Identification and Characterization of Risk Factors in Alzheimer's Disease

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Identification and Characterization of Risk Factors in Alzheimer's Disease

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MASTER THESIS WORK

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ABBREVIATIONS

AD	Alzheimer’s disease
Aβ	Amyloid-beta
sAD	Sporadic Alzheimer’s disease
fAD	Familial Alzheimer’s disease
rpAD	Rapidly progressive Alzheimer’s disease
PSEN1	Presenilin 1
PSEN2	Presenilin 2
APP	Amyloid Precursor Protein
ROS	Reactive oxygen species
TREM-2	Triggering receptor expressed on myeloid cell 2
TIA-1	T-cell intracellular antigen-1
ER	Endoplasmic Reticulum
TAU	Tubulin associated unit
pTAU	Phosphorylated tubulin associated unit
SFPQ	Splicing factor-proline and glutamine rich
RBP	RNA-binding protein
MAPK	Mitogen activated protein kinase
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
IgM	Immunoglobulin M
IgG	Immunoglobulin G
BSA	Bovine Serum Albumin
TBS-T	Tris buffered saline
PBS-T	Phosphate buffered saline
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
IAA	Iodoacetamide
GAPDH	Glyceraldehyde 3-phosphate dehydrogen-ase

ddH2O	Double distilled water
MW	Molecular weight
FBS	Fetal bovine serum
kDa	Kilo Dalton
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

ABSTRACT

Alzheimer's disease poses a steady loss of neurons, disruption of synapses and, aggregation of proteins such as tau and amyloid-beta. It influences more than 50 million people around the globe, with no preventive treatment or early diagnosis. Drugs targeting amyloid-beta accumulation have failed in clinical trials. Tau hypothesis and oxidative stress are the next strong candidates of the therapeutic and diagnostic target. This study has investigated the role of oxidative stress and related pathways involved in post-translational modulation of the ptau and prp through in vitro analysis. The three-phased study included oxidative stress induction through NaAsO3 and human CSF (cont., sAD, rpAD) in the 1st and 2nd phase respectively. The induction of stress was analyzed through immunoblotting and co-immunoprecipitation in 1st phase and cell-viability assay (MTS) and co-immunofluorescence in the 2nd phase. The third phase was to characterize the oxidative stress as a risk factor through 2D-proteomic analysis comparison of in-vitro stress-induced cell lines with that of human brain lysates (cont., sAD, rpAD). Results showed enhanced modulation and change in localization of AD-related proteins i.e., ptau, prp and SFPQ with oxidative stress marker TIA-1. A decrease in the viability of cells under oxidative stress was also observed. This confirms the disease progression role of oxidative stress linking it to the hallmark of AD through its aggregation. It also explains the pathway of neuronal cell death through cytotoxicity by means of co-localization analysis of essential aggregating proteins in AD and stress granule marker TIA-1. Further identification and characterization of important molecular factors contributing to the pathways of oxidative stress in AD as well as other neurodegenerative diseases will add in to a better understanding of its mode of action and mechanism. A mass-spectrometric analysis of the interacting partners of oxidative stress markers will give further insights into new therapeutic and diagnostic targets.

CHAPTER 1

INTRODUCTION

A pre-symptomatic diagnosis and cure of Alzheimer's disease (AD) are still elusive after more than a century of its discovery. According to a report of Alzheimer's disease International, more than 50-million people all over the world suffer from dementia and a rise to 152 million is expected by the year 2050. Most of these people live in Asia (29.23 million), in the low-income population. Only in south Asia, 8.41 million people are suffering from dementia (International et al., 2020). AD is the widespread cause of dementia including gradual loss of memory and disruption of routine life activities making the patient a social burden (Weller & Budson, 2018). Only in America the reported estimated cost of caregivers for the year 2018 was 277 billion dollars as stated in a 2018 report of Alzheimer's Association. Also, there is an increased risk to the emotional and mental health of the caregivers increasing the cost and social burden of the country ("2018 Alzheimer's Disease Facts and Figures," 2018). Thus, it's imperative to find not only a cost-effective treatment but also, early preventive measures. The two main risk factors of AD that have been under extensive research since its discovery are the accumulation of neurofibrillary, hyperphosphorylated tau and Amyloid-beta (Aβ). Even today presence of these hallmarks is essential for the AD diagnosis. Based on age, mode of onset, and risk factors AD is classified into an early onset or familial AD (fAD) which follows an autosomal dominant mendelian order, and a late-onset sporadic AD (sAD) caused by multiple polymorphisms and environmental risk factors. sAD accounts for most of the cases of AD however fAD accounts for only 1% of the cases and through genetic pedigree at-risk individuals can be identified (Lista et al., 2015; Rossor et al., 1996). Three of the gene mutations found in fAD are PSEN1, PSEN2 and APP or amyloid precursor protein. All these mutations are linked to an abnormal accumulation of Aβ (Sepulveda-Falla et al., 2020). Amyloid hypothesis is essential in explaining fAD, however, sAD is now explained as a product of the tau hypothesis, where tau initiates glutamatergic neuronal cell death and increases Aβ accumulation by trapping endosomes containing APP on microtubules (Arnsten et al., 2021). Rapidly progressive AD (rpAD) is a specific form of fAD with PSEN1 mutation, early onset in the 20s and progression time is very rapid as compared to other classes of AD.

However, the neurological changes are atypical and an increased tau and Aβ ratio in CSF is identified (Mendez, 2019; Raimundo et al., 2021). Among two hallmarks Aβ hypothesis has been vastly studied as the preceding cause of AD. fAD related mutations also give a strong link to the Aβ hypothesis, however, it only accounts for 1% of the cases. In the case of sAD observations report an imbalance between production and clearance of Aβ (Selkoe & Hardy, 2016). Aβ hypothesis has been in the limelight of research for both diagnostic and therapeutic targets, considering it a preceding risk factor of AD (Hardy & Selkoe, 2002; O'Brien & Wong, 2011b). Various Aβ targeting therapies including, anti- Aβ antibodies, vaccination, intravenous immunoglobulins, and immunotherapy have been tried (Y.-H. Liu et al., n.d.). The drug development targeted Aβ production, clearance, anti-plaque formation to balance the Aβ homeostasis (Kung, 2012). However, the failure of therapies based on the Aβ hypothesis makes it evident that there is another risk factor involved in AD progression and initiation. A microtubule-associated protein tau is considered as the key contributing factor of AD, after failure of Aβ targeted therapies (Kametani & Hasegawa, 2018). The gene coding for human tau protein is present on chromosome 17 providing a genetic link to tau accumulation and propagation (McMillan et al., 2008). Abnormal tau accumulation is also boosted by other risk factors such as oxidative stress (OS), polymorphisms or gene mutations linked to inflammation response proteins such as ApoE gene, and TREM2. Both genes are associated with astrocytes of microglia inducing inflammation responses. Inflammation responses mediated by microglia support tau mediated neuron damage without involvement of Aβ (Bejanin et al., 2017; Leyns & Holtzman, 2017; Shen & Kelleher, 2007). Tau has 6 isoforms and experiences numerous PTM such as phosphorylation (Martin et al., 2011; Morris et al., 2015). OS, now considered as another important risk factor in AD shows pathophysiology coupled with hyper-phosphorylated tau protein, disrupting its normal function (Melov et al., 2007a). OS, results by an imbalance of production and clearance of reactive oxygen species (ROS) by antioxidants. Inflammatory responses by microglia are a key source of ROS giving another link of mutations in ApoE and TREM2, inflammation and OS to tau pathology (Alavi Naini & Soussi-Yanicostas, 2015; Egaña et al., 2003). Colocalization of ptau with stress granules under inflammatory responses or OS gives a clear link of tau as an important modulatory protein in AD-related pathology (Younas et al., 2020). Under normal conditions there is minimum interaction of tau with stress-granule protein TIA-1, however, after

phosphorylation of tau, it is found to be colocalizing TIA-1 in stress granules (Asadi et al., 2021; Brunello et al., 2016). OS also alters the protein responses involved in Aβ degradation, resulting in Aβ cellular toxicity. Aβ plaques are implicated in the formation of reactive oxygen species that further oxidize proteins, nucleic acids, and membrane lipids creating a cyclic response between the two (Chakrabarti et al., n.d.; Cheignon et al., 2018). Modulation of hallmarks of AD under OS and its increase under various genetic and environmental conditions, e.g., AD-related gene mutations, environmental factors such as head injury, ageing, metallic load, infections or inflammation and poor or unhygienic diet plan make it a strong candidate as a major risk factor of AD. Low-income/ developing countries that are exposed to such environmental conditions are hence more prone to AD. OS is also a link to explain the relation of AD with co-morbidities such as obesity and diabetes (Newcombe et al., 2018; Pugazhenthi et al., 2017). This study identifies OS, as a risk factor implicated in increased post-translational modification of tau-protein and its colocalization with TIA-1 a stress marker. The study is conducted on human neuroblastoma cells (SHSY-5Y) and HELA cells. There are three phases of the study. In phase-1, artificial OS is induced by NaAsO3 that ameliorates tau post-translational modification. In phase-2 human CSF of control, sAD, and rpAD is used to induce OS to study apoptotic responses and colocalization of tau and prion protein with stress markers. Prion protein acts with Aβ resulting in synaptic failure and neuronal loss (Zhou & Liu, 2013). Although, prion protein has an essential role in pathogenesis of AD, the study shows that the expression of it is decreased in brain sample of AD as compared to the control brain samples (Velayos et al., 2009). In phase-3, 2-dimensional proteomic analysis of ptau (S199) and total tau (tau-5) on human brain lysate of control, sAD and rpAD is conducted in comparison with that of NaAsO3 treated SHSY-5Y cells to characterize the OS as a major risk factor in AD.

According to the available literature, it is hypothesized that OS enhances phosphorylation of tau that leads towards cell apoptosis recruiting stress granules and pathways related to it. understanding the role of OS as a preceding risk factor in AD pathogenesis, new therapeutic and diagnostic targets can be achieved. There were two objectives of the study:

1. To Identify risk factors involved in AD progression.
2. Characterization of AD-related signal transduction pathways.

This thesis comprises of 5 chapters. Chapter-2 discusses the background of AD with its risk factors, diagnostic techniques and therapeutic techniques that are being used so far. Chapter-3 gives a thorough view of the experimental work that has been done. Chapter-4 consists of the results and chapter-5 discusses the observations of the study in comparison to the literature.

CHAPTER 2

LITERATURE REVIEW

2.1. Alzheimer's disease:

The disease was first presented by a clinical psychiatrist Alois Alzheimer, to a conference in 1906, which he labelled as "peculiar severe disease process of the cerebral cortex". The early observations included loss of memory, sleep, and coordination that accelerated to a degree resulting in the death, of a 51-year-old woman, within 5-years. Loss of neurons results in gradual memory loss and problems related to the routine activity such as coordination, sleep, judgment, impulsivity, recognition, and the sensibility of direction. AD, being a rapidly developing disease coupled with neuron deterioration, is the most frequent type of dementia (Finder, 2010). More than 43-million people were estimated to live with dementia in (2016) with Alzheimer's disease being one of the leading causes (Nichols et al., 2019). A later histology report of the brain indicated plaques and tangles present in the patient's brain (Hippius & Neundörfer, 2003). Hence, establishing the key players in the disease pathology as tangles comprising tau and plaques consisting of Aβ. The research indicates that disease pathology begins years before any visible clinical symptoms in patients aged 70 and above (Scheltens et al., 2016). Being multifactorial various risk factors involved in disease pathology are genetics, environment, comorbidities, ageing, and trauma (Gatz et al., 2006; Newcombe et al., 2018). Genetic risk factors include an early disposition of up to 50 genes loci coding enzymes and proteins that regulate essential pathways involved in immunity, endocytosis, ubiquitination, transport, Aβ, and Tau processing (Sims et al., 2020). Several environmental factors include pollution, sedentary lifestyle, diet leading to obesity, alcohol consumption, smoking, injuries, and infections (Campdelacreu, 2014). Other factors include disruption in metabolic pathways and diseases such as diabetes, cardiovascular disease, hypertension, hypercholesterolemia, depression, and gastrointestinal disorders (Santiago & Potashkin, 2021). AD is divided into two subtypes based on the age at onset and factors behind the pathophysiology. Familial Alzheimer's disease (fAD) is an early-onset AD, and genetic disposition is its major risk factor. Genes mainly include PSEN1, PSEN2, and APP follow the Mendelian rule of transmission and are autosomal dominant (Santiago & Potashkin, 2021).

Sporadic Alzheimer's disease (sAD) is referred to as late-onset AD and involves polymorphisms such as APOE along with environmental influence (Lin et al., 2018).

2.2. Disease-Related Risk Factors:

Ever since the discovery of AD in 1906, Aβ clusters outside the neurons and threads of tau tangles inside the neurons are the two hallmarks of the disease. Research to understand risk factors of AD always included these two hallmarks not only as therapeutic targets but also for diagnosis. Research has shown many other contributory factors to disease pathology.

2.2.1. Amyloid Beta (Aβ):

To define the stage of the disease and its severity, the density of Aβ plaques and that of tau tangles is observed (Butterfield & Boyd-Kimball, 2004; Ittner et al., 2010). Hence, Aβ deposits are one of the diagnostic markers for AD, and the density of insoluble Aβ deposits implies the stage of the disease (Murphy & Levine, 2010). Aβ is a 4-kDa, 37-43 amino acid long peptide produced by cleavage of Amyloid precursor protein (APP) by β and γ-secretase. The length of the peptide depends on the cleavage site of γ-secretase. It exists in two forms Aβ-40 and Aβ-42 which, are more common in the Aβ plaques (Zhang et al., 2010). Nuclear magnetic resonance (NMR) spectroscopy has revealed that Aβ is a combination protein with an α-helical structure from amino acid 15 onwards, and from peptide 1-14, it is unstructured. In the presence of membrane-like-fluids, it undergoes β-sheet conversion that can be seen in AD as well. 1-14 residues being primarily polar are affected by water. The acidic amino acids deprotonation in the helix of Aβ encourages the helix to a coil that leads to aggregation of Aβ (Chen et al., 2017)

APP is a single α-helix transmembrane protein with an extracellular domain, originated in the endoplasmic reticulum (ER) of the cell and shipped for packaging to the Golgi complex (Brien & Wong, 2011). Once matured, it translocates to the plasma membrane and is cleaved in a sequential pathway by two processes. Non-amyloidogenic processing involves sequential cleavage of the peptide by α-secretase and γ-secretase that produces an 83 amino acid long peptide chain (Allinson et al., 2003). It leads to the generation of neuroprotective APPα and is retained in the CSF (Palmert et al., 1989). The amyloidogenic process involves cleavage by β-secretase producing a 99 amino acid peptide. Final cleavage by γ-secretase leads to the

production of Aβ (Zhu, 2006). Aβ was discovered in 1984 from amyloid plaques surrounding the neurons. Once generated, Aβ assembles itself into soluble oligomers, insoluble large-fibrils, and protofibrils. Oligomers being soluble can translocate all over the brain and are more dangerous in disease pathology (Chen et al., 2017). The Aβ is grouped based on molecular weight, length, and dimension. Aβ was discovered in 1984 from amyloid plaques surrounding the neurons. Once generated, Aβ assembles itself into soluble oligomers, insoluble large-fibrils, and protofibrils. Oligomers being soluble can translocate all over the brain and are more dangerous in disease pathology (Chen et al., 2017).

