Proteome Wide Alterations and Impact of Identified Risk Factors on the Progression of Parkinson´s Disease.

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MASTER THESIS WORK

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I, Rizwana Fatima, certify that this research work titled **"*Proteome Wide Alterations and Impact of Identified Risk Factors on the Progression of Parkinson´s Disease*"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged / referred.

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Iqbal Masood

Contents

1. List of Abbreviations

AMC - Arthrogryposis Multiplex Congenita

PD - Parkinson’s disease

AD - Alzheimer's disease

PBST - Phosphatase buffer saline with tween

TBST - Tris-buffered saline with tween

WB - Western Blot Analysis

ELISA - Enzyme Linked Immunosorbent Assay

α-Syn - α-Synuclein

SHBG - Sex hormone binding globule

CST3 - Cystatin C Cst3

ABCF1 - ATP-binding cassette sub-family F member 1

Robo1 - Roundabout homolog 1

AB-A4 - Amyloid Beta A 4

ECL - Enhanced Chemiluminescence Solution

OD - Optical Density

2. Abstract

Parkinson's disease is the second most common neurodegenerative disorder. Parkinson's disease is a heterogenous disorder which means more than one causative agent contributes to the disease onset. Malfunctioned proteostasis, protein accumulates, protein over and under expression are the characteristic features of Parkinson's pathology. It is notable that body fluid proteomics has the potential to complement histopathology in disease diagnosis. In this research study we utilized proteomic approach to seek out clinically valuable serum biomarkers for PD. Assessment of changes in protein expression and quantification of disease related proteins was performed through techniques such as 2D gel electrophoresis, Elisa, western blot analysis, and dot blot analysis. Moreover, MTS assay performed to analyse cell viability by exposing healthy cells to patient serum and to healthy control serum. The maximum number of samples used in these protocols were 17 samples of PD and AMC each and 4 controls. The findings of this research study indicated increased alpha synuclein levels in PD serum compared to controls. Differential expression analysis of samples supported the above-mentioned outcomes for alpha-synuclein expression in PD serum vs healthy control. Cst3 levels were upregulated in PD serum compared to AMC and control whereas SHBG levels were downregulated in PD serum than in controls. Rab 9 a Ras GTPase protein increased relatively in PD serum, PrP Saf 32 significantly increased in PD samples compared to controls and higher expression of Amyloid Beta A4, 14-3-3 protein was observed in PD serums. Like the other biomarkers of PD these proteins are yet non-specific but can be potential indicators for PD diagnosis. It is envisioned that further advance in proteomic analyses will provide better opportunities for the diagnosis of PD.

3. Introduction

3.1 Neurodegenerative disorder:

Human central nervous system is responsible for most of brain and body functions. It comprises of brain and spinal cord where most of the electrical communication and signalling originate and pass on to the body. Neurons carry the information produced inside the brain and send it to other parts of body there are 100 trillion neuronal networks in the average human brain. Neurodegeneration involves the impairment in the structure of the neurons, anomality in the function of the neurons and death of the neurons. Thus, the disorders which involve any of these abnormalities in the neuronal networks are called neurodegenerative diseases. Alzheimer's disease (AD), Parkinson's disease (PD), prion diseases are few examples of neurodegenerative disorders.

Most neurological diseases affect patients later in life, abundant evidence suggests the presence of a phase of "pre-clinical" which could begin years before a person can be clinically diagnosed. Diagnosis of neurodegenerative disorders is tough due to complex patterns of disease onset. For a disease like Parkinson’s there are multiple factors taking part in establishing a multi-organ disease with clinical symptoms appearing only in later stages of life. This complex nature of neurodegenerative disorders makes diagnosis and treatment difficult. That is the reason no drug could be discovered till date to cure Parkinson’s disease completely. Therapeutic measures known to date only mild the symptoms and relief the pain but cannot stop the progression of the disorder.

