed to confirm this relation, given that RR and OR were obscured by the fact that the reference group was not devoid of NAb prevalence, given the presence of environmental exposures.

4. Age and Sex as Determinants of Titers

Age.

It is not surprising that given the population under study, with attendant risk factors of diet, occupation, and environment, that age would prove to be a factor in modulating the prevalence of NAb or titer levels. Age is a risk factor for cardiovascular complications and stroke. Duration of exposures to occupational and/or environmental chemicals, such as heavy metals determined in the present study, are likely to manifest a life-long impact, particularly with their persistent presence. Organic solvents and/or pesticide exposure can also not be ruled out, considering that many of the individuals assayed reside in rural or semi-rural communities. Aging is known to be associated with immune alterations, including a reduced production and function of T and B lymphocytes, and a predisposition to developing autoimmune responses. A general greater prevalence of IgG in the present study (figures 6A-F) with direct and significant correlation with

age would tend to confirm that the NS insult is well established in the immunological memory of the subjects, whereas, IgM responses tended to be inversely correlated. Among other NAb which increase with age and have a significant correlation coefficient (Table 13) are anti-GFAP IgG (figure 6D), and anti-a-SYN IgG (figure 6F). Association of the latter with age has been also reported by Shalash and co-investigators (2017) in older PD patients and serum anti-α-SYN IgG compared to younger patients.

Sex.

The differences in the immune system between males and females is due to multiple factors which are mainly hormonal, genetic and environmental (Klein & Flanagan, 2016). Autoimmune diseases are more common in females (Lotter & Altfeld, 2019). T cell numbers, CD4+ T cells and CD4+/CD8+ ratio, and antibody response are greater in females than males (Klein & Flanagan, 2016). Females also have higher basal immunoglobulin levels and B cells (Klein & Flanagan, 2016). Our results showed that females have higher levels of IgG antibodies against NF-L and anti-IgM against NF-H compared to males (Figures 7A-E). Males had higher IgM antibody level against NF-L, α-SYN and IgG antibodies against GFAP (figures 7A, C-D). Our study populations had similar sex ratios except for the ET group, which had more males. Males had a higher level of anti-MBP IgG (figure 7C-D), and anti-GFAP IgG (Figure 7C) as well as anti-NF-L IgM (Figure 7A). All of which have the highest concentrations in the autoantibody pattern in the ET group. Sex difference in levels of heavy metals were present as well. Figure 8A-D show that males had significantly higher levels of Zn and Pb. Pb levels in an Egyptian population from the same region as our study participants showed an increased levels of Pb in males compared to females (Mortada et al., 2002). This might be due to higher exposure to these metals at the workplace. Men are more likely to work in construction and heavy industries and spend more time outside the home, where

the exposure to certain heavy metals is elevated, explaining the elevated levels compared to females found in our study. Aluminum and Chromium on the other hand were higher in females (Figure 9). Because we did not have any data on the BMI of our subjects, we can't determine whether fat deposition of some metals affected the corresponding levels in the serum.

5. Heavy Metals as Determinants of NAb Titers

Heavy metals levels differed according to the different disease groups. Table 14 presents this data. It showed that NMS patients present with significantly high levels of serum Zn and lower levels of serum Cd, Cu, As, Al and Mn. Research about this syndrome are lacking in defining environmental factors other than medications, so this is the first study which tackles these heavy metals levels in NMS. For bipolar disease, a study by Siwek at al. (2016) showed no difference in the Zn concentration in patients with mania compared to controls. Zn has been shown to act as an inhibitor of the NMDA glutamate receptor. It also impacts the limbic system which is important in the mood disorders such as schizophrenia and bipolar disorder (Chen et al., 2018; Petrilli et al., 2017; Strik et al., 2018). Zn deficiency during childhood has been linked to schizophrenia development in adulthood (Petrilli et al., 2017). A meta-analysis about Zn and schizophrenia showed no specific pattern, except that dysregulation in Zn homeostasis may be involved in the pathogenesis (Joe et al., 2018). Zn showed a strong negative correlation with anti-NF-H IgM (Table 14), which was discussed before as being specific to the NMS group. This association of heavy metals and NAb in the pathogenesis of NMS warrants further investigation. Another meta-analysis showed an increase in Cu, and decrease in Zn and Mn in schizophrenic patients (Saghazadeh et al., 2020). In a research report, plasma Al and Zn in schizophrenia patients were shown to increase after 6 weeks of treatment (Sussulini et al., 2018). Mn levels were specifically