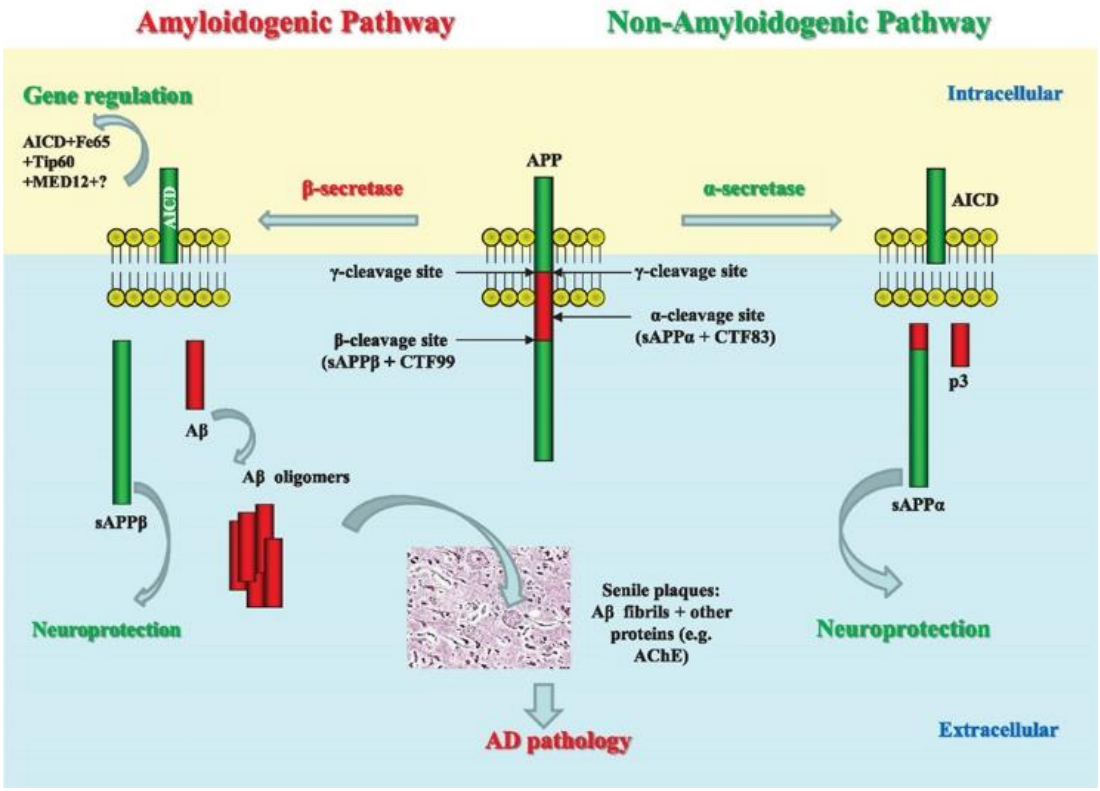

Figure 1: Schematic diagram of APP processing through amyloid-beta producing pathway and non-amyloid beta producing pathway and role in AD pathology (Adapted from Hicks et al., 2012).

The Aβ is grouped based on molecular weight, length, and dimension. Short oligomers are also either dimmers or hexamers. However, other soluble oligomers range in size of 17-42 kDa. Protofibrils are named pre-fibrils as they lead to mature Aβ fibrils. These peptides lead to self-aggregation, synaptic disruption and stimulate nitric oxide (NO) production by calcium influx. NO leads to the formation of peroxynitrite radicals triggering neuronal death. The soluble form of Aβ can impair

long-term potentiation and be detected in CSF of AD patients as a diagnostic marker (SADigh-Eteghad et al., 2015). Aβ amassing triggers macrophages of the brain called microglia. That activates many inflammatory responses and cell signals such as NF-kB and MAPK involved in cytokine and chemokine production (Ridolfi et al., 2013). Aβ based neurotoxicity provokes both cellular and humoral immunity. The presence of anti- Aβ antibodies in the CSF of AD patients is available in the literature (Weksler et al., 2005). Fibrillar Aβ stimulates the production of inflammatory cytokines by microglia through toll-like receptors TLR4, TLR5, and TLR6 (Boutajangout & Wisniewski, 2013). In the brains of healthy or young controls, Aβ readily degraded and cleared maintaining, an equilibrium between its generation and clearance. Insulin degrading enzyme (IDE) degrades insulin, amylin, few other peptides as well as Aβ. Aβ competes with insulin as a substrate for degradation by IDE (Qiu & Folstein, 2006). Neprilysin is another peptidase along with IDE that is involved in Aβ degradation. P-glycoprotein and other cellular clearance pathways such as "low-density lipoprotein receptor-related protein 1 (LRP1)" are also important in the clearance of Aβ. Aβ either degraded within the brain through neprilysin and IDE or transported out through the blood-brain barrier with the help of P-glycoprotein (SADigh-Eteghad et al., 2015).

With ageing, metabolic dysfunction such as type II diabetes, and genetic or pathological conditions, the processes of Aβ formation and clearance get disrupted. In genetic diseases, an additional copy of the APP gene causes excess production of Aβ. While, in metabolic disorders, high insulin in blood competes with Aβ for the IDE enzyme leading to accumulation of Aβ. IDE has 20x more affinity for insulin than Aβ. Studies report that deletion of LRP1 and p-glycoprotein from mouse model lead to Aβ build up in the brain cortex region. Also, some studies disclose a diminished activity of neprilysin in AD patients (Qiu & Folstein, 2006; SADigh-Eteghad et al., 2015). The toxicity caused by Aβ build-up has deleterious effects on brain cells, such as OS due to ROS , synaptic disruption, and cell signalling due to plaque formation around neurons. The figure shows the procedure of cell death and the role of Aβ in AD pathology.

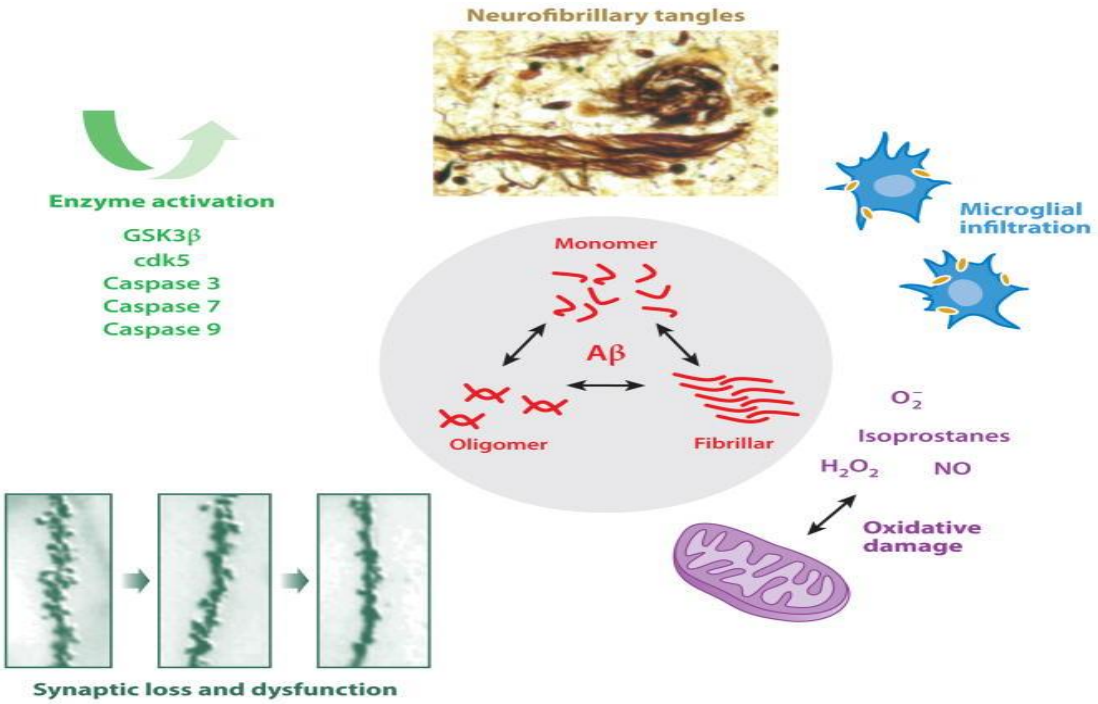

Figure 2: Role of Aβ in AD pathology through various mechanisms i.e., OS, inflammation, NFTs, synaptic disruption, and enzyme activation that are involved in apoptosis and phosphorylation (Adapted from O'Brien & Wong, 2011a).

2.2.2. Tau and phosphorylated Tau (pTau):

Tau is a microtubule-associated protein, is a fundamental part of the cytoskeleton. It mostly resides into cytoplasm that is involved in the stability of the microtubules and cellular trafficking through neuronal extensions called axons. The gene coding for tau resides on chromosome 17, consisting of 16-exons of which 3 undergo alternate splicing resulting in the 6-isoforms of the protein in the adult brain (Jesús Avila et al., 2004; Kanaan et al., 2015). Exon splicing depends on the stage of neuron maturation and the type of neuron (Wang & Mandelkow, 2016). The molecular weight of the tau isoforms varies between 37-46 kDa that appears to be 55-62 kDa owing to the mobility of tau isoforms in SDS-PAGE gels. Tau differ based on either presence or absence of a region coded by exon 2 and 3 at N-terminal as 0N or no insert, 1N for amino acid insert coded by exon 2, and 2N for insert coded by both 2 and 3 exons. Exon 3 always exist concurrent with exon-2 and never alone. Another criterion to distinguish the isoforms is the manifestation of microtubule-binding repeats at the C-terminal due to the absence (3R) of exon-10 or the presence (4R) of exon 10 (Guo et al., 2017). Tau protein is more hydrophilic with an α-helix or β-sheet secondary structure achieved on phosphorylation or post-translational modification. Tau protein

structure resembles a paperclip due to folding back of C-terminal over microtubule-binding-repeats, and N terminal also folds back in reverse (Jesus Avila et al., 2016). However, upon binding with the microtubule, the N-terminal extends away which, gives a linear structure. It also changes upon phosphorylation of the proline-rich region present upstream of C-terminal microtubule-binding repeats (Guo et al., 2017).

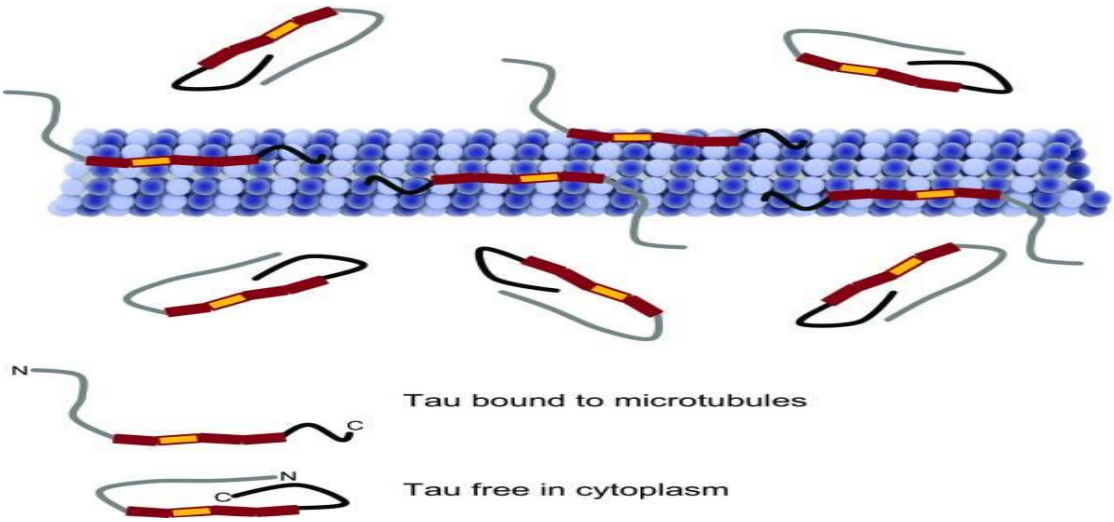

Figure 3: Structure of Tau protein: paperclip, and unfolded upon binding with microtubule (Adapted from Guo et al., 2017).

Tau interacts through microtubule-binding repeats with several other proteins such as filamentous actin, presenilin-1, apolipoprotein E and alpha-synuclein etc. It binds the actin with at least two microtubule-binding-repeats that make it bind concurrently to the actin and microtubule. Hence, tau is fundamental for normal synaptic function, proving its role in AD through aggregation and synaptic disruption (Elie et al., 2015).

The presence of the insoluble tau fibrillary tangles is essential as a diagnostic criterion for AD. Tau undergoes several PTM that include phosphorylation for several protein interactions and functions. Tau consists of at least 85 sites for phosphorylation, making it essential for the physiological role of tau. However, increased phosphorylation reduces the affinity of tau for microtubules binding, disrupting its normal function (Hanger et al., 2009). Thus, aberrant phosphorylation of proline-rich domain leads to tau dissociation from microtubule, and that of C-terminal stimulates self-aggregation of tau, as seen in tauopathies (Von Bergen et al., 2000). Hyper-phosphorylation stimulates aggregation, disrupts synaptic functions, and stimulates microglia by kinase activation, leading to neuron cell death. Also, tau protein assembles itself in paired helical filaments (PHF), an essential component of AD

pathology. Research shows that all 6-tau isoforms present in PHF were aberrantly phosphorylated (Barthélemy et al., 2020; Goedert, 1993). Like Aβ plaques, tangles also consist of a heterogeneous mixture of tau monomers, oligomers, straight filaments and PHFs. The complexity of hyper-phosphorylation is reflected by the fact that it leads to aggregation, pre-tangle formation, inhibition of cleavage of tau and phosphorylation of some sites can stimulate the phosphorylation of others. Pathology can also be indicated by an aberration in kinases or phosphatases, resulting in a lack of tau regulation (Congdon & Sigurdsson, 2018). Several kinases implicated in tau phosphorylation can be grouped based on region-specific phosphorylation. GSK-3 is an essential kinase both in AD pathology and in the normal functioning of the protein (Avila et al., 2004).

2.2.3. Oxidative Stress:

Thorough research on pathological pathways and hallmarks of developing AD has shown a link of OS with tau phosphorylation and accumulation, Aβ deposits, and specific neuronal cell death (HUANG et al., 2016; Perry et al., 2002; Smith et al., 2000). OS is not only a causative factor in the accumulation of altered proteins but also a resultant factor of those altered proteins as evident by the presence of byproducts of oxidization in Aβ plaques and Tau tangles (Gella & Durany, 2009; Markesbery, 1999). OS is mainly caused by a burden of reactive oxygen species (ROS), because of a failure in the clearance of ROS, mitochondrial disruption, external injury or infection that activates inflammation response in the body, excessive production of ROS as in old age or decreased production of antioxidants (Tönnies & Trushina, 2017).Iron and copper are such metals that are also a major source of ROS and hence OS (Persson et al., 2014). OS has an important role in brain disease since there is more involvement of oxygen in brain functions and brain lipids are readily oxidized. It affects the brain lipids, proteins, and mitochondrial DNA (Zhao & Zhao, 2013). The brain has an enormous number of polyunsaturated fats, more vulnerable to oxidization producing aldehydes as a byproduct. These aldehydes can also be found in CSF as OS markers. Carbonyls are released as a byproduct of protein oxidation, and it also causes filament formation of proteins resulting in aberrant accumulation and hyperphosphorylation. DNA oxidation products can also be found in CSF as biomarkers both in AD patients and in Ageing models (Markesbery, 1999). Aβ oligomers as potent neurotoxins also cause the synthesis of

ROS and OS (Mattson, 1997). Similarly, it is also evident through studies that OS enhances the activity of β and γ-secretases and lowers α-secretase activity, thus promoting Aβ production and accumulation (Behl et al., 1994; Quiroz-Baez et al., 2009; Tamagno et al., 2002)s.