3.2 Parkinson’s disease:

Parkinson’s disease (PD) is most prevalent neurodegenerative disorders, 2% people above age 65 and 4-5% people above age 85 suffer from PD. (Reeve, 2014)

Parkinson's is an untreatable incapacitating neurodegenerative disorder clinically characterized by pervasive accumulates of Lew bodies in both the Peripheral and Central nervous system. (Jellinger, 2012) There are almost 5-10% known genetic causes of Familial PD and 90% unknown causes of idiopathic or sporadic Parkinson's disease. (Nuytemans, 2010)

The neuropathological indicator of this disease is depletion of dopaminergic neurons in substantia nigra. Dopamine is the neurotransmitter essential for smooth running of functions like motor movement, support, and motivation. Lack of dopaminergic neurons push the body towards movement disorders and other Parkinson's related symptoms. Onset of the clinical syndrome Parkinsonism can be due to environmental toxins such as MPTP, genetic mutations, protein misfolding and dysfunction, oxidative stress, mitochondrial damage, and few more. Better understanding of all these phenomena's is therefore crucial for therapeutic and prognostic interventions for PD.

PD is a progressive disorder affecting multiple functions of the body. Pathological changes within the nervous system during Parkinson's onset when topographically imaged are mapped into six stages. In the initial stages (1-2), inclusion bodies are limited to olfactory bulb and medulla oblongata. Stage (3-4) disease progresses to substantia nigra where death of dopaminergic neurons worsens the condition and disease symptoms appear. In the final stages (5-6), clinical symptoms increase when the disease enters the neocortex which deals with critical functions like cognition, sensory perception, reasoning, and motor commands. (Braak, 2004)

Clinical manifestations of PD are classified into 2 categories: cardinal/motor and behavioural/non-motor symptoms. Motor symptoms or movement symptoms comprise of rigidity or freezing in place, bradykinesia (slowness of movement), and postural

instability. Motor symptoms are considered cardinal symptoms of Parkinson's disease. The behavioural symptoms of PD are sleep problems, constipation, fatigues, depression, and mood changes. Other symptoms of PD include cognitive deficiencies due to dementia, impaired thermal regulation, and sexual dysfunction. (Sveinbjornsdottir, 2016)

Epidemiological discoveries, genetic studies, and pathological annotations have contributed to the significant increases in the understanding of PD pathogenesis. Molecular pathways involved in onset of both idiopathic and genetic PD are better understood by fitting disease related genes into normal intracellular networks. (Trinh J, 2013). Parkinson's disease is a complex multi-system disorder with several factors being responsible for its onset. Pathogenesis of Parkinson's disease involve impairment in cellular mechanism responsible for protein homeostasis which leads to abnormalities in protein clearance pathways, protein trafficking and protein accumulation. (Bezard E, 2011)

Parkinson's is a heterogenous disorder which means along with genetic mutations other factors like aging and environment play key roles in disease development. Meta-analysis studies revealed eleven environmental factors that have role in alteration of the risk of PD. Among the factors that increase chances of disease were pesticide exposure, former head injury, agricultural work, and β-blocker consumption. Factors associated with a reduced risk were anti-inflammatory drugs use, calcium channel blockers use, coffee drinking and tobacco smoking. (Iranzo, 2013)

Parkinson's disease (PD) involves a diverse set of causative agents and clinical manifestations. Identification of genetic causes and risk factors in Parkinson's has developed extensively in the past 2 decades. The genetic risk factors for PD can be apportioned into those linked with a high-level risk for PD and those that have moderate risk towards PD onset and progression. Monogenic or mendelian mutations (bring about

5 to 10% of PD cases) are those that are correlated with a greatest probability for PD occurrence. (Kim C. Y., 2017)

3.3 Genetic mutations in Parkinson's disease:

Genome wide alteration studies for PD suggest six genes LRRK2, DNAJC13, SNCA, EIF4G1, CHCHD2, and VPS35 that regulate autosomal dominant form of disease. LRRK2 codes the leucine-rich repeat kinase 2, is a bulky multi-domain protein which plays role in various cellular functions including synaptic morphogenesis. LRRK2 is known for its dual enzymatic activity: serine-threonine kinase and GTPase. As much as 8 mutations in LRRK2 gene's catalytic domain (Paisán-Ruíz, 2004) (Aasly, 2010) have been recognised playing great role in genetic or familial Parkinson disease. The LRRK2 gene is found to be responsible for 4% of familial PD and account for 1% of sporadic PD. (Healy DG, 2008)