low in the NMS group, which correlates with a common finding in schizophrenia patients. Reduced Mn levels are associated with a reduced Mn superoxide dismutase and arginase activity which may play a role in the pathogenesis (Cao et al., 2019). Low Cu levels are also a common finding in schizophrenia, especially after the administration of antipsychotic treatment. It's main effect is due to its role in dopamine metabolism mentioned earlier (Cao et al., 2019). Heavy metal levels, medication and duration of disease are likely to be interacting factors in NMS presentation and should be further investigated.

The profile of responses and heavy metals in MCI and ET displayed a similar pattern. As for As, a study on mice showed the loss of neurons in the Purkinje layer and occurrence of autophagy by transmission electron microscopy (TEM) in the cerebellum when exposed to high dose of As (Manthari et al., 2018). These are the same targets in ET, which may explain arsenics contribution to ET, given that levels were highest in this group. As reduced tyrosine hydroxylase (TH) level in the corpus striatum in rats and subsequently dopamine concentration in the striatum. TH is the rate limiting enzyme for dopamine synthesis (Chandravanshi et al., 2019). This may be implicated in the effect of As on the ET group, especially because it correlated positively with serum IgM antibodies against α-SYN and negatively with serum IgG antibodies against NF-M (Table 14). A study by Abou-Donia et al. (2013) measured the autoantibody level against neurofilaments in individuals with neurological deficits who were exposed to As. They showed a correlation with NF-L and NF-H that could differentiate between organic damage, and subjective complaints.

MCI patients had a significantly lower As serum concentration to references (Table 14). This might be because of accumulation of As in the brain (Baum et al., 2010; Benramdane et al., 1999; Gong & O'Bryant, 2010). A study by (Gong & O'Bryant, 2010) explains how As exposure my increase the risk for AD and the hypothesized pathways and the effect of As on the white matter, astrocytes

and axons (Abou-Donia et al., 2013). Arsenic also inhibits acetylcholinesterase and choline acetyl transferase enzyme levels in rats' brains. Plasma cholinesterase activity is also diminished with As exposure. All of these effects lead to the accumulation of acetylcholine in the brain, and downregulation of cholinergic receptors in the frontal cortex and hippocampus. This likely leads to difficulty in memory and learning abilities (Chandravanshi et al., 2019). The cholinergic system is a primary target in the pathogenesis of AD (H. Ferreira-Vieira et al., 2016).

Our results showed significantly elevated serum Zn levels in the MCI group (Table 14). Previous studies showed mixed results. Iqbal et al. (2018) showed results similar to ours, with increased levels of Zn in cognitively impaired patients. A study on occupational workers showed no effect of Zn on cognitive function (Mohammed et al., 2020). Not many studies were found on MCI patients, so we looked at AD studies as well. A meta analysis in China also showed that serum Zn levels were lower in AD patients compared to references (D. D. Li et al., 2017; Z. X. Wang et al., 2015). This is contradictory to our results. Zn excess is related to Cu deficiency which can lead to cognitive dysfunction (Nuttall & Oteiza, 2014). In our MCI population we can see that Cu concentration is especially low compared to the other disease groups and the references (Table 14). Our MCI group had previous hemorrhagic strokes, so we looked at past studies relating to this and found that acute hemorrhagic stroke patients in previous studies showed lower levels of Zn, unlike our study, but also lower levels of Cu as in our study (Karadas et al., 2014). High Zn levels were also associated with paresthesia and numbness, and it is also linked to paralysis (Afridi et al., 2011). This correlates with our MCI study population where most of the patients suffer from paralysis due to stroke.