2.3. Diagnosis of Alzheimer's disease:

The development towards the diagnosis of Alzheimer's disease started after 1984. Since the discovery of AD in 1906, only post-mortem studies showed the buildup of neurofibrillary tangles and plaques along with neuronal cell death. The early diagnosis was by neuropsychological diagnostic criteria. Further development led to identifying different biomarkers, and research is ongoing on developing biosensors for AD diagnosis.

2.3.1. Neuropsychological Diagnosis:

2.3.1.1. NINCDS-ADRDA:

It is a criterion established by the "National Institute of Neurological and Communicative Disorders and Stroke- Alzheimer's Disease and Related Disorders Association" in 1984. It is not a laboratory test but a neuropsychological test that confirms the incidence of dementia based on a decline in 8-cognitive functions. It linked the severity of dementia with underlying AD-related pathology, and to verify a biopsy or an autopsy of the patient was needed. A person with AD pathology exhibits impairment of functional abilities, memory, problem-solving skills, orientation/coordination, attention, language, perceptual skills, and constructive ability. The criteria also exclude the possibility of any other brain disease or comorbidities that can lead to dementia (Dubois et al., 2007; Khachaturian, 1985; McKhann et al., 1984).

2.3.1.2. DSM:

It was first published in 1952 by the American psychiatric association, and its most recent revision is called diagnostic and statistical criteria for mental disorders (DSM-5). As suggested by the name DSM is not specific only for AD, but it gives the idea of the presence of dementia or neurological disorder based on the impairment of cognition that affects routine activities. It diagnoses two diseases named Major cognitive impairment and mild cognitive impairment. Hence, it is applied with NINCD-ARDA criteria (Apostolova, 2016; Khachaturian, 1985).

2.3.2. Neuropathological diagnosis:

Neuropsychological diagnosis cannot diagnose AD in its initial stages before dementia has developed and has less specificity, often confused with mild cognitive impairment. So, the utilization of biomarkers specific to structural and molecular changes was essential for further development in the disease. The neuropathological criteria are based on the examination of autopsies and can group the patient as healthy, probable, or definite AD. Thal Aβ phase criterion groups the disease into 5 phases based on the spread of Aβ deposits in various brain regions (Thal et al., 2002). BRAAK-NFT staging is a criterion that groups the AD brains into six stages based on the spread of neurofibrillary tangles and neuropil threads throughout different regions (Braak & Braak, 1991). Another criterion is "the consortium to establish a registry on Alzheimer's disease" (CERAD), which groups the brain into 4 phases, from healthy to definite AD brains based on neuritic plaques deposition (Mirra et al., 1991). Currently, all three criteria are employed concurrently to group the brains into ABC stages (Dallaire-Théroux et al., 2019; Deture & Dickson, 2019).

2.3.3. Brain imaging:

2.3.3.1. MRI and CT-Scan:

So far, AD diagnosis employs the concurrent use of different techniques available. Magnetic resonance imaging (MRI) and Computed tomography (CT scan) are diagnostic techniques and measure volume loss in the grey matter of the brain and demyelination of the axon process in the white matter. The accuracy of the two is 87%. The volume loss due to neuronal cell death is reciprocal to NFT density which is a requirement of BRAAK stages. Hence, these can be used in combination to define the stage of AD in any patient. However, neuronal cell death is not an early stage of AD, so MRI or CT scan cannot be termed as an early diagnosis (Vemuri & Jack, 2010).

2.3.3.2. PET and SPECT Scan:

Nuclear medicine has brought development in AD diagnosis criteria. It measures brain amyloid build-up, tau-accumulation, neural cell inflammation, synaptic or receptor changes and metabolic abnormalities. Positron emission tomography (PET) is not only a relatively early diagnosis criterion, but it also gives information on disease progression and the effectiveness of the given treatment on relative targets.

PET used to quantify the accumulated Aβ peptides involves binding of agents to the insoluble Aβ-40 and Aβ-42. Pittsburgh compound B (PiB) was the first imaging agent and had a high affinity for the insoluble Aβ. It is US-FDA approved. To date, many other FDA approved imaging agents are in use for amyloid-PET (Marcus et al., 2014). FDG-PET is an imaging technique based on glucose metabolism in different regions of the brain. It also differentiates AD from other dementia and detects AD-related initial brain changes. Tau-PET uses agents that bind with the neurofibrillary tau tangles and get deposited in specific brain regions. However, Tau-PET is not specific to AD and can also give deposits in other tauopathies. (Schilling et al., 2016). single-photon emission computed tomography (SPECT) can also detect underlying biochemical and physiological changes. Hence, both PET and SPECT are molecular neuroimaging biomarkers for the early diagnosis and differentiation of AD (Jagust, 2004; Valotassiou et al., 2018).

2.3.4. Fluid Biomarkers:

Although neuroimaging techniques could be early diagnosis biomarkers, fluid biomarkers are both suitable and essential in the pre-symptomatic diagnosis of AD. Among fluid biomarkers, urine, blood, and CSF based biomarkers are noteworthy. Urine biomarkers are more specifically used to rule out vitamin deficiencies and to provide information on AD progression. Neural thread protein is a urine biomarker, the expression of which is proportional to disease severity. CSF is a fluid that is in immediate interaction with physiological changes of the brain and is a more specific biomarker (Maji et al., 2010). The pathophysiology of AD is the accumulation of Aβ-42, total tau and hyperphosphorylation of tau. Hence, these three are the basis of CSF-based biomarkers. The soluble form of these biomarkers is also found in the CSF of AD patients. However, because of the complex nature of AD, like other biomarkers, none of these biomarkers is enough to be used alone but concurrently with other biomarkers (Blennow & Zetterberg, 2009; Zou et al., 2020). As blood is easy to sample fluid, it is a more convenient way to diagnose AD. Many proteins have been identified as specific to AD-related changes and have shown an increased or decreased expression. For example, Complement-factor-H, and α2-macroglobulin proteins identified in senile plaques, α1 antitrypsin, and α1-antichymotrypsin show an enhanced expression in the blood sample of AD as matched to the healthy sample. Other protein biomarkers include apolipoprotein A1 and Aβ-40/42. Although, Blood-

based biomarkers are convenient to use but lack reproducibility of results, low sensitivity, and specificity (Maji et al., 2010).

2.3.4. Biosensors:

The diagnosis method using biosensors is cost-effective, environment-friendly, quick, and convenient. Biosensors convert physiological changes or reactions into signals that are easy to analyze. It involves a transduction technique to covert biological signals into detectable or readable signals. It can either be an electrochemical or optical biosensor. The technique also needs a biological element as an interface for the biological fluid. The technique aims at the utilization of easily sampled biofluids and detection of main diagnosis biomarkers as well as genetic mutations (Brazaca et al., 2020).

2.4. Development of therapeutic targets in AD:

Therapeutic targets of disease are built on the pathophysiological interactors at the molecular level. Since the discovery of the disease, extensive research conducted to understand the molecular pathology of AD. There are two accepted interacting hallmarks through post-mortem studies of AD brains. So, research is ongoing to understand the two hallmarks, i.e., Aβ plaques and tau tangles as biomarkers for diagnosis and therapeutic targets. Studies have shown that AD is a homogenous, multifactorial disease influenced by genetics, environment and metabolic pathways disruptions, and neurochemical changes among many. With the development in diagnostic markers, several novel therapeutic targets are also under consideration.

2.4.1. Amyloid β as a therapeutic target:

The amyloid cascade hypothesis makes Aβ a key factor in AD pathology. It proposes that Aβ is the core cause of AD through disrupting various pathways with subsequent neuronal degeneration (Barage & Sonawane, 2015). change in Aβ regulation due to genetic aberrations, disruption of degradation pathway or increased production leads to excess deposition of Aβ in dimers or oligomers. This disrupts neurochemical synaptic transmission. The deposition ultimately forms amyloid plaques resulting in metabolic alterations that enhance tau phosphorylation. Intracellular hyperphosphorylated tau deposit into PHF, neurofibrillary tangles and neuropil threads that surround Aβ. Hyper-phosphorylation of tau also stimulates apoptotic pathways causing neuronal cell death (Coman & Nemeş, 2017).

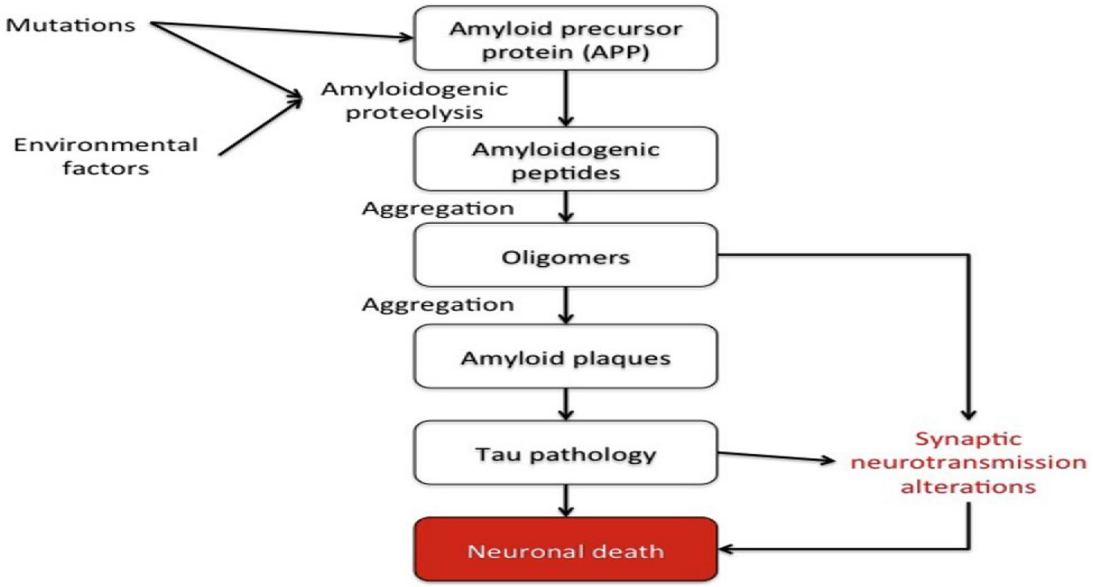

Figure 4: Amyloid cascade hypothesis is a primary cause of AD. Pathway shows how APP is involved pathaology of AD according to amyloid cascade hypothesis (Adapted from Coman & Nemeş, 2017).

So, research has been focused on Aβ as the primary cause of AD in one way or another. Genome-wide association studies also verify the role of Aβ in both familial and sporadic AD (Bateman et al., 2011; Kunkle et al., 2019). Various therapeutic agents that target pathways involved in Aβ production, stimulation or clearance have been developed.

2.4.1.1. Alteration of Secretases involved in Aβ synthesis:

Amyloid precursor peptide (APP) is processed through two pathways as mentioned in section (2.2.1). The amyloidogenic pathway involves β-secretase that cut App to form a 91 amino acid (AA) peptide. The 91 AA peptide is further cleaved through γ-secretase to produce Aβ-40 and Aβ-42. By inhibiting β-secretase Aβ production can be reduced. β-secretase inhibition either through drugs or gene silencing or deletion resulted in decreased Aβ levels in the plasma of transgenic mice. BACE (β-secretase aspartyl protease) type-1 is another target to reduce β-secretase activity (Chaudhary et al., 2018). Several β-secretase inhibiting drugs are underway in their trials (Ghosh & Tang, 2015). However, studies also report that complete inhibition of β-secretase although decreases Aβ levels but also diminishes synaptic neurotransmission having deleterious effects (Munise Satir et al., n.d.).

γ-secretase can either be inhibited or modulated to produce Aβ-38 that has a less neurotoxic effect. However, since γ-secretase is also important in non-amyloidogenic

processing of APP, a neuroprotective pathway, and has a role in other hepatic or splenic associated molecular pathways (Kukar et al., 2008; Wolfe, n.d.). α-secretase stimulation to enhance the non-amyloidogenic pathway is another way to decrease the production of Aβ. Muscarinic receptor M1, responsible for cholinergic neurotransmission, alteration of tau-hyperphosphorylation and stimulation of protein kinase C to stimulate α-secretase activity (Chaudhary et al., 2018; Francis, 2008).

2.4.1.2. Modulation of Aβ clearance pathways:

Aβ clearance can be achieved by therapeutic agents that bind with the peptide and inhibit its accumulation and promoting its clearance through various pathways. Receptor for advanced glycation end products (RAGE) expressed in membranes near the blood-brain barrier. In disease conditions, its expression is increased thus letting Aβ from the blood back into the brain. It also stimulates neuronal inflammatory processes and neuron death (Rafii & Aisen, 2009). Thus, an important therapeutic target is to reduce the accumulation of Aβ in the brain. LRP or low-density lipoprotein receptor-related protein-1 is another blood-brain barrier associated receptor involved in Aβ clearance. However, its expression decreases with ageing. Therapeutic agents specific to LRP expression could be beneficial in Aβ clearance (Zlokovic, n.d.).

2.4.2. Tau as a therapeutic target:

Though amyloid-cascade-hypothesis refer to Aβ as the main cause of AD progression, over the year therapeutic agents targeting Aβ have failed. Tau being one of the two hallmarks is another main therapeutic target. Agents targeting the accumulation of tau or inhibiting its hyperphosphorylation can be employed as therapy. NAP (AL-108) and methylene blue have been shown to decrease phosphorylation of tau and its accumulation respectively (Rafii & Aisen, 2009). Inhibition of glucogen-synthase-kinase-3 can inhibit hyper-phosphorylation through the M1 receptor which can also inhibit Aβ synthesis (Cai et al., 2012).

2.4.3. Immunotherapy:

Antibodies targeting tau hyper-phosphorylated strain and Aβ-42 could be effective therapeutic agents. AADvac1 is a vaccine that targets hyperphosphorylated-tau and is under the clinical trial phase. However, many vaccines target Aβ-42 and are named as Solanezumab, Gantenerumab, and Bapinezumab etc. (Coman & Nemeş, 2017)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents and Antibiotics of Cell Cultur:

Reagents	Catalogue and Company
DMEM, high glucose	D6429/ Sigma-Aldrich
Fetal bovine serum FBS	F9665/Sigma-Aldrich
Penicillin/ streptomycin	P06-07100/ PAN Biotech
Plasmocure	PCL- 39-01A/InvivoGen
Trypsin/EDTA 0.5%/0.2% (w/v)	L2153/Merck Biochrom
Trypan Blue solution	93595/Sigma-Aldrich
Dulbecco's Phosphate buffered saline (PBS)	D8537/ Sigma-Aldrich
GlutaMAX supplement	Gibco 35050038/Thermo Fischer Scientific
OptiMEM, reduced serum medium	11058021/Thermo Fisher Scientific
MTS Reagent	Ab197010/ Abcam

3.1.2. Immunoglobulins:

All the immunoglobulins used in experiments mentioned in this document are listed in the table-.