The genes associated with autosomal recessive form of PD are DJ-1, PINK1 and Parkin and disease onset with this mode of transfer can occur at early age usually less than 40 years. The genes responsible for autosomal dominant transmittance of PD just like the sporadic form of PD tend to occur in later stages of life. (Schrag, 2006) In autosomal recessive PD mutations in Parkin are the most common factor whereas mutations in DJ-1 (1-2%) and PINK1 (1-8%) are less common. (Singleton, 2013) Parkinson's patients with age below 45 years Parkin mutations are observed up to 50% of familial cases and 15% of sporadic cases. (Lücking C. B., 2000) Mitochondrial dysfunction and anomalies in synaptic form and function play a role in PD pathogenesis, especially toxin models of PD, as reported in genetic studies. (Bezard E, 2011) The genetics of Parkinson's disease is a thoroughly examined area of research in understanding of the pathophysiology of PD. Studies suggest that mutations in no less than 17 genes lead to the onset of familial form of PD. It is to notice that these genes

encode proteins which are linked to the molecular pathways occurring in other idiopathic or sporadic forms of PD. (Dexter, 2013)

3.4 Prevalence:

Incidence of Parkinson's disease is higher in Europe, North America, and South America as compared to that in Africa, Asia, and Middle East. Age and gender are two well established risk factors for Parkinson's disease. Research studies suggest occurrence of PD in ratio of 3:2 from male to female. The frequency and incidence for PD increases exponentially with age and risk is greatest after 80 years of age. (De Lau, 2006) (Driver, 2009) Probable elucidation for greater risk in men is neuroprotection from estrogens, X-linked genetic factors in women and extra occupational exposure in men.

3.5 Diagnosis:

Parkinson's disease affects the life quality of its patient to a vast extent as it disturbs the normal functioning of multiple organs. Research on this disorder is being conducted significantly in many countries to discover early therapy and disease prevention measures and for this early diagnosis is the most crucial factor. Lewy body formation and dopaminergic neurons depletion are the hallmarks of PD. (Yasuda, 2013) Till date PD treatment is only symptomatic as disease is diagnosable only after clinical symptoms begin to appear. After loss of significant bulk of dopaminergic (DA) neurons, the neuroprotective therapies have poor effects. To make therapies effective and manage disease progression diagnostic interventions are necessary.

Progress in the search of early diagnostic methods for PD depends on discovery of specific and sensitive biomarkers of the disease. These biomarkers must have the potential to highlight the level of PD progression in the patient and to evaluate the effectivity of current and upcoming therapies. (Brit Mollenhauer, 2010)

Presently the diagnosis of PD is rendered based on the cardinal motor characteristics and the linked non-motor features of the disease. (Goldstein, 2011) Neuroimaging, MRI, and PET scans are a means to identify the onset and progression of PD in patient's brain. Diagnosis of Parkinson's is established on the results of neuroimaging, neurocognition, clinical symptoms and proteomic analysis of csf, blood and urine. Discovery of an economical, easy to use, and specific biomarker can replace the expensive brain imaging methods. Like most of the infectious diseases detectable from a blood sample the idea is to make PD diagnosis as simpler as possible.

3.6 Biomarkers in PD:

Biomarkers for PD are classified broadly into two categories: preclinical (pre-motor) and clinical (motor) biomarkers. However, a detailed classification divides biomarker into following classes: genetic, clinical, physiological, biochemical, biophysical, morphological, pathological, and immunological.

Identification of early risk factors for PD is research area of great interest as it is claimed that a long asymptomatic phase may exist in PD. For example, loss of striatal dopamine and degeneration of substantia nigra occur way before clinical symptoms appear. PET scan can help detect the reduction in striatal dopamine in individual at risk of PD, but this diagnostic method is a high-priced and scarcely available method. These facts further emphasize on the need of economical and novel biomarkers to detect preclinical PD. (Koller, 1992)

There are many clinical biomarkers of PD few to mention are: bradykinesia, postural irregularity, incontinence, muscular rigidity, body tremors, drooling and neurobehavioral effects such as depression, nocturia and dementia. (Sharma, 2013) Many studies on clinical and experimental animal models suggest that progesterone, estrogens and androgens have the neuroprotective properties. (Butterfield, 2010) The sex difference

in neurodegenerative disorders is the central point of several studies and it is asserted that the difference is not only due to genetic differences but also due to steroids influence in the brain.