Serum Al level was found to be significantly elevated in the MCI group only (Table 14). It did not correlate positively with any of the autoantibodies (Table 14).

No studies were found analyzing Zn levels in ET patients, so we looked at studies relating to PD. Zn levels were found to be lower compared to references in PD patients (Mezzaroba et al., 2019; Sun et al., 2017). Another study showed serum Zn levels to be similar between PD patients and controls (Ajsuvakova et al., 2020). Other studies support the idea that Zn levels correlate negatively with age and disease severity (Ajsuvakova et al., 2020), which is why in our population (which is younger and is at early stages of PD) the level of Zn is higher than found in the literature. Zn at high levels causes demyelination (Iqbal et al., 2018), this is supported by the positive correlation of IgG antibodies against MBP with Zn in our study (Table 14 and figure 8B). Serum Zn levels also correlated positively with anti-GFAP and anti-α-SYN antibodies (Table 13). Both Zn and Pb had similar patterns when comparing the levels of autoantibodies according to stratification (figure 8A-B). Higher levels of these heavy metals in the serum correlated with higher levels of autoantibodies, specifically IgG antibodies against NF-H, GFAP, MBP and α-SYN and IgM against NF-L. In PD, Cu increases oxidative stress leading to Lewy body formation. Zn and Cu are both found in the rim of senile plaques of autopsied brains of AD patients in higher amounts compared to controls (Lovell et al., 1998). This might explain the low level of Cu in the serum of our MCI patients (Table 14).

As for Pb, our results show that serum Pb concentrations were higher in both MCI and ET patients, compared to the reference group (Table 14). Pb is known to cause cognitive impairment, especially in early childhood exposure. High blood Pb concentration was associated with impaired cognitive function. This correlation was even stronger with higher blood concentrations and with age (Iqbal

et al., 2018). Also, acute hemorrhagic stroke patients have shown increased serum levels Pb in previous studies (Karadas et al., 2014). Serum Pb concentration had a significant positive correlation with IgG antibodies against NF-H, MBP, α-SYN and GFAP, and IgM antibodies against NF-L (Table 14) especially when at high concentration (>27.8 ug/dL) (Figure 8A). This was regardless of the disease groups. A study comparing workers exposed to Pb in a battery factory to references showed an association between antibodies against GFAP and NFL and Pb exposure (Abdel-Moneim et al., 1999). This is plausible because of the proven effect of Pb on the NS. Pb intoxication in humans causes axonal degeneration (Mitra et al., 2017), which leads to neuronopathy and myelinopathy, increasing the levels of GFAP and MBP. Pb affects astrocytes directly through intracellular accumulation. Astrocytes have limited antioxidant capacity, and are therefore prone to damage (Shefa & Héroux, 2017).

Pb crosses the BBB as calcium ion substitute and interacts with NMDA receptors. (Karadas et al., 2014; Mitra et al., 2017). NMDA is the receptor for the neurotransmitter glutamate, which is the main excitatory neurotransmitter in the brain. Pb affects calcium signaling by altering the NMDA receptor expression. Eventually this leads to calcium depletion and reduction in brain derived neurotrophic factor (BDNF) which is linked to cognitive deficits (Mitra et al., 2017). This is how it may be implicated in the MCI pathology, as seen in our results. Pb also affects synapse formation and neuroplasticity (Shefa & Héroux, 2017). This directly affects neurotransmitter release which subsequently affect the cholinergic and dopaminergic systems in the brain which are implicated in AD and PD pathology respectively (Shefa & Héroux, 2017). This effect can be implicated in the MCI and ET groups.