Table 1: List of primary antibodies used in the study

Primary Antibody	Origin	Dilution	Catalogue/Brand
Phospho-Tau (S-199)	Rb.	1:1000	ab81268/Abcam
Tau-5	Ms.	1:1000	ab80579/Abcam
Tau- E178	Rb.	1:1000	ab32057/Abcam
TIA-1	Ms.	1:200	Sc-48371/Santa-Cruz
SFPQ	Rb.	1:200	Ab38148/Abcam

SAF-70	Ms	1:1000	A03206/Cayman chem
GAPDH	Ms	1: 2000	G8795/Sigma-Aldrich

Secondary Antibody	**Origin**	**Dilution**	**Catalog/Brand**
α-Mouse-HRP (IgM)	Goat	1: 5000	AP128P/Merck Millipore
α-Mouse-HRP (IgG)	Goat	1:5000	115- 035-062/Jackson IR Lab
α-Rabbit-HRP (IgG)	Goat	1: 5000	11-035-144/Jackson IR Lab
α-Rabbit-A546 (AlexaFluor)	Goat	1: 200	A-11010/Invitrogen
α-Mouse-A488 (AlexaFluor)	Goat	1: 200	A-11001 /Invitrogen

3.1.3. Reagents and standards:

Reagent/Standard	**Catalogue/Company**
Dynabeads™ Protein G	10003D/ Invitrogen
Protein Assay Dye Reagent concentrate	5000006/Bio-Rad
Complete™ Protease inhibitor cocktail	4693116001/Roche
Phosstop EASYpack	04906837001/Roche
Precision Plus Protein Standard	161-0374/Bio-Rad
Bovine serum albumin (BSA)	P0914/Sigma-Aldrich
RedDot™2 200x in DMSO	40061-1/ Biotium
Immu-Mount™	9990402/Thermo-scientific

3.1.4. Chemical Reagents:

All the reagents used in the study were ordered from ROTH, BIO-RAD, Sigma-Aldrich, Merck, Roche and thermo-scientific as listed below:

Chemical	**Company**
CHAPS	Roche
Bio-Lyte® 3/10, 40 %	BIO-RAD
Dithiothreitol, DTT	Thermoscientific
Iodoacetamide	BIO-RAD

Milk powder	ROTH
Sodium chloride	ROTH
Sodium carbonate	ROTH
Sodium dodecyl sulphate	Sigma
Silver nitrate	ROTH
Glycine	ROTH
Ammoniumperoxidesulphate APS	ROTH
Bromophenol Blue	Sigma
TEMED	ROTH
Roti®-Load 1 4x	ROTH
TRIS	ROTH
Thiourea	Sigma-Aldrich
Tris-hyddrochloride	ROTH
TWEEN® 20	Sigma
Rotiphorese® Gel 40 (37, 5:1)	ROTH
Albumin Fraction V	ROTH
Methanol	ROTH
Ethanol	ROTH

3.1.5. Apparatus and Instruments:

The following list of instruments and apparatus were used in the study.

Apparatus	**Model/description**	**Company**
ChemiDoc XRS+ system	170-8265	BIO-RAD
Electrophoresis apparatus	Mini-ProteanSarstedt III	BIO-RAD
Filtopur V50 0.2 (Vacuum filter)	83.1823.001	SARSTEDT
Heated magnetic stirrer	iKAMAG RCT	IKA-Labortechnik
Centrifuges	5415C Optima TL 100	Eppendorf
Ice machine	-	Ziegra

Incubator	IFE 400	Memmert
Microscope	Leica TCS SPE	Leica Microsystem
Microscope	Zeiss LSM 510 Meta	Carl Zeiss
Microscope	Zeiss 667183 Axio-vert 25C	Carl Zeiss
Microplate reader	Perkin Elmer Wallac 1420 Victor	GMI
Power supply	Power Pac 200	BIO-RAD
Safe-Lock tubes	0.2, 0.5, 1.5 and 2ml	Eppendorf
Semi-Dry transfer Cell	Transblot Turbo transfer system	BIO-RAD
Serological pipettes	2, 5, 10, 25ml	Sarstedt
plastic tubes	15 and 50ml	
pH strips (6.5-10)	1.09543.0001	Merck Millipore
Shakers	KS 501 digital	IKA® WERKE
Spectrophotometers	EL808	Bioteck instruments,
TC Flask T75, vented Cap	83.3911.302	SARSTEDT
Thermomixer comfort	5436	Eppendorf
Tissue-lyser LT	85600	Qiagen
Weighing machine 0.001g-310g	TE313S-DS	Sartorius
Weighing machine 3200g	CP 3202 P	Sartorius
Protean IEF Cell	10747067	BIO-RAD
Extra thick Blot Paper	1703967	BIO-RAD
Shaker small	CERTOMAT R	Sartorius
Ready-strip IPG Strips	1632002 nonlinear pH 3-10	BIO-RAD
PVDF Transfer Membrane	0.45µm 88518	ThermoScientific

Software and Tools		
ImageLab (6.0.1)	Densitometric Analysis	BIO-RAD
Image J (1.8.0)	Immunofluorescence Analysis	Nation Institute of Health USA
Prism (5.0.1)	Statistical Analysis	GraphPad Software, Inc.

3.1.6. Buffers and Solutions:

Blocking solution for immunoblotting: 5% milk powder (fat -free)/BSA in TBS-T/PBS-T.

2X Laemmli Buffer: 10% (w/v) SDS 4ml, Glycerol 2 ml, 1 M Tris-Cl (pH 6.8) 1.2 ml, H2O 2.8 ml, Bromophenol blue (1%) 200ul, 200mM DTT added Freshly.

Tissue lysis buffer: 7M urea, 2M thiourea, and 4% CHAPS (freshly added 2% ampholytes, 1% DTT)

Transblot-buffer: 192 mM glycine, 10% methanol, 25 mM Tris-HCl, pH 8.3

Rehydration Buffer: 7M Urea (42g), 2M Thiourea (15.2g), and 4% CHAPS (4g) in 100ml ddH2O.

Equilibration Buffer: 6M Urea(36g), 2% SDS (2g), 30% glycerin (30ml), 0.375M Tris (0.05M Tris-HCl 1.4ml)

Cell-lysis buffer: 50 mM Tris-HCl, pH 8, 1% Triton X-100, 0.5% CHAPS, 1 mM DTT

Citrate buffer: 10 mM sodium citrate (pH 6.0)

Tissue lysis buffer: 7 M urea, 2 M thiourea, 4% CHAPS (freshly added 2% ampholytes, 1% DTT)

Transblot-buffer: 192 mM glycine, 10% methanol, 25 mM Tris-HCl, pH 8.3

PBS-T buffer: PBS and Tween-20 (0.05% Tween)

Resolving gel buffer: 1.5 M Tris, 0.4% SDS, pH 8.8

SDS-running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3.

Stacking gel buffer: 0.5 M Tris, 0.4% SDS, pH 6.8

TBS-T buffer: 50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.6

3.1.7. Study Subject:

3.1.7.1. Human CSF: Samples of Control, rpAD and AD patients were obtained from the Department of Neurology at the University Medical Centre, Göttingen Germany.

3.1.7.2. **Human Brain Lysate**: Samples of Control, rpAD and AD patients were obtained from the Department of Neurology at the University Medical Centre, Göttingen Germany.

3.1.7.3. Cell Culture:

1. **Hela Cells:** HeLa cells were provided by Dr. Neelam Younas, Department of Neurology, Universitätsmedizin Göttingen. The cells were propagated in DMEM, including 10% FBS and 1% P/S at 37°C with 5% CO2 and 95% humidity.
2. **SH-SY5Y cells**: SH-SY5Y cells were obtained from Dr. Aneeqa Noor, Department of Neurology, Universitätsmedizin Göttingen. The cells were maintained in a medium in DMEM with 10% FBS, 1% Glutamax supplement and 1% P/S at 37°C with 5% CO2 and 95% humidity

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3.2. Methods

3.2.1. Cell culture Optimization and passaging:

Cell culture medium (DMEM, 10% FBS, 1% PS) was pre-warmed at 37ºC, in the water bath. The cells were taken from dry ice and incubated in a 37°C water bath until nearly 80% thawed, and gently mixed with 10 ml of culture medium and centrifuged at 400xg for 5 min. After discarding the supernatant, the pellet was suspended in 14 ml of the culture medium in T75 Flask. The cells were cultured at 37°C, with 95% humidity and 5% CO2. Media was changed if required till the cells reached 80% confluency. Confluent cells were washed once with 5ml PBS and then put to trypsinization using 3 ml of 1% trypsin/EDTA solution. After 3-5 mins of trypsinization, 10ml of culture medium was added to stop the reaction. In a new flask for 1:2, concentration 7.5ml of fresh pre-warmed medium and 7.5ml of trypsinized cells were added. cells were maintained till passage 4 for further experiments. Then after trypsinization cells were centrifuged at 400 xg for 5 min and washed again with PBS. For plating, cell counting was performed using a hemocytometer (0.1 mm sample depth) with trypan blue in 1:5 concentration. The cell pellet was re-suspended in a fresh pre-warmed culture medium and the different numbers of cells were plated in a 97 well and 24 well plate. Observations were taken every day for 14 days to note the confluency period for the different numbers of cells. All cell lines were cultured between 5 and 14 passages.

3.2.2. Cell Preservation:

To be stored for longer, the cell stocks were preserved in DMSO at -80ºC. For stocks, Cells were trypsinized when 80-90% confluent and centrifuged at 400×g for 5 min. The cell pellet was resuspended in 3 ml of cryoprotective medium (10% DMSO in culture medium) and immediately transferred to 1 ml cryogenic vials on dry ice, followed by storage at -80°C.

3.2.3. Oxidative Stress Induction:

Cells were cultured in a flask labelled as control and treated. Confluent cells (Hela, SH-SY) were treated by adding a warmed culture medium containing 0.6 mM-NaAsO3s. The culture medium of the control flask was also changed with a fresh pre-

warmed medium without NaAsO3. Cells were then incubated for 1 hr. at 37°C with 95% humidity and 5% CO2. After 1-hour cells were harvested for protein extraction and immunoblotting.

3.2.4. Protein Extraction and Quantification.

After harvesting the pellet was resuspended in 0.3-0.5ml cell lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS (freshly added 2% ampholytes, 1% DTT, protease & phosphatase inhibitor 50µl/ml). after 1-hour incubation at 4ºC cells were centrifuged at 14000rpm for 30 mins at 4ºC. The supernatant was separated into new Eppendorf tubes and subject to protein quantification. The protein Dye reagent was diluted to 1x from 5x concentration to make a working solution. Standards were prepared using 1mg/ml aliquots of BSA stored at -20ºC. The standards are given in the table:

Table 2: standards volume for optimization of protein-dye reagent for Bradford assay

Concentration µg/ml	**Preparation**	**Total volume**
1000	Use the stock solution	60µl
750	45µl stock+15µl dH2O	60µl
500	50µl stock + 50µl dH2O	100µl
250	30µl of S500 + 30µl dH2O	60µl
100	20µl of S500 + 80µl dH2O	100µl
50	30µl of S100 + 30µl of dH2O	60µl

In 20µl of standards, 980µl of dye reagent working solution was added and incubated at room temperature for 10 mins in dark. Absorbance was recorded at 595nm. Protein samples were diluted to 1:20 in ddH2O. after incubation with 980µl of dye Reagent for 10 mins at RT in dark absorbance was also recorded at 595nm. Two dilutions of each sample were used and a difference less than 0.05 was considered correct. Water was used as a Blank and the value was subtracted in the spectrophotometer.

3.2.5. Immunoblotting:

Proteins were separated based on the molecular weight in 12% SDS-PAGE polyacrylamide resolving gels. Samples were prepared using 100µg protein of each using ddH2O to equalize volume. Roti®-load 4x was added to samples to make a final concentration of 1x. Samples were subjected to thermal digestion at 95ºC in

thermomixer for 5 mins at 700rpm. Prepared samples were loaded on resolving gel. 8µl of protein marker (Precision plus protein dual-colour standards from Bio-Rad) was used to visualize the precise molecular weight of the proteins and to verify the separations of bands. Proteins were resolved at 100 V in 1x running buffer till the loading dye reached the bottom of the gel. After that, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45µm) pore size using a semi-dry blot chamber (Transblot turbo BIO-RAD) for 1 hr. at 14V and 1mA. Then membranes were blocked in blocking reagent (5% milk powder/BSA in TBST/PBST) for 1 hr. at RT, followed by primary antibodies (5ml) incubation at 4°C overnight shakings. Primary antibodies were diluted in blocking buffer in dilutions given in the table. The next day, membranes were subjected to washing in PBST/TBST for 1 hr. and incubated with secondary antibodies of the same origin as primary antibody, coupled to horseradish peroxidase (HRP) for 1hr. at RT. Protein bands were visualized using the enhanced chemiluminescent (ECL) method in a ChemiDoc (Bio-Rad). The results were analyzed with Image Lab software (6.0.1) (BIO-RAD).

3.2.6. Co-Immunoprecipitation:

Before Co-IP cells were subjected to stress with NaAsO3 treatment. To harvest the cells without denaturing proteins cells were washed with ice-cold PBS before trypsinization. The pallet was also centrifuged at 4ºC and stored on ice. To extract proteins in their native conformation and minimum denaturation of antibody sites, lysis buffer (Section 3.1.6.) was used instead of urea-thiourea lysis buffer. 500ug of protein was used and volume was adjusted with PBS (pH 7.4). 30ul of Dynabeads™ protein G (10003D/ Invitrogen) were washed once with PBS (200µl) and twice with 0.3% CHAPS (200µl). Anti-PrP antibody (SAF-70) was diluted in 200µl of PBS as 1:50 and added to the Dynabeads and put on a rotator for 30 mins at 4ºC. Lysates were added to antibody/beads complex along with 50ul/ml protease and phosphatase inhibitors and the total volume was adjusted to 500ul with PBS. The tubes were again put on the rotator at 18rpm overnight at 4ºC. After the collection of supernatants, the complex was washed with 0.3% CHAPS (300µl) 4-times. All the washes were collected in tubes. To avoid background contaminants complex was then diluted in 100µl CHAPS (0.3%) and shifted in fresh tubes. After removal of supernatant 30ul of Lameli buffer (Section 3.1.6) was added and denatured at 95º C for 5 mins in a

thermomixer. The elutes were collected and the process was repeated with another 30ul of lameli buffer. The elutes were then separated on an SDS-PAGE gel.

3.2.7. Cell viability assay after treatment with CSF:

In two different experiments, 2.5x10^4 and 3125 no. of cells were plated on a 96 well plate. Treatment was given with 30% (v/v) CSF of control, rpAD and AD patients in triplicates. In the first experiment, cells were diluted in a 200µl DMEM medium which was changed with OptiMeM medium containing 30%(v/v) CSF after 24 hours. one plate was incubated for 48 hrs. and other for 72 hrs. 10ul of MTS Reagent was added as described in the kit protocol and incubated at 37ºC for 1 hour and absorbance was recorded at 490nm on a microplate reader (Perkin Elmer Wallac 1420 Victor). In the second experiment, cells were diluted in a 100µl DMEM medium containing 30µl of CSF (30% v/v). One of the plates was incubated for 24 hours and the second plate was incubated for 10 days at 37ºC with 95% humidity and 5% CO2. MTS Reagent was added 1 hour before taking the readings at 490nm.

3.2.8. Immunocytochemistry:

Confluent cells were diluted in 500ul DMEM containing 30% (v/v) CSF after trypsinization and plated in a 24-well plate on glass coverslips (13mm). Duplicates of control, rpAD and AD samples were used. Incubation was done for 10 days at 37ºC with 95% humidity and 5% CO2. The cells were grown in T75 flasks with a culture medium (DMEM, 10% FBS, 1% PS). At confluency (70–90%), cells (5 x 10^4) were trypsinized and transferred on glass coverslips (13 mm) in 24-well plates. Following the stress treatment as described above, the cells were fixed with 4% PFA for 20 min at room temperature, followed by 3x-washes with ice-cold PBS for 5 min each. Permeabilization was achieved by adding 0.2% Triton X-100 in PBS for 10 min, followed by 3x-washes with PBS for 5 min each. To avoid non-specific binding, cells were incubated with blocking buffer 1% BSA, 10% FBS in PBS for 30 min at room temperature. The primary antibody antibodies i.e ptau and TIA-1 and SFPQ and SAF-70 as a double-label were diluted in 1% BSA in PBS, followed by overnight incubation at 4°C. The cells were washed three times with PBS 5 mins each, preceding incubation with secondary antibodies diluted in 1% BSA in PBS for 2 hr at room temperature in the dark. Three washed of 5 mins each were given with PBS in the dark to remove non-specific immunoreactivity. The cells were then counterstained

with a RedDot 2 Far-red nuclear stain for 20 min. After nuclear staining, the cells were washed three times with PBS, followed by mounting with one drop of mounting medium (immuo-mount, Thermo Fisher Scientific). The slides were stored in the dark at +4°C and imaged upon drying.