In the last decade, research has increased on the role of extra-and intracellular proteins that have irregular function or expression in PD. Impaired protein expression of tau, alpha-Synuclein and amyloid beta and neuroinflammatory markers is observed extensively in AD, PD, and other neurodegenerative disorders. To improve the diagnostic process of PD exploration and validation of potential protein biomarkers is the need of the hour. (Martí, 2013)

Search for biomarkers is the most queried topic in field of proteomics. Preferably a biomarker should be assayable from a biofluid noninvasively collected such as plasma, serum, urine, and saliva. Proteomic technologies have advanced so much to scan the individual target proteins in a variable complex medical biofluid. (Meng, 2007) In neurodegenerative disorders understanding of molecular mechanisms such as protein-protein interaction, oxidative stress, mitochondrial dysfunction, protein aggregation and others provides foundation for diagnostic and therapeutic interventions.

To analyse the pathology behind dopaminergic neuron degradation metabolomics and proteomics have an integral role. Pathways associated with dopaminergic neurodegeneration include synaptic neurotransmission and proteasomal, mitochondrial function. The omics techniques have greatly helped in identification of novel pathway e.g axon-guidance and increased oxidative stress to be involved in pathogenesis of PD. (Caudle, 2010) Numerous potential csf, blood, clinical, genetic, and imaging biomarkers identified to be useful in the diagnostic, progression, and therapy response analysis of PD patients. (Morgan, 2010) There is a need to assess these potential biomarkers for their specificity and sensitivity in a large cohort of PD patients' samples. (Robinson, 2010) A

single biomarker may not be enough sensitive and specific to PD diagnosis as Parkinson's is pathogenetically heterogenous and possesses common etiological elements with other neurodegenerative disorders. The search for effective biomarker for surveillance and diagnosis is still in progress. (Shtilbans, 2012)

3.7 Proteomics in Parkinson's disease:

Revolution in the field of genomics led to successful completion of the genome sequencing of humans and various other species. This has provided researchers with knowledge about gene therapy for genetic diseases, for example haemophilia. This revolution has highlighted the need to accelerate the pace of proteomic research. Proteomics has displayed substantial value in various facets of medical research including discovery of biochemical markers, disease pathogenesis, diagnostic and prognostic approaches, and last but not the least is detection of vaccine and drug targets. (Petricoin E. F., 2003)

Extensive research studies provided substantial evidence the major indicator among common age- associated neurodegenerative disorders including PD is protein aggregates in the brain tissues. (Ross, 2004) The mechanisms responsible for coordination between rate of protein production and degradation in a healthy individual are effective and thus protein aggregation does not occur. Whereas, in Parkinson's patients' mutations in genes disrupt the ubiquitin proteosome system thus disturbing the harmony in protein synthesis and protein clearance mechanism. (Balch, 2008) There is a need to understand the pathogenic mechanisms behind protein aggregates accumulation in dopaminergic neurons to take therapeutic measures to minimize disease progression. It can be predicted that degree of protein accumulation is directly related to neuron death and dysfunction in the brain.

3.8 Serum for Parkinson's diagnosis:

As soon as the disease begins to manifest its pathological features in the human body the expression of proteins changes in various biological fluids of the body e.g blood, urine, cerebrospinal fluid, and saliva. Determining the changes in protein expression in biofluids can aid in early diagnosis and in monitoring of pathogenesis throughout the course of the disease. Proteomics of body fluids has the potential to complement histopathology in disease diagnosis. (Petricoin E. F., 2002)

Collection of blood samples from patients is minimally invasive. Rapid variations in the protein expression in the blood occur when an intruder (infectious organism) enters, or pathological condition happens in the human body. This makes blood a plentiful source of observation for disease progression. (Ray, 2011) Another reason blood can prove valuable for the discovery of Parkinson's disease related protein biomarkers because level of hydroxyl radicals increases as the disease progresses. Augmented levels of hydroxyl radicals lead to oxidative stress which is a key factor in the progression of Parkinson's. (Ihara, 1999)

3.9 Proteins of Interest:

3.9.1 Alpha-synuclein:

In the central nervous system α-Synuclein is expressed in dopaminergic neurons, noradrenergic neurons, platelets, and endothelial cells. α-Synuclein has been discovered in the plaques in the brain of AD and PD patients. α-SN is released into the csf by neurons during regular cellular processing or neuronal death. (Takeda, 1998) Pathological aggregation of α-Synuclein leads to neurodegeneration. Now it is well known that presence of α-SN in Lewy neurites and Lewy bodies is a hallmark of Parkinson's disease. (Lücking C. B., 2000)