Whole blood Pb concentration correlated positively with IgG antibodies against NF-L in the MCI and ET individuals. This is consistent with a study by (Louis et al., 2003) where blood Pb

concentration correlated with the diagnosis of ET, especially sporadic cases. In addition, a study by Ong et al. (2019) showed increased blood lead concentrations compared to controls in ET patients. Pb and Zn serum levels were found in high concentrations in the MCI and ET groups (Table 14). Their correlation coefficients were highest for anti- NFH, -GFAP, -MBP, and -α-SYN IgG, all of which were the most significantly elevated and prevalent in the ET and MCI groups (Table 14). Zinc and Pb highly correlated with anti-NF-L IgM, which was included in the autoantibody pattern of the ET group (Table 13; Figures 8A-B).

GFAP is a strong indicator of astrocytic scar formation which happens after neuronal loss. So, when serum Zn and Pb show a positive correlation with anti-GFAP IgG antibodies (Table 14), this is consistent with their neurotoxicity, particularly that they are both elevated in the MCI and the ET groups. In addition, the presence and significant correlation of α-SYN antibodies in these disease groups with both of these heavy metals support the role of this protein in the pathogenesis of these diseases which later result in neurodegeneration.

Serum Cd levels were significantly low in the MCI group (Table 14). Cd cannot enter the brain readily, so the occurrence of the stroke and barrier disruption will have aided the accumulation in the brain. It was also positively correlated with IgM antibodies against NF-L, NF-H, GFAP, and α-SYN (Table 14). This is concordant with the fact that it is correlated with chronic and acute neurological disease, like MCI. As mentioned in the introduction, Cd has many effects on the brain. It may well be one of the neurotoxins that plays a role in the pathogenesis of dementia. Previous studies showed that acute hemorrhagic stroke patients have increased serum levels of Cd (Karadas et al., 2014).

When investigating the serum heavy metals levels and their correlation to the autoantibodies using the correlation coefficient, there appears to be a general trend of negative association of heavy

metals with IgG and positive association with IgM titers, except for anti-NF-L. This is not true for Pb and Zn, which show the inverse association. Here it is important to remember that IgM not only indicates a primary immune response, but also a chronic one, while IgG indicates memory (secondary response) and acute damage.

Stratification of titers based on tertiles of metals directly correlated with NAb (Osuna eta l., 2014) was used to determine the association of NAb levels with internal dose as determined by serum levels. Significant dose-dependent increases in NAb were evident for Pb, Cu, Zn and Cd (Figures 8A-D). Both Cd and Cu showed a dose-dependent increase in IgM NAb titer against NF-H, GFAP, and α-SYN (figures 8C-D), and IgM NAb titers against NF-L (figures 7A-B). Increase in IgG antibody titers against NF-H, GFAP, MBP and α-SYN were similar in both Pb and Zn (figures 8A-B).

6. Summary and Conclusion

This preliminary study sought to test whether the use of detection of autoantibodies directed against neural antigens, referred to as neurantibodies (NAb), be used to indicated nervous system damage in conditions with varying (and confounding) etiologies.

Eight of the NAb assayed had a greater prevalence in three conditions. Titer levels of the IgG isotype were elevated more than IgM antibodies for the NF-H, GFAP, MBP and α-SYN antigens. This suggests that these subjects had a damage with a more prolonged duration as to have well established immunological memory. However, it should be borne in mind that with progressive insult or degeneration that IgM will also be elaborated (Mason et al., 2013; El-Fawal, 2014). The results also suggest that anti-NF-H, IgG, may prove to be a reliable biomarker of NS damage given the consistency of the response displayed in the present study. This is further supported by the RR and OR (Table 11) which shows that these antibodies were found to have an odds ratio above 8

with a significant p value <0.02 in all three disease groups, for the IgG against NF-H and a RR>7 in all disease groups (p= 0.0001). The Receiver Operating Characteristic (ROC; Appendix) for NF-H, IgM and IgG, as for 10 other NAb, showed a significant sensitivity and specificity.

When we compare the antibody level in the serum, we find that IgG antibodies against GFAP, α-Syn and MBP are also elevated compared to reference. Also, IgM against NF-L was elevated in disease groups compared to references. These antibodies may, therefore, provide a panel of potential biomarkers for NS damage.