3.2.9. Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE):

To understand the proteome alterations in AD, rpAD and control tissue lysates 2D-PAGE was employed and a non-linear IPG strip (7cm) of pH range 3-10 was used. 1-D PAGE was run on a BIORAD protean IEF cell after rehydration in rehydration buffer (section 3.1.6). For a 7cm strip, 60ug of protein is added to the rehydration buffer to raise the volume to 150ul. After thorough mixing, the sample was loaded in a reswelling cassette track carefully to avoid any bubble formation. The lamination from IPG-strip was removed and the strip was loaded in the well such that the gel part was facing the sample and the positive end of the strip facing the positive terminal of the cassette. 1ml or mineral oil was loaded above to protect the strip from burning. For active rehydration protean IEF cell was set at room temperature for 12 hours on steady 50μV. The system was programmed as 500V for 1-hr, 1000V for 1-hr, 8000V for 1-hr and 10000V till 35000Vh for brain lysate. For cell lysate, the programming was as 500V rapid for 1-hr, 1000V rapid for 1-hr, 5000V slow for -2hr and 8000V till 50000Vh and 100μg of protein were used. The process took almost 24-hr to complete. To further degrade and stabilize the protein isoforms before 2^{nd} dimension PAGE, strips were put to equilibration with equilibration buffer-1 and equilibration buffer-2 for 25 mins each on shaking. Equilibration buffer-1 was replenished with 2% DTT and equilibration buffer-2 was replenished with2.5% iodoacetamide (IAA) in stock (section 3.1.6). The strips were then Loaded on 12% polyacrylamide gel and western blotting was perform

CHAPTER 4

RESULTS

It is evident from research that Aβ and tau are two hallmarks of AD and multiple factors are influencing the disease. Genetics is one factor and environmental stress or OS being the other. AD puts a huge economic and social burden not only on developing countries but also in developed countries. But in developing countries, different environmental factors can induce stress responses in individuals including air and water pollution, food shortage and unhealthy or unhygienic diet plans (Escher et al., 2019). OS is linked with both the hallmarks of AD. It is reported that aggregation of tau and Aβ stimulates OS-producing pathways. The brain has many lipids that are vulnerable to reactive oxidative species (ROS), thus rendering the brain vulnerable and assisting in disease progression (Chen & Zhong, 2014; Mravec et al., 2018; Smith et al., 2000).

In this study, we have identified AD-associated markers that were being modulated by OS induced by NaAsO3 in cell culture. Literature shows that NaAsO3 can successfully induce OS in cell culture as well as animal models (Lii et al., 2011; Nassireslami et al., 2016). Post Translational modifications were identified through immunoblotting of the cell lysate. In second phase characterization was performed with Co-immunoprecipitation and Co-localization of proteins under stress conditions. In third phase further modification of those markers was related to the stress induced by CSF of sporadic AD (sAD) and rapidly progressive AD (rpAD) patients. To characterize the results further proteome analysis is done by 2D-PAGE.

4.1. *In-Vitro* Analysis of Protein Modification under NaAsO3 induced Oxidative Stress

4.1.1. Cell Culture Optimization

Through cell plating of the different number of cells in 94-well and 24-well plates, the number of cells to be plated to achieve full confluency in 14 days were optimized. Cells were cultured as 5x10^4, 2.5x10^4, 12500, 6250, 3125 in a 94-well plate. Where 2.5x10^4 cells reached their 80% confluency in 3 days, and 3125 reached their 80% confluency within 10 days of incubation in a 95-well plate.

4.1.2. Protein quantification:

After stress induction with 0.6mM NaAsO3 as mentioned in section (3.2.3) cells were harvested and subject to protein extraction and quantification protocol (section 3.2.4). The Bradford Assay graph with the absorbance of the unknown sample at y and concentration of the unknown sample at x was plotted and R-value was 0.9957. So, the given dye reagent was further used in the quantification of samples and values are mentioned in the table.

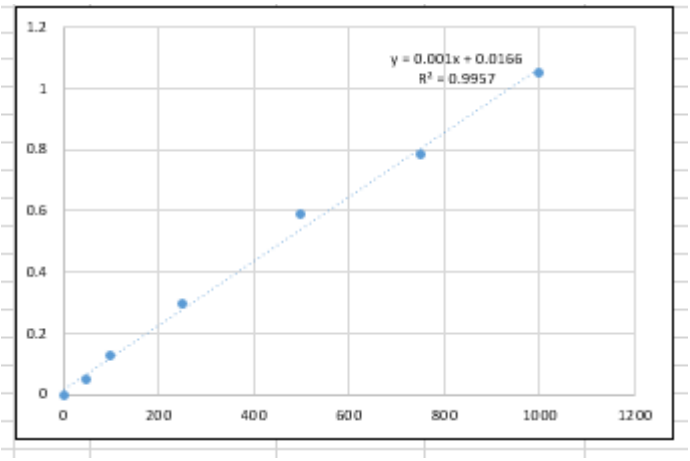


Figure 5: Bradford Assay standards graph with Y= absorbance and X= concentration. Quantification of protein was performed using these standards.

Table 3: Quantity of protein in the given volume of the sample

Sr.NO.	**Samples**	**The volume required for 50µg**	**The volume required for 75µg**
1	healthy 1	14.25313569	21.37970353
2	healthy 2	34.29355281	51.44032922
3	healthy 3	13.74381528	20.61572292
4	treated 1	12.66464032	18.99696049
5	treated 2	7.144898542	10.71734781
6	treated 3	12.08313195	18.12469792

4.1.3. Identification of Post Translational Modification under Oxidative Stress Induced by NaAsO3:

After quantification of the protein in cell lysates harvested from control and NaAsO3 treated Hela cells, samples were subject to immunoblotting. 100µg of protein was

loaded for each sample. Volume was adjusted with ddH2O after adding rotiload followed by thermal digestion. OS results in hyperphosphorylation of tau (Alavi Naini & Soussi-Yanicostas, 2015; Kang et al., 2017; Z. Liu et al., 2015; van der Harg et al., 2014). To identify the proteins that were specifically upregulated or downregulated under stress conditions antibodies for total tau (TAU-5), phosphorylated tau (PTAU-S199), SAF-70 and SAF-32 were incubated with the immunoblot of the samples (Table 3). The results showed an upregulation or increased expression of phosphorylated tau in stress-induced cells as compared to healthy or control cells. No change was observed in Tau expression, and PrP expression was downregulated under OS. Change in expression of phosphorylated tau was reproducible as it is also evident from the literature that stress influences hyper-phosphorylation of tau (Melov et al., 2007b; Mondragón-Rodríguez et al., 2013; Su et al., 2010).

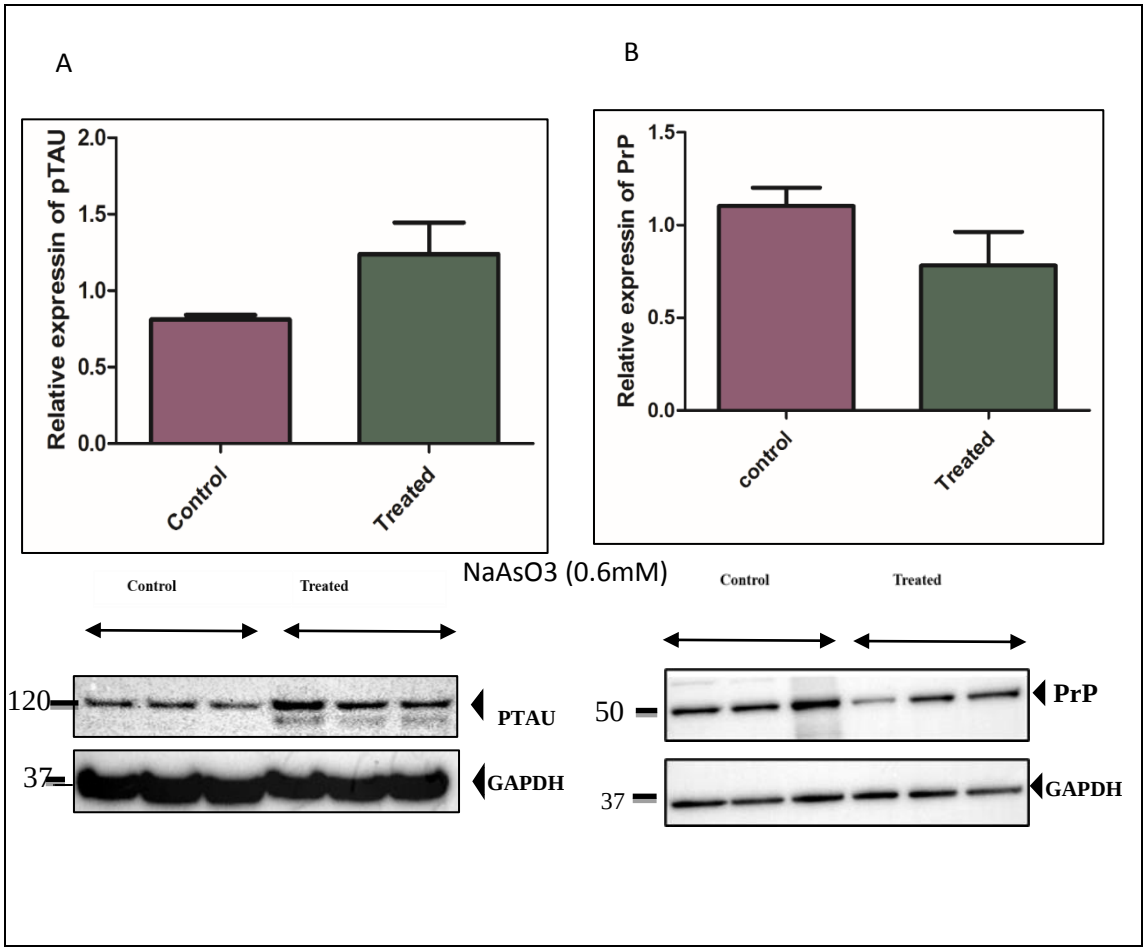


Figure 6: Relative Expression of phosphorylated Tau and Prion Protein under OS Induced by NaAsO3 in Hela cells. (A) Relative expression of Ptau is increased in cells that were treated with NaAsO3 in comparison to control cells (B) Relative expression of prion protein was decreased in hela cells treated with NaAsO3. The

Quantification analysis was performed using Imagelab and statistical analysis was done using student t test.

4.1.4. Cell Viability Assay shows Human CSF Induce Apoptosis in Neuroblastoma Cells via Oxidative Stress

As in the current study, to understand the role of OS in AD and rpAD cell viability assay was performed after exposure with CSF. The experiment was performed in two different methods with CSF exposure was 30% v/v for different days. The results showed a significant decrease in cells exposed with rpAD CSF for 48 hours as compared to control CSF. The results however were not reproducible and did not show a significant decrease in the number of viable cells after exposure for 24, 72 hours and 10 days. The reason could be the age-matched CSF control and the presence of ROS and other neurodegenerative factors in old age. Also, the trend was reproducible and remained same over the time. A further detailed study to understand the propagation of neurodegeneration through CSF exposure would also help in the identification of biomarkers and possible therapeutic targets. One-way ANOVA was performed followed by Tukey's post-hoc analysis to compare all the groups with each other.

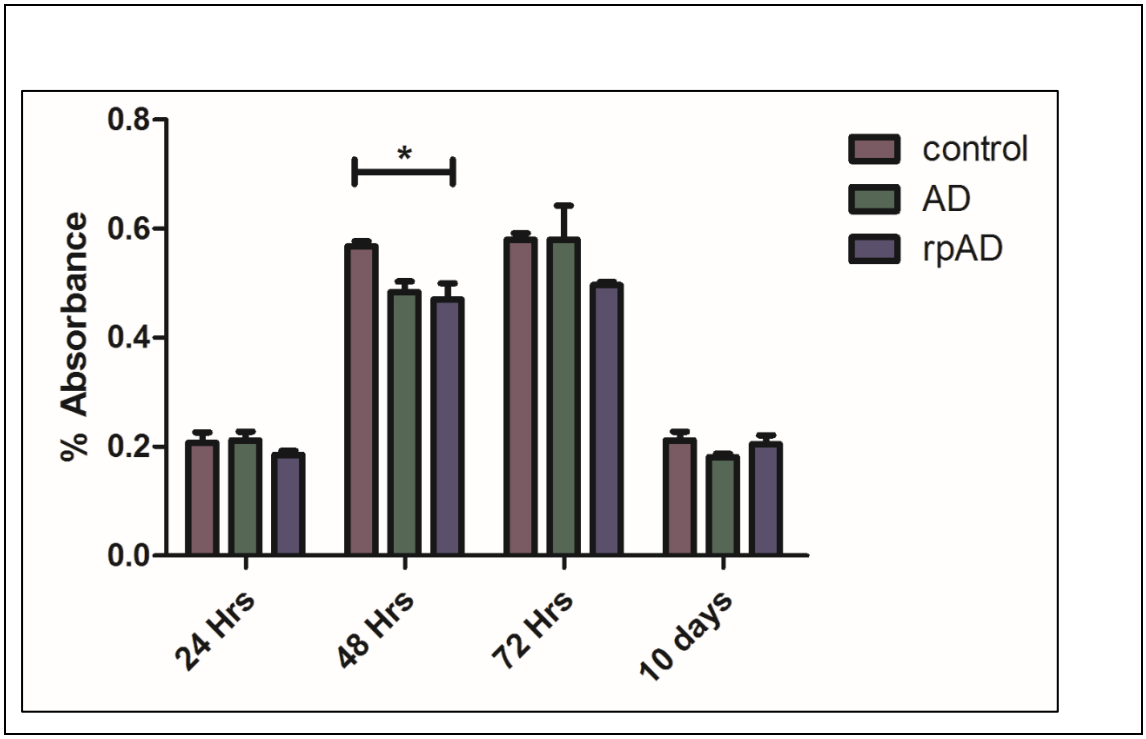


Figure 7: Cell viability assay as performed after exposure of cells with 30% v/v Human CSF of Control, AD, and rpAD for 24 hours, 48 hours, 72 hours, and 10 days.

Absorbance recoded at 490nm on plate reader. Two-way Anova was performed for multiple comparisons over time. *$p < 0.05$.

4.2. Role of Oxidative Stress-Induced by Exposure with 30% v/v Human CSF (cont, rpAD and AD)

Studies suggest a link between exposure of neuroblastoma cells with cerebrospinal fluid (CSF) of a patient with a neurodegenerative disorder. Exposure of neuroblastoma cells (SHSY) for 14 days to CSF of multiple system atrophy induced stress-related pathways and apoptosis in the cells (X. Wang et al., 2015)(Ratan et al., 1994). A similar study on rat model exposure with CSF of amyotrophic lateral sclerosis patients gave neurodegenerative results (Shanmukha et al., 2018).