The physiological role of this protein is not certainly known yet however it is observed that this protein manages cell death. Research studies suggested that PD-linked mutations in alpha synuclein make the cells prone to apoptosis. The accumulates of mutant and wild type alpha synuclein have resulted in stimulation of apoptosis in the neuroblastoma cells. (El-Agnaf, 1998) Immune abnormalities have been reported in PD patients including the generation of autoantibodies against neuronal structures and increased microglial cells presenting the HLA-DR in substantia nigra. In a study to determine the relation between peripheral immune system and CNS, α-synuclein expression was observed in peripheral blood mononuclear cells (PBMC) to investigate the functional role of this protein in peripheral immune system. The outcome indicated significant upregulation in the α-synuclein expression in PBMC of PD patients in contrast to the healthy controls. (Kim S. S., 2004)

3.9.2 SHBG:

SHBG is a glycoprotein with homo-dimeric structure and molecular weight of 90kDa. Two N linked and one O-linked oligosaccharide are attached to the top side of each monomer. (Walsh, 1986) Serum levels of SHBG depend on several hormonal and non-hormonal factors e.g Rab GtPases

R levels are boosted by oestrogens and reduced by androgens. Sex hormones estrogen, progestogens and androgens regulate SHBG levels in serum through either posttranscriptional modification or alterations in its clearance mechanism. Serum levels of SHBG are regulated by prolactin, insulin, thyroxine, and insulin growth factor 1 (IGF-1). (Selva, 2009)

SHBG does not merely regulate the balance of sex hormones but also possesses the ability to amplify the effects of sex hormones in the body. (Anderson, 1974) Current research on SHBG provides substantial proof that this protein in the body is not only

produced peripherally but also originates from the pituitary and the hypothalamus in the brain. SHBG in brain is spatially directly associated with oxytocin releasing neurons. (Toljan, 2016)

PD is twice more prevalent in men than in women according to large meta-analysis studies. Apart from difference in prevalence PD, reports suggest disease onset is 2 years later in females than in males. (Haaxma, 2007) Estrogen is a sex hormone involved in functions beyond reproduction such as memory, synaptic plasticity, neurogenesis, cognition, and neuroprotection. Sex hormones play major role in functional and structural differentiation of brain thus also an important driver in sex differences in disease predisposition. (Gillies G. E., 2014)

3.9.3 Rab GTPases:

Rab GTPases play the role of carriers between organelles. It is a large family of small GTPase proteins that regulate receptor identification and vesicle budding on the cell membrane. Rab GTPases make sure that the spatiotemporal vesicle traffic runs smoothly via signalling among various Rab GTPases through shared effectors. Anomalies in Rab pathways cause diseases such as cancer, neurological disorders and immunodeficiencies. (Stenmark, 2009) Rab 9 GTPase has multiple roles such as lysosome biogenesis, CI-MPR recycling, retromer dependent transport, transport of lipids, and release of viral particles. (Matheoud, 2016)

Snx9 and Rab9, cytoplasmic proteins, are essential for Lipopolysaccharide induced mitochondria derived vesicle (MDV) formation and mitochondrial antigen presentation (MitAP). Rab9 regulates vesicle release from late endosomes and plays role in the formation of MDVs. LRRK2, genetic mutation in familial PD, controls vesicular trafficking when it phosphorylates a subgroup of Rab proteins. Rab 9 has significant role to play in mitochondria dysfunction during PD.

3.9.4 PrP Saf 32:

Prion protein (PrP) with a molecular weight of 33-35kDa is encoded by PRNP endogenous gene. PrP proteins are resistant to physical or chemical therapies against the infectious agents. Naturally, these protease sensitive proteins are expressed in a diverse range of infected or non-infected cells. (Quek, 2017) (Le, 2015)

PrP^c is located ubiquitously but dominantly in neurons, on the cell membranes. PrP^c a glycosylphosphatidylinositol (GPI) attached protease sensitive protein, plays role in copper/zinc homeostasis, signal transduction and as a receptor. Although, the exact biological roles of PrP^c are yet unknown. (Tamgüney, 2018) The function of copper attachment sites in the maintenance of neurogenesis in PrP^c has been proven crucial. (Nguyen, 2019) Emergent research underlines that various neurodegenerative disorders including PD share similarities with prion diseases in the prion like progression. Self-aggregation, templated misfolding of proteins (especially alpha synuclein) and transmission between the cells are key similarities of PD with prion diseases. (Ma, 2019)