Prompted by the high prevalence of NAb against NF-M, GFAP and MBP in the reference group, which suggested subclinical neurological involvement, and given the industrialized/rural environment of the population, heavy metal, many of which are neurotoxic, levels were determined for all participants. Several heavy metals, most notably Zn, Pb, CU and As, appeared to have an association with the detection of NAb. This provides a cautionary tale in selection of reference groups when validating biomarkers. It also underscores the significance of environmental factors as contributing factors to neurodegenerative conditions, particularly in light of their sporadic nature.

A second cautionary note is that while the present study demonstrates the potential for NAb use and translation into a clinical setting, larger cohorts need to be used to investigate and validate their utility, coupled with more objective measures of neurological function, and a longitudinal study design. Of note is that the threshold of damage leading to NAb formation is much lower than needed for neuronal loss to cause clinical sign, thereby providing an early alert (El-Fawal, 2014; Salama et al., 2018)

While this study represents a proof-of-concept and the versatility of the assay, a team effort between neurologists, biochemists, and public health professionals needs to be incorporated in this type of study, prospectively. The present study relied on the generous willingness of patients and clinicians to provide samples to test a first-tier approach to answer the question: can NAb detect insult to the nervous system. In that regard, the study is a success.

The present study also succeeded in shedding some light on the importance of environmental factors to nervous system health. By no means are heavy metals the only environmental factors capable of inducing neurotoxicity. Industrial solvents, pesticides, nutritional deficiency and life style are factors that should be taken into consideration, both in patient history and analytically in the laboratory.

This present study represents the first of its kind in defining the role NAb detection may play using a population with as yet unidentified etiologies, diagnoses, and pathophysiology. Particularly interesting is the suggestion that NMS may have an organic basis and the ET may have a neurogenic component.

In short, this study lays the groundwork for a promising avenue of investigation towards translational value. In the future this should be coupled with genomic and environmental assessment towards understanding the human exposome.

Limitations and advantages of the study

While it would have been a more specific and clear study if we were to examine only one population with a specific disease, it was not in the options. The study population was the one available to us, and we decided to use it to our advantage. In a tier 1 study, we look for any marker which can represent nervous system damage, regardless the cause. So, looking at these 3 different

diseases/populations gave us more opportunity to examine non-specific markers, which can then guide us to pursue the hunt for a more specific marker. Looking for a specific marker from the beginning will be tedious and exhausting, as we would be looking for a needles in a mountain of hay. This research is our starting point for more thorough research.

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Appendix I

The Mini-Mental State Exam (MMSE) tests cognitive function in the elderly. It is comprised of a number of tests, each of which has a score. The testing includes orientation, attention, memory, language and visual-spatial skills. The lower the MMSE score, the more likely it is that cognition is impaired in this individual. Education level in the patient is a major determinant of the cut-off value for the MMSE score.

Diagnostic Criteria

- For each NMS patient, the duration of disease, the antipsychotic responsible, somatic pathology and the current symptoms were identified in addition to meeting the criteria of DSM-5 classification.

-According to (Lorenz & Deuschl, 2007) ET by definition, is an action tremor or a bilateral postural tremor of the hands or head. Diagnosis usually requires 3 years, to assess the slow progression. There is still no consensus on the criteria of diagnosis for ET, but Deuschl et al. 1998 has set a definition for definite, probable and possible ET.

For diagnosing ET, one should assess:
-the anatomic distribution of tremor;
-the activation condition of the tremor (rest, posture, intention);
-the tremor amplitude and frequency;
-the muscle contraction pattern as agonist- antagonist interaction;
-the degree of functional disability and handicap;
-the psychosocial problems and the impact on the patient's life;
-the duration of tremor;
-the history of disease progression;
-the family history of tremor;
-the sensitivity of tremor to alcohol;
-the coexistence of other neurological signs or symptoms;
-the current medication and the response to current and previous medications.