4.2.1. Identification of Prion Protein Interactome in Stress-Induced Cells

Evolving studies on sAD and rpAD suggest a link between prion protein and cytoskeletal associated proteins. It is reported that prion protein has a damaging effect on cytoskeletal in early-onset AD. Interactome and co-localization studies also report the presence of prion protein with hyperphosphorylated tau in rpAD (Schmitz et al., 2014; Shafiq et al., 2021; Zafar et al., 2017). However, studies also suggest a protective role of cellular prion protein (PrPc) and upregulation

of it under OS conditions and interacting with both the hallmarks of AD (Brown, 2001; Voigtländer et al., 2001). To understand that interaction of prion protein with phosphorylated tau under OS, Co-immunoprecipitation using Saf-70 specific antibody with subsequent elution of the prion protein and its interactome was performed. Samples used were NaAsO3 treated Hela cells and non-treated controls. There was a significant decrease in a mono-glycosylated isoform of prion protein in NaAsO3 treated samples. A student t-test was performed to analyze the difference. According to studies, a 1.2 fold decrease has been observed in the di-glycosylated isoform of prion protein in rpAD as compared to healthy controls (Zafar et al., 2017).

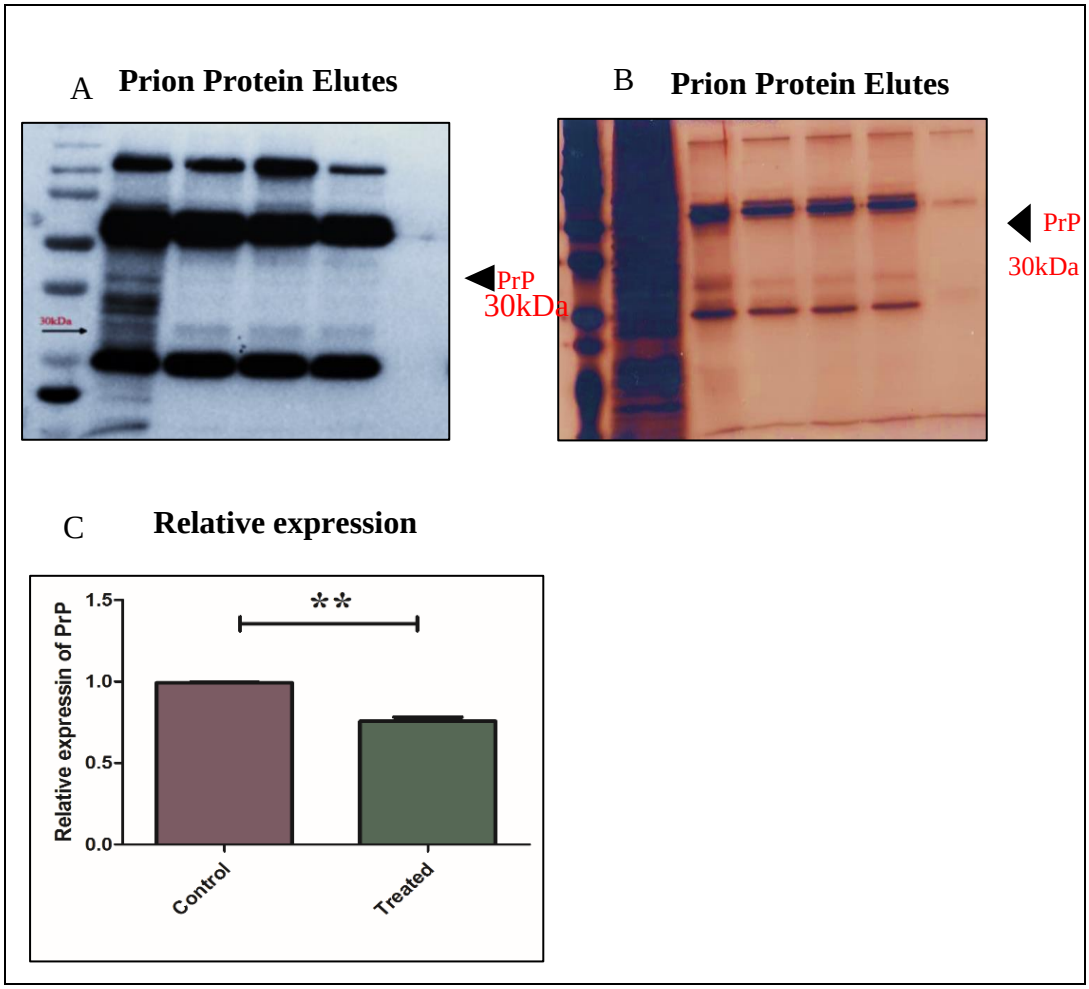
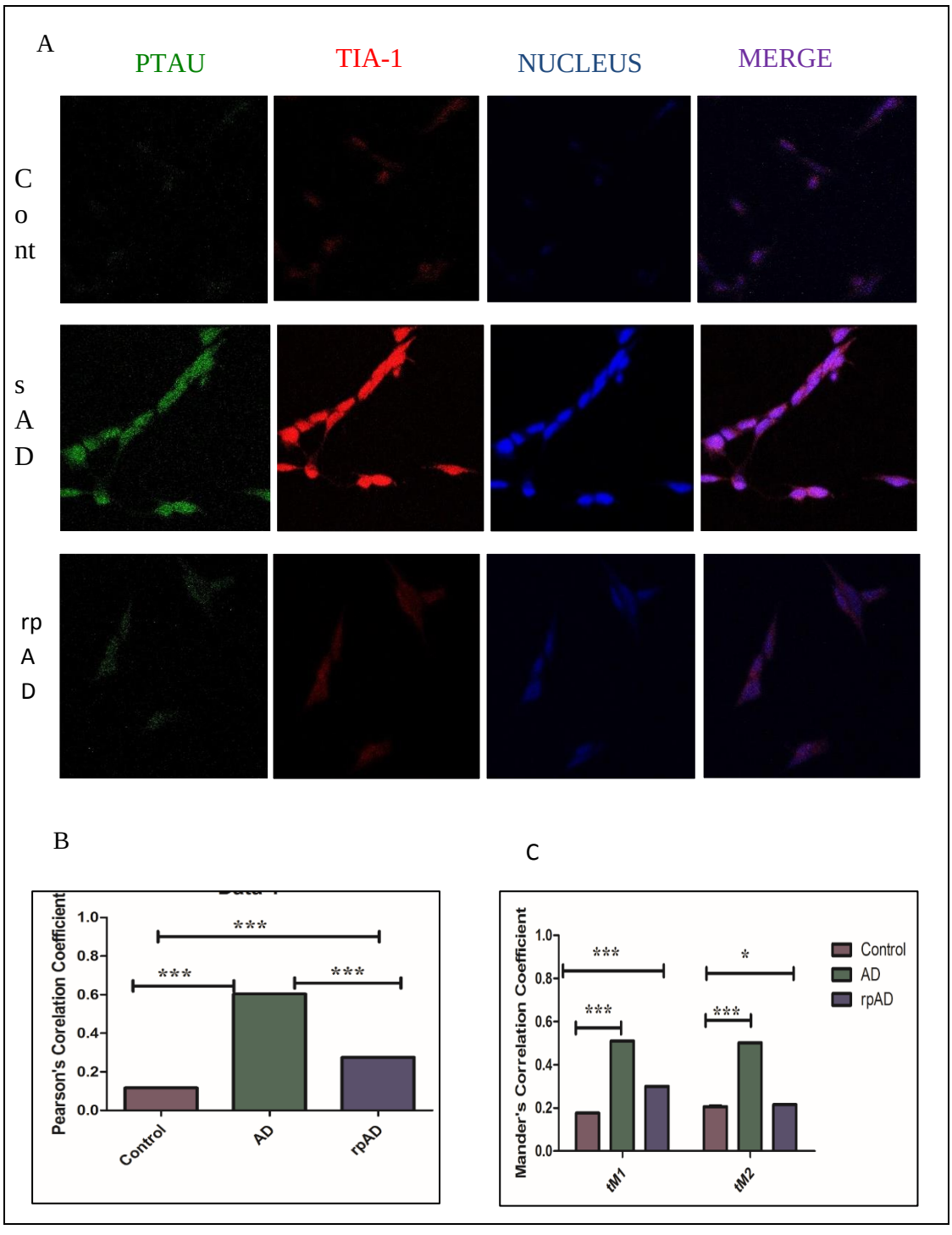


Figure 8: Expression of prion protein isoforms in Co-immunoprecipitation elutes and decrease in expression of monoglycosylated form. (A)Western blot image (B) silver stain image (C) Quantitaive Analysis was performed using student t test. **p <0.01.

4.2.2. Role of Oxidative Stress-Induced by Human CSF on Localization of Stress-Modulated Proteins by Immunofluorescence of Cells:

Cell viability assay performed after stress induction with human CSF showed that CSF induces OS leading to induction of apoptotic pathways and cell death. The stress response is the confiscation of superfluous RNA-binding proteins such as TIA-1 into stress granules that exhibit reversible aggregation. Studies show that TIA-1 mediated the aggregation of tau through hyperphosphorylation under OS. it also helps in the misfolded protein aggregate stabilization. Although stress granule accumulation is a reversible process, aggregation of misfolded proteins is not and further leading to apoptosis of the cell (Piatnitskaia et al., 2019; Vanderweyde, 2015; Wolozin & Ivanov, 2019). In the present study, a human neuroblastoma cell line (SHSY-5Y) was

cultured with 30% V/V of CSF for 10 days. Co-immunofluorescence was performed using anti-ptau (s199) and anti-TIA-1 antibodies. The results were analyzed with ImageJ. Pearson's correlation coefficient and threshold Mander's coefficient were used to measuring the quantitative colocalization of ptau and TIA-1. In sAD, there was high colocalization of ptau and TIA-1 as compared to control and rpAD. A significant colocalization was also visible in rpAD, however, less than that found in sAD.



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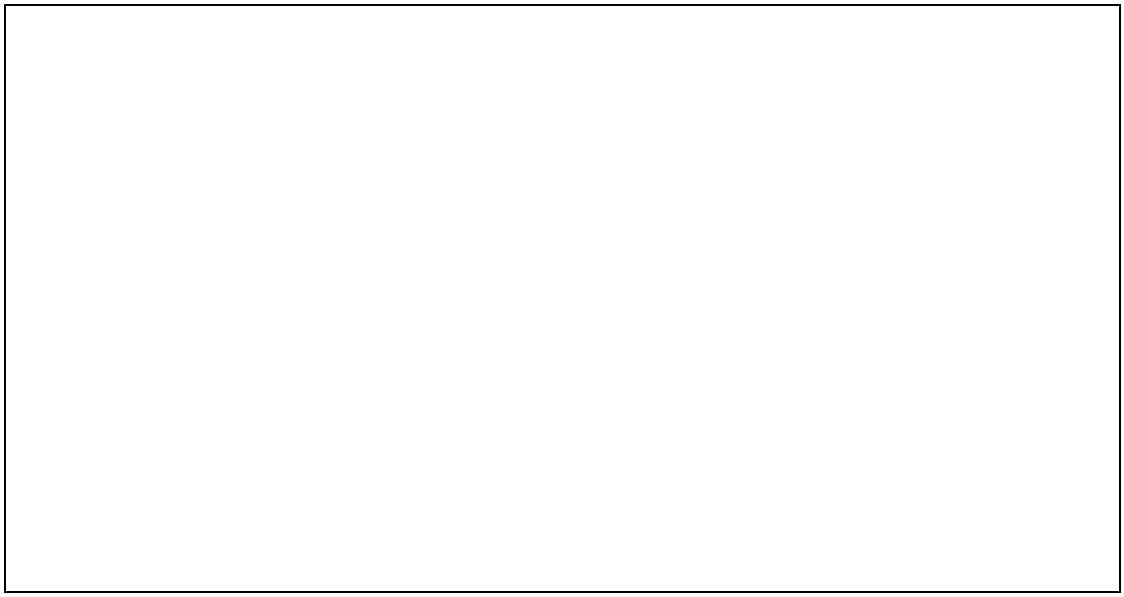
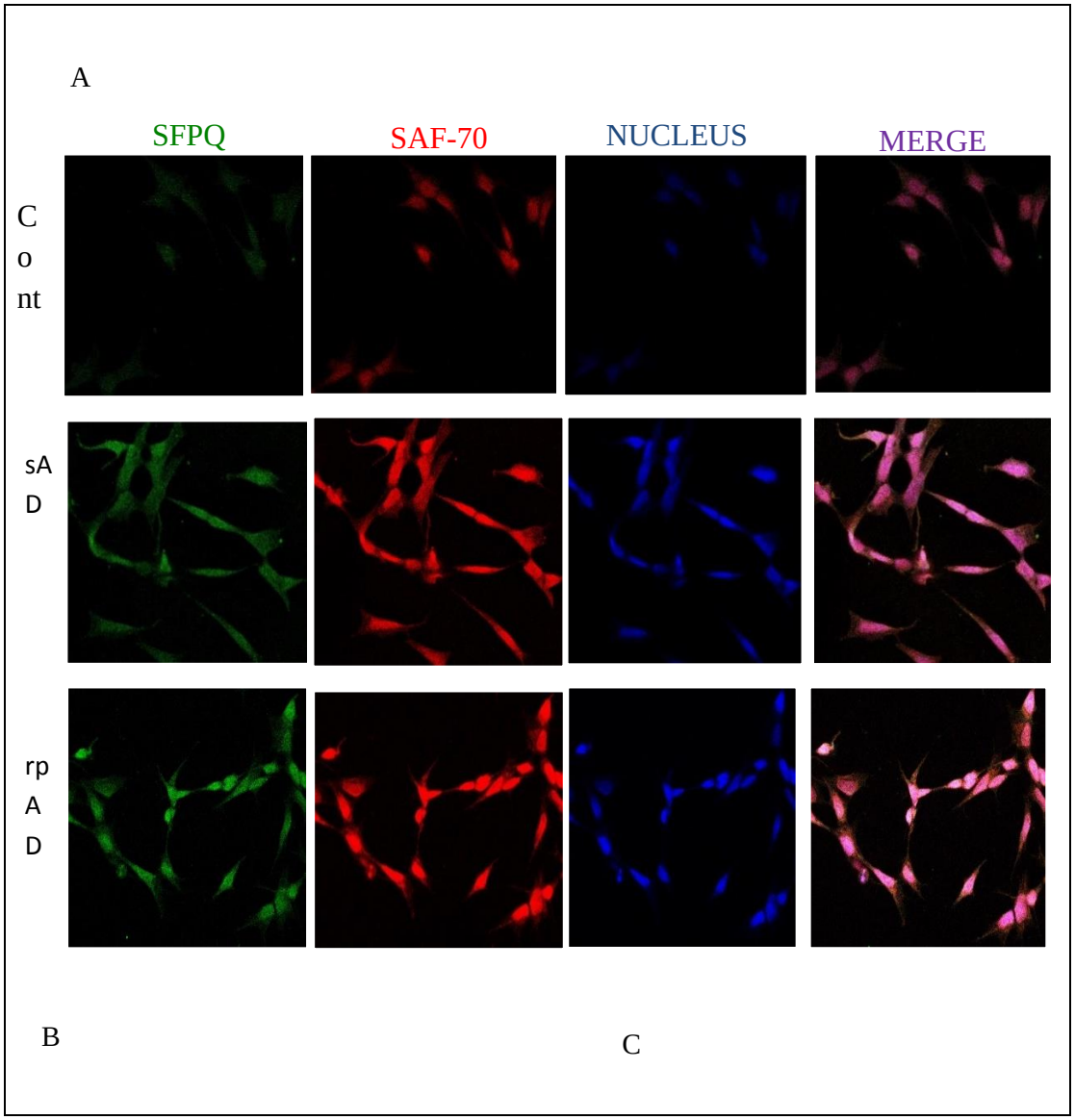


Figure 9: Colocalization of phosphorylated tau (s199) and Stress granule marker/ RNA-binding protein TIA-1 after Co-immunofluorescence. (A) Immunofluorescence images of SHSY cells treated with the 30% V/V CSF of control, rpAD and AD images (B) pearson's correlation coefficient (C) Mander's correlation Coefficient. The analysis is performed on ImageJ and Graphpadprism. ***P<0.0001

Splicing-factor-proline and-glutamine-rich (SFPQ) is another RNA-binding protein like TIA-1 and studies show colocalization of TIA-1 and SFPQ in rpAD and sAD (Younas et al., 2020). In this study, colocalization of SFPQ with Prion protein is observed after co-immunofluorescence of human CSF induced stress cells. Thus, predicting the role of prion protein interaction between tau, SFPQ and TIA-1 under OS. the study shows more colocalization of SFPQ and Prion protein (SAF-70) in rpAD as compared to sAD and control. The analysis is performed on ImageJ. Pearson's correlation coefficient and Threshold Mander's coefficient were utilized to quantify the correlation of the two proteins.