3.9.5 Cystatin C (Cst3):

Cystatin C (Cst3) belongs to the protein superfamily of cysteine-proteinase inhibitors. Cystatin C is found abundantly in csf, blood plasma, synovial fluid, and urine of uraemia patients. Cystatin proteins play role in the regulation of cysteine proteinases and shield cells from incorrect proteolysis. (Green, 1984) CST3 gene is localized on the human chromosome 20 and encodes human cystatin C protein. (Abrahamson, 1990)

Cystatin C (CC) is a cysteine protease inhibitor expressed in abundance in the CNS. Bunina bodies, exact composition not known but possibly an abnormal accumulation of proteinaceous material, are the histological hallmark of ALS. Bunina bodies have been proven to be stain positive for cystatin C. (Yamamoto-Watanabe, 2010)

4. Objectives of Study:

Identification of the risk factors and validation of protein biomarker of PD in the serum samples of patients. The aim of this research is to contribute to the research related to early diagnosis of PD with a biofluid that is conveniently available. To establish a thorough understanding of the types of proteins and frequency of these proteins involved in PD. To look for reliable biomarkers for the diagnosis of familial as well as idiopathic or sporadic PD and diagnosis of pre-clinical and clinical forms of PD.

Primary aims of the research were:

1. Identification of potential risk factors and biomarkers of Parkinson's disease from human serum samples
2. Verification and characterization of identified risk factors and protein biomarkers specific to PD

5.Materials and Methods:

5.1 Materials

5.1.1 Chemicals:

Chemicals used in this study were purchased from Thermoscientific (Canada) Biochrom (Germany), Sigma Life Science (Germany), Roche Diagnostics (Switzerland), Carl Roth (Germany), Bio Rad (Germany).

5.1.2 Kits:

Name	Description	Company/Catalogue no.
BCA kit	Protein concentration measurement	Thermo fisher Scientific/ 23225
MTS Assay kit	Cell viabikity/toxicity assay	Abcam/ab197010
Human 14-3-3 protein eta, YWHAH ELISA Kit	Elisa	ImuGex/BT-E3410Hu
SNCA/alpha Synuclein Bioassay Elisa Kit	Elisa	ImuGex/BT-E1313Hu
Amyloid Beta A4 Elisa Kit	Elisa	ImuGex /BT-E4942Hu

5.1.3 Antibodies:

List of primary antibodies:

Name	Source	Company	Catalogue No.	Dilution (WB)
Anti-ABCF1 antibody	Mouse	Abcam	Ab50976	1:100
α/β-synuclein antibody (3B6)	Mouse	Santa-Cruz	Sc-69699	1:100
SHBG	Rabbit	LifeSpan BioSciences	LS-B12124	1:300
Slit 2	Rabbit	Abcam	Ab 7665	1:500
CST3	Mouse	My BioSource	MBS 9700331	1:500
Saf 32	Mouse			1:500
Robo 1	Rabbit	Abcam	ab7279	1:500
Rab 9	Rabbit	Cell Signaling Technology	D52G8	1:1000
ABCF1	Mouse	Abcam	Ab 89109	1:100

List of secondary antibodies:

Name	Source	Company	Catalogue No.	Dilution (WB)
Rabbit-HRP	Goat	Jackson IR lab	111-035-062	1:2000-5000

Mouse-HRP	Goat	Jackson IR lab	115-035-062	1:2000-5000

5.1.4 Protein marker and loading buffer:

Standard	Company	Catalogue No.	Volume used (WB)
Precision Plus Protein Standard	Bio-Rad	161-0374	8ul
Roti-Load (4X)	Roth	K929.1	1/3 of sample volume

5.1.5 Cell culture reagents

Reagent	Company/Catalogue No.
Dulbecco's Phosphate Buffer Saline	Sigma Aldrich/D8537
Fetal Bovine Serum (FBS)	Sigma Aldrich/F9665
Penicillin-Streptomycin	PAN Biotech/P06-07100
GlutaMAX supplement	Thermo Fischer Scientific/ Gibco 35050038
Media DMEM	Sigma Aldrich/ D6429
Trypsin/EDTA solution	Sigma-Aldrich/T4174

5.1.6 Mammalian cell line and culture media

SH-SY5Y cells were acquired from Prof. Walter SchulzSchaeffer, Department of Neuropathology, University Medical Center (UMG), Göttingen, Germany. The cells were

cultured in a T75 flask, covered in DMEM, supplemented with 10% FBS, 1% GlutaMax add-on and 1% PS incubated at 37°C, 95% humidity with 5% CO2.