A
SFPQ
SAF-70
NUCLEUS
MERGE
Cont
sAD
rpAD
B
C

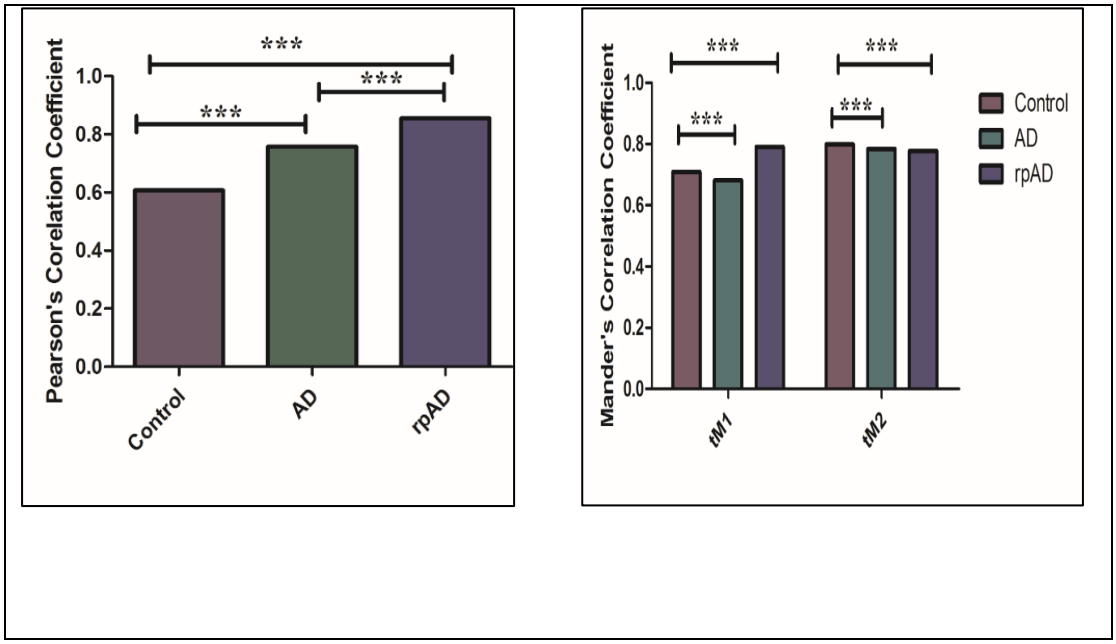


Figure 10: Colocalization of RNA-binding protein SFPQ with prion protein, after Co-immunofluorescence. (A) Immunofluorescence images of SHSY cells after 10 days incubation with 30% V/V CSF of Control, rpAD and AD (B) pearson's correlation coefficient (C) Mander's correlation Coefficient. Analysis was performed in ImageJ and Graphpad Prism softwares. With ***P<0.0001

4.3. Characterization of Ptau and Total Tau in Oxidative-Stress Induced Cells and Brain Lysates of Control, AD and rpAD.

To characterize the altered expression of phosphorylated tau upon OS induction and to relate it to that of human samples of the control and disease, proteomic analysis using 2D-PAGE was performed. As mentioned earlier tau has 6-isoforms and undergoes various PTM (Y. Wang & Mandelkow, 2016). 2D-PAGE can separate the proteins based on their isoelectric points and molecular weights. In healthy conditions, tau has a mixture of neutral, acidic, and basic amino acids. To understand how post-translational changes that include phosphorylation alter the pH of the tau protein. 2D-PAGE of control and stress-induced neuroblastoma cells was also performed to relate the stress as one of the factors influencing PTM. The total tau expression analysis of control and diseased brain lysate shows a clear difference in altered expression of certain isoforms of tau and expression replicated in neuroblastoma cells resembles that in rpAD brain lysate.

4.3.1. Expression analysis of pTau:

The expression of phosphorylated tau in the brain lysate of control, rpAD and AD show a decrease in expression of certain isoforms in disease as compared to the

control sample. Also, in stress-induced neuroblastoma cells expression of few new isoforms can be observed which is not present in control samples.

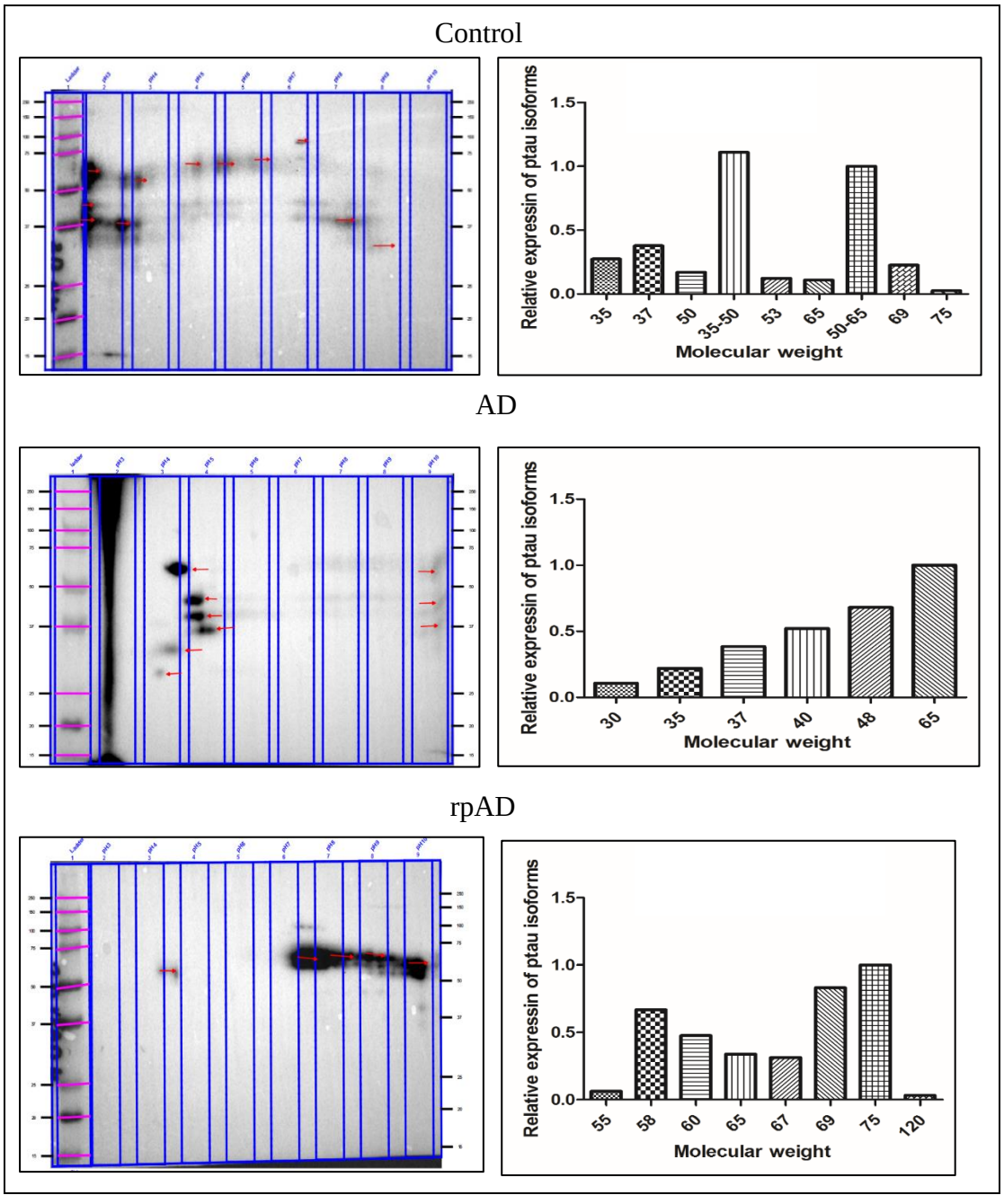


Figure 11: Two-Dimensional proteomic analysis of human brain lysate of control, AD and rpAD as incubated with anti-ptau (s199) antibody. The Statistical analysis was performed with Graphpad Prism.

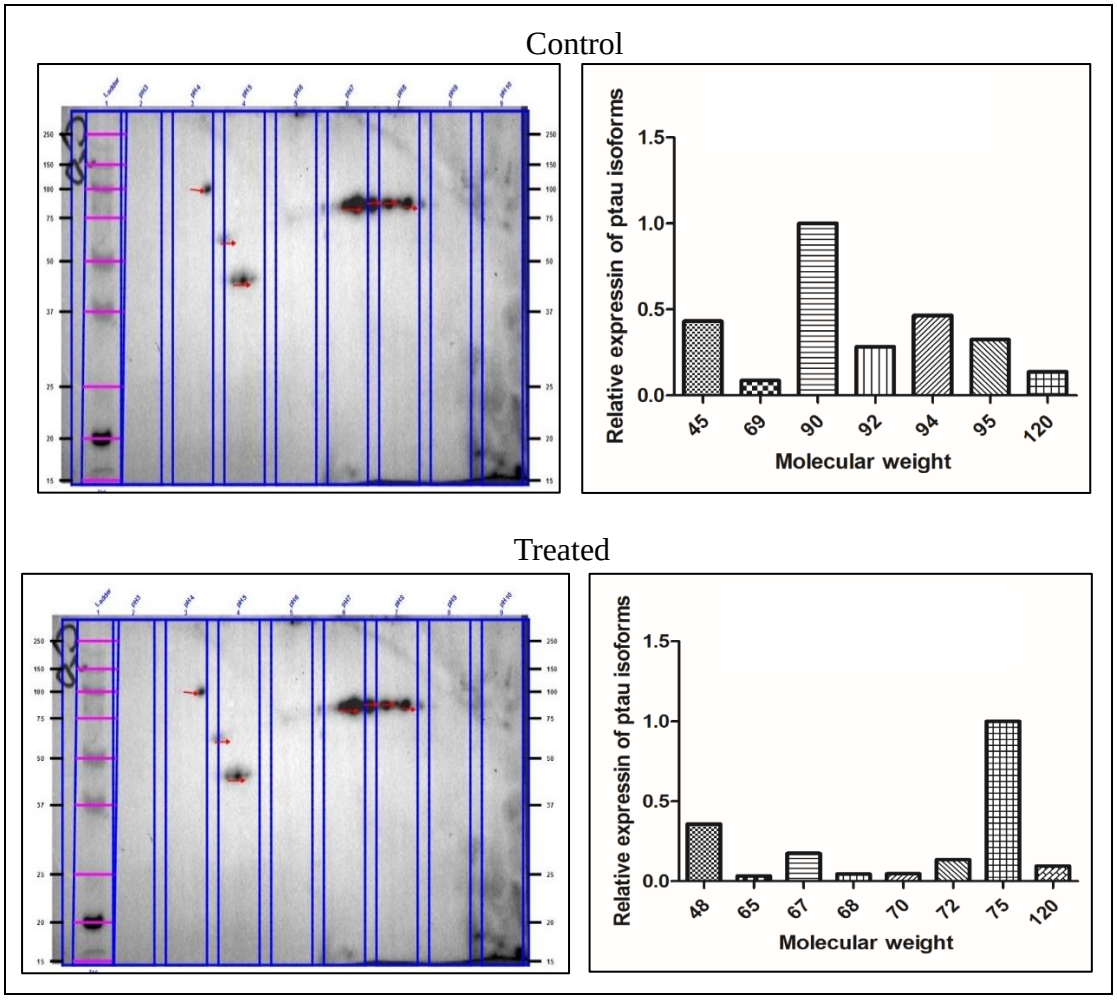


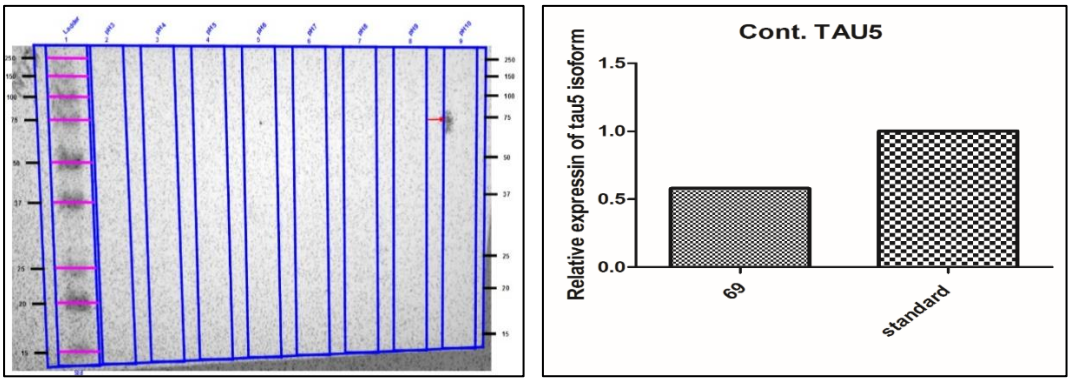
Figure 12: Two-Dimensional proteomic analysis of control SHSHY cells as compared to sodium-arsenite treated stressed neuroblastoma cells after incubation with anti-ptau

(s199) antibody. The densitometric analysis was performed in ImagLab and statistical analysis was performed in Graphpad prism software respectively.

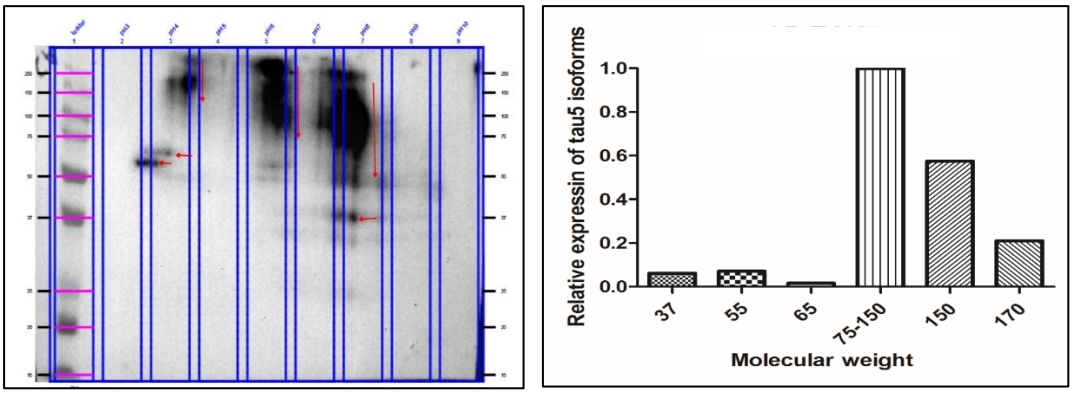
4.3.2. Expression Analysis of Tau-5:

Two-dimensional proteomic analysis of human brain lysate (cont., sAD, rpAD) with tau-5 antibody as compared to NaAsO3 treated and normal neuroblastoma cells show some similar isoforms in cells and brain lysate. The pH of tau-5 isoforms present in controls is basic in both human brain lysate and SHSY-5Y cells. However, there is a difference in molecular weight (MW) of both. The tau-5 present in human brain lysate has a high MW as compared to that present in cells. Similarly, there is an abundance observed in low MW isoforms in NaAsO3-treated cells, while high MW isoforms are more abundant in sAD and rpAD. However, a few low MW isoforms are also present in sAD and rpAD human brain lysates.