5.1.7 Laboratory Equipment:

Equipment	Company
Centrifuge 5810 R	Eppendorf, Germany
Microscope Leica TCS SPE	Leica Microsystems, Germany
Trans-Blot Turbo	Bio-Rad
Extra thick blot paper	Bio-Rad
Power Supply 200/2.0	Bio-Rad
Protean IEF Cell	Bio-Rad
IEF trays and electrode wicks	Bio-Rad
IPG strips	Bio-Rad
Weight machine CP3202 P TE313S-DS	Sartorius
Incubator IFE 400	Memmert, Germany
Shaker	Sartorius, Germany
Microplate reader Perkin Elmen Wallac	GMI USA
Safe-Lock tubes (0.2, 0.5, 1.5 and 2ml)	Eppendorf
The Protean II electrophoresis chamber	Bio-Rad
Cell culture TC Flask T75	SARSTEDT
Thermomixer comfort	Eppendorf
Chemi Doc XRS + system	Bio-Rad
pH strips	Merck Millipore, Germany

5.1.8 Stock solutions and Buffers:

Running buffer 10x (1L) :144 g Glycine, 30 g Tris, 10 g SDS in ddH2O

Transfer buffer 10x (1L): 48 mM Tris (58.2 g), 39 mM Glycin (29.3 g), 1 mM SDS (0.375 g) in ddH2O

Resolving Gel buffer: 181.7g/l Tris Base, 4g/L SDS (pH: 8.8)

Stacking Gel buffer: 60g/L Tris, 0.4% SDS (pH 6.8)

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

10x PBS-Tween (1L): 95.5g/L PBS, 5ml Tween-20

10x TBS-tween (1L): 24.23g Tris, 87.66g NaCl, 10ml Tween (pH 7.4 – approximately 15ml of 37%HCL)

Blocking solution for immunoblotting: 5% non-fat dry milk in PBS-T/TBS-T

5.1.9 Software's and Online Tool:

Name	Use/Description	Reference
Image Lab 6.1	Densitometric analysis	Kapelan, GmbH/Halle, Germany
GraphPad Prism 7.04	Statistical analysis	GraphPad Software, Inc. California, USA
My Assays	Data analysis	https://www.myassays.com/

5.2 Methods

5.2.1 Determination of protein concentration:

Protein concentration in serum samples of PD, AMC and Control was determined through Bicinchoninic acid assay (BCA) kit. Sample were diluted 1:25 (1ul of disease sample diluted in 24ul of ultra-pure water) with a dilution factor of 25.

Working reagent volume calculated by:

No. of standards + no. of samples × no. of replicates × volume of WR per sample = total volume

Amount of WR required is (9+14) × (2) × (200ul) = 9200ul

WR A: WR B = 1: 50. Which means to prepare working reagent add 184ul of Reagent B in 9052ul of Reagent A.

Microplate Procedure:

Standard or unknown sample replicate in a concentration of 25ul is pipetted into the microplate well. 200ul of the working reagent is poured into the wells and it is placed on shaker for half minute. Incubation at 37°C for 30 minutes after covering the plate. After cooling samples down to RT, absorbance is measured at 562nm on plate reader. Protein concentration in the serum samples determined through BCA assay was then statistically analysed via four parameter logistic curve.

5.2.2 Gel electrophoresis:

Sample preparation:

dH2O was added in each sample of controls 50ug and diseased 100ug serum protein to equalize sample volumes. Loading buffer was added to every sample making 1x final dilution of buffer. Then samples were subjected to thermal digestion in the

thermomixer at 95^{o}C for 5 minutes following vortex and brief centrifugation to settle down the volume in tubes.

Gel casting:

To carry out molecular weight-based proteins separation gel was prepared in laboratory with 12.5% resolving gel and 4% stacking gel buffer. Gel casting apparatus from Bio Rad was used to cast gel.

Components	Bottom gel (ml) Volume for 2 gels	Upper gel (ml) Volume for 2 gels
Buffer (ml)	3.70	1.05
Acrylamide 40% (ml)	4.40	0.84
dH2O (ml)	6.80	2.60
10% APS (ul)	150	45
TEMED	6	5

Table 1: Recipe for 12% resolving gel and 6% stacking gel

Resolving gel (7ml) was poured between the glass plates, covered it with isopropanol and left it to polymerize for 20 minutes. Isopropanol was removed and plates were cleaned briefly with dH2O. Then stacking gel was poured and comb was inserted immediately.