Control



AD



rpAD

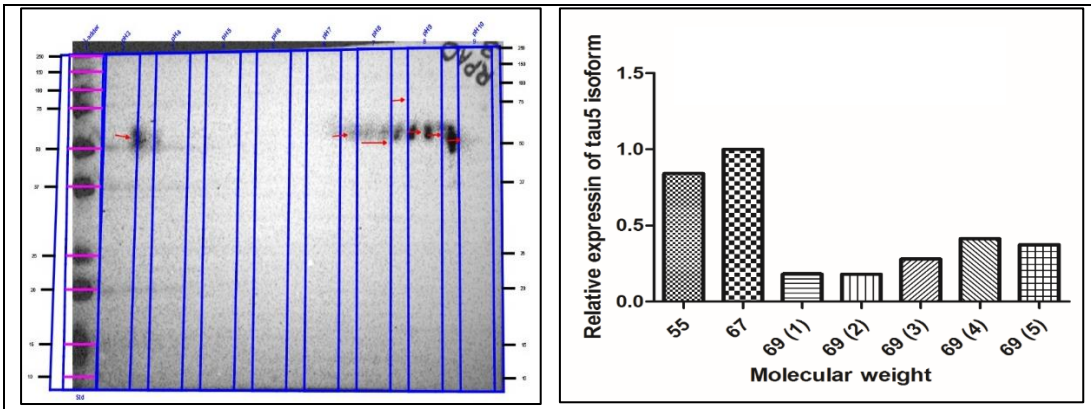
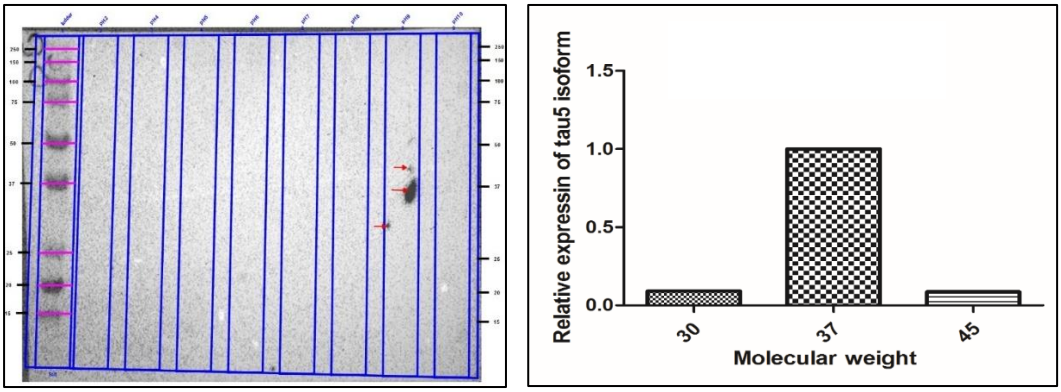


Figure 13: Two-Dimensional proteomic analysis of human brain lysate of control, AD and rpAD as incubated with the tau-5 antibody. The densitometric analysis was performed in ImagLab and statistical analysis was performed in Graphpad prism software respectively

Control



Treated

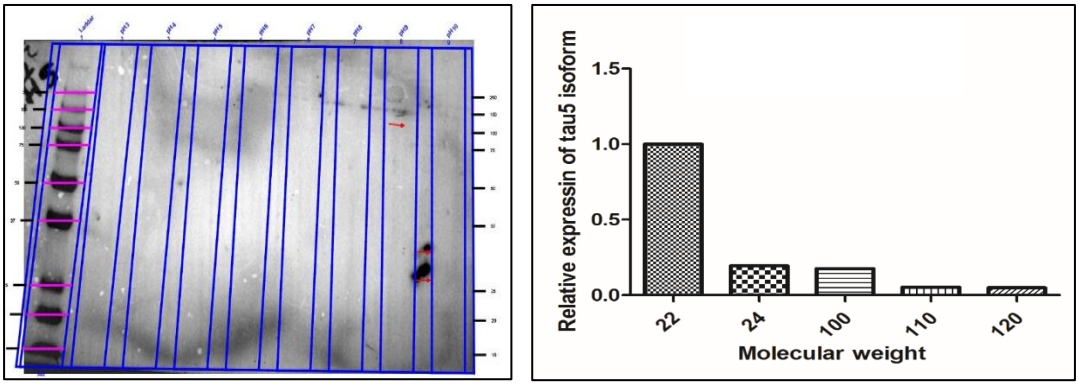


Figure 14: Two-Dimensional proteomic analysis of control SHSY cells as compared to sodium-arsenite treated stressed neuroblastoma cells after incubation with anti-tau-5 antibody. The densitometric analysis was performed in ImagLab and statistical analysis was performed in Graphpad prism software respectively

CHAPTER 5

DISCUSSION

The initiation of AD and its progression in the human brain is elusive because of the multifactorial risk associated with it. The typical classification of AD somehow, categorize it based on risk factors that play the most part. The fAD involves genetic risk factors and sAD is more associated with environmental risk factors and some polymorphisms. However, the atypical class of AD that is rpAD which belongs to a class of rapidly progressive dementias, although involve genetic mutation as a risk factor but, shares OS pathways with sAD. The present study verifies that NaAsO3 induces OS that enhances the expression of phosphorylated tau in the cell lines. The recent failure of Aβ targeting therapies after years of extensive research has raised the question about other major risk factors involved in disease progression and initiation (F et al., 2019). The co-immunoprecipitation (CO-IP) analysis of stress treated, and non-stress treated control Hela cells show a decrease in the mono-glycosylated isoform of the prion protein, a phenomenon linked with prion related neurodegenerative disease. Studies show, that glycosylated isoforms of the prion protein are localized at the cell membrane and have a lesser tendency to aggregation, as compared to un-glycosylated isoforms (Velayos et al., 2009; Yi et al., 2018). Thus, a decrease in mono-glycosylated prion protein upon artificial OS induction shows the role of prion protein and OS in the progression and initiation of AD. Further mass spectrometric analysis of the CO-IP elutes of prion protein will show the interacting partners of prion protein under OS conditions. Further, the present study shows how human CSF from control, sAD and rpAD propagate OS cell death in the SHSY-5Y cell lines. The cell viability assay conducted on cell lines incubated for a time duration of 24-hr, 48-hr, 72-hr and 10 days show a reproducible pattern of a decrease in cell viability in rpAD cases. This reflects the prion-like nature of pathophysiological changes involved in rpAD. The co-immunofluorescence analysis of the cell lines from human CSF incubation of 10 days with TIA-1 as an OS marker shows a significant correlation or colocalization with ptau. Tau is a hallmark of AD and its strong correlation with TIA-1 under OS shows and essential role of OS in the initiation and propagation of the disease. Prion protein co-localization with another RNA-binding protein SFPQ was observed. As mentioned earlier, prion protein that

localize in cytoplasm has an increased expression in AD cases (Yi et al., 2018). Previous studies also show a significant correlation and co-localization of SFPQ with TIA-1 and an increased co-localization with phosphorylated tau in the cytoplasm of the cell (Younas et al., 2020). To further elucidate the role of prion protein and its interacting partners colocalization analysis of prion protein with SFPQ after human CSF induced OS was performed. The results showed a significant colocalization of prion protein with SFPQ in both sAD and rpAD as compared to control. However, there was more colocalization in cells incubated with rpAD human CSF as compared to those with sAD human CSF. Thus, providing another link of prion protein involvement in rapid progression and death in rpAD cases. To further characterize the in vitro analysis model, 2-dimensional proteomic analysis was performed on cell lines treated with NaAsO3 to induce OS and compared with 2-dimensional proteomic analysis of human brain lysates. Antibodies against phosphorylated tau and total tau showed many similar isoforms being expressed in NaAsO3 treated cell lines and sAD and rpAD human brain lysates. However, more isoforms were observed in human brain lysates, which can be explained by the complex nature of the disease and the time duration of stress induction as compared to the brain tissue. The objectives of the study were fulfilled by the identification of OS as a strong candidate of AD initiation and progression both in sAD and rpAD. Pathways related to eukaryotic stress response incorporating RNA-binding proteins and prion proteins are also essential as a further diagnostic and therapeutic target.

5.1. Conclusion:

The cellular response to stress by sequestering RNA-binding proteins into stress granules and their aggregation and colocalization of stress granule marker TIA-1 with that of phosphorylated tau in the cytoplasm provides a strong link of OS as a risk factor of AD. The hyperphosphorylation of tau under OS, elucidate the role of OS in the aggregation of tau through incorporation in stress granules. The evident role of SFPQ and prion protein aggregation in neurodegenerative diseases from previous studies and their colocalization in cytoplasmic stress granules of OS models further confirms the role of RNA-binding protein and OS. Further study of OS in animal models and therapies targeting proteins involved in OS pathways and RNA-binding proteins is needed. The presence of OS markers in the CSF can also lead to new diagnostic markers. Further study of these markers into other fluids such as blood

plasma, urine and saliva could lead to the identification of cost-effective and easy to approach diagnostic markers.

Chapter 6

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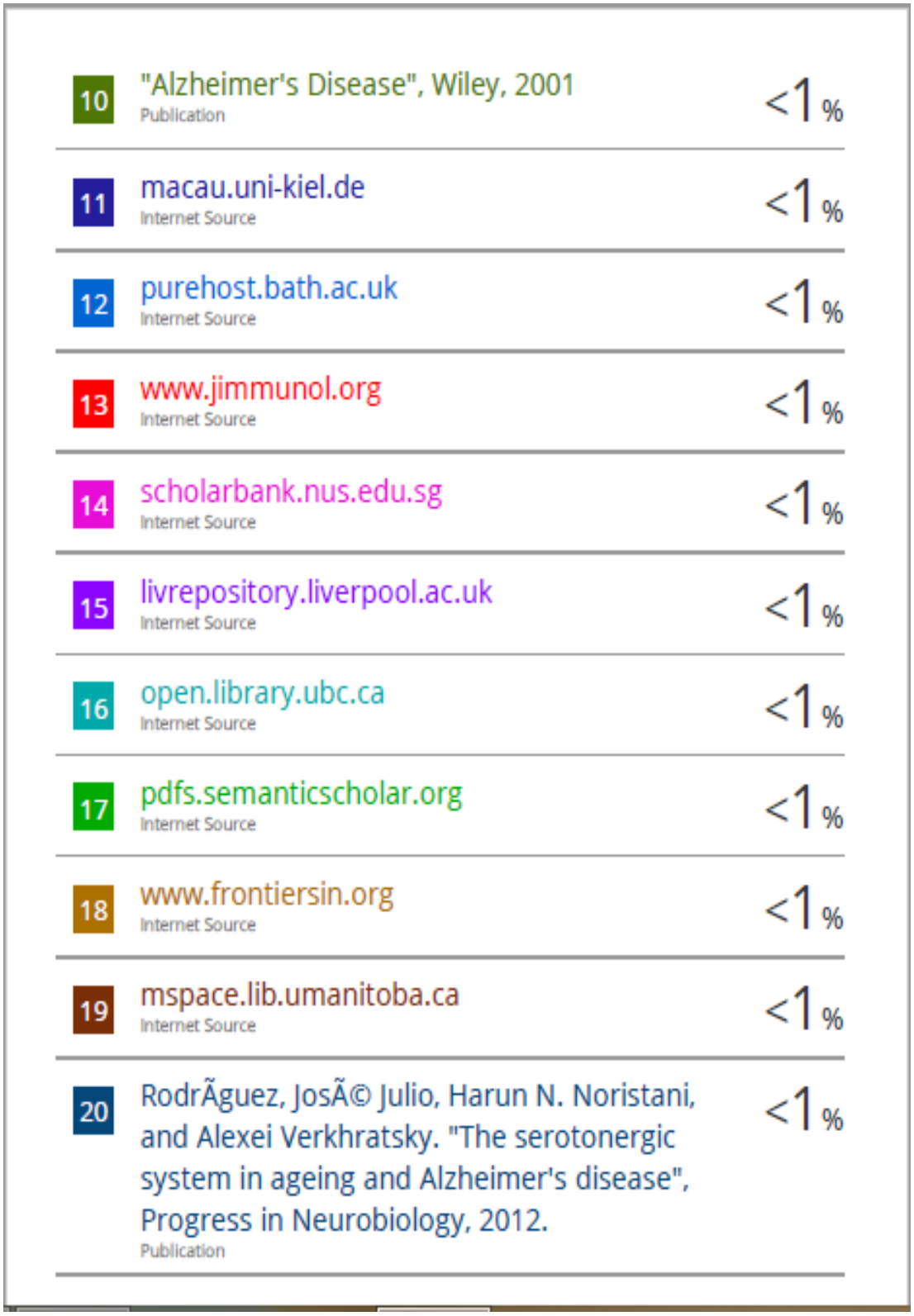

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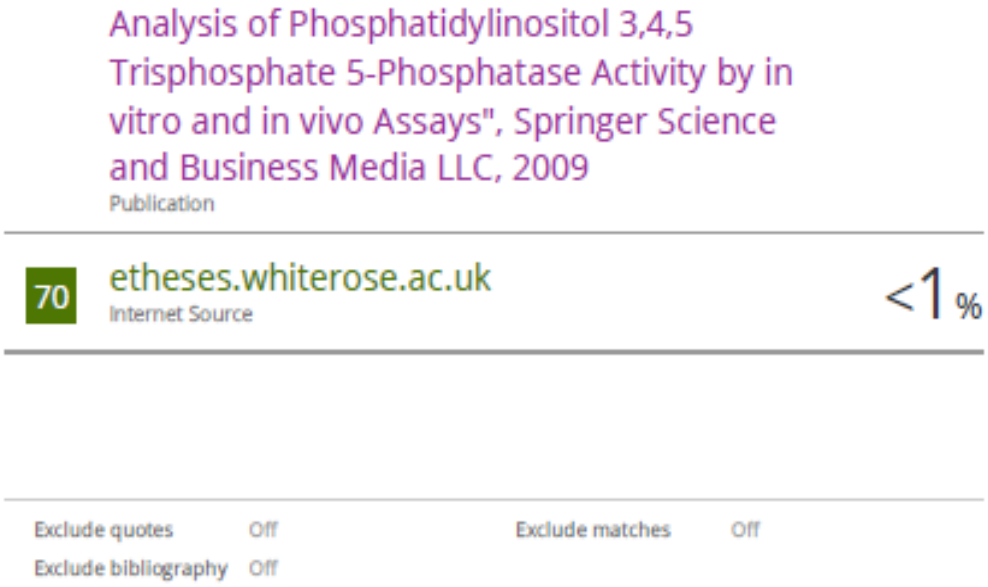
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