Running buffer 1x was prepared by diluting 10x running buffer in dH2O. The samples were loaded as follows 50ug and 100ug of controls and diseased, respectively. When the assembly is set up gels are subjected to electrophoresis at 100V till the loading reached bottom of the gel.

5.2.3 Western Blot:

To immunoblot the proteins PVDF membrane (pore size 0.45um) was cut as per gel size. PVDF membrane was pre-soaked in methanol and 1x transfer buffer for 1 minute each, respectively. Recipe for 1x Transfer buffer is following:

Transfer buffer 1x (1L): 100ml 10x transfer buffer, 200ml methanol, 700ml water

The resolving gel was removed and put in 1x transfer buffer for 1 minute. Thick blot paper (pre-soaked in 1x transfer buffer for 1 minute) were used to make sandwich: membrane at the top of first blot paper, gel above the membrane then covered with second blot paper. This setup was put in the trans blotter and processed at 14volts constant for 60 min.

The membrane is blocked in blocking buffer for 1 hour with shaking. To prepare blocking buffer: 5% non-fat dry milk added in a wash solution (1x PBST or 1x TBST). After this the membrane was incubated with the main antibody of desire and left on a gentle shaker (4^{o}C) overnight.

Washes were given with the relevant wash buffer and incubation with respective secondary antibody is done (5% milk in PBST/TBST with Ab dilution) for 60 minutes. Membranes are treated with ECL solution for 1 minute in absence of light.

Bio-Rad Chemidoc was used for obtaining images. Re-blotting of same membrane was done after stripping.

Stripping buffer: 15 g glycine, Adjust pH to 2.2, 1 g SDS, 10 ml Tween 20

in Distilled water up to 1 L

5.2.4 Western blot of CST3:

Sample proteins settled on SDS page are moved onto pvdf sheath using blotting protocol. Membrane is blocked in milk in TBST (5%) for 1 hour. Then membrane was incubated in primary antibody cystatin C (cst3) left on mild shaking overnight at 4^{o}C.

Cst3 primary antibody diluted in milk with TBST in a ratio of 1:500. Next day after giving washing according to the protocol secondary antibody was applied and incubated membrane in it for 1 hour with shaking. Secondary antibody prepared in same blocking buffer in a ratio 1:2000. After the remaining washes membrane was incubated in ECL solution for 1 minute and images were scanned using the chemidoc.

5.2.5 Western blot of Rab 9:

SDS page and western blot analysis done to transfer proteins of PD, AMC samples and controls. Membrane blocked in 5% milk in TBST for 1 hour prior to overnight incubation primary antibody with gentle shaking at 4^{o}C. Rab 9 antibody prepared in 5% milk with TBST in a dilution 1:500. Washes were given the next morning as mentioned in the protocol above. Secondary antibody prepared in 5% milk in TBST in ratio 1:2000. Images of the membrane were scanned post washing off the secondary antibody followed by 1 minute incubation in ECL solution.

5.2.6 Western blot of SHBG:

Sample proteins resolved on SDS page and transferred onto pvdf membrane using western blotting protocol. Membrane blocked in 5% milk in PBST for 1 hour. Sex hormone binding globulin (SHBG) was prepared in the same blocking buffer in a dilution 1:300. Then membrane was incubated in primary antibody SHBG and left on mild shaking overnight at 4^{o}C. Secondary antibody prepared in the same blocking buffer in ratio 1:2000 was poured in the container containing the membrane and left on the shaker for 1 hour. Washes were given with 1xPBST according to the protocol mentioned above. Incubation in ECL in the dark for 1 minute. Membrane was scanned in the chemi-doc using Imag lab 2.0 program to save images taken after every 10 seconds until bands turn red due to complete saturation.

5.2.7 Western blot of Saf 32:

SDS page and western blot analysis done to transfer proteins of PD, AMC samples and controls onto a PVDF membrane. Membrane blocked in 5% milk in TBST for 1 hour then incubated overnight with the primary antibody saf 32 (continuous gentle shaking) at 4^{o}C. Saf 32 antibody prepared in 5% milk with TBST in a ratio of 1:500. The next morning membrane is washed in 1x TBST buffer according to the protocol and then incubated in 1:2000 diluted secondary antibody for 1 hour on a shaker at room temperature. Then incubated in ECL Solution in dark for 1 minute before scanning in chemi-doc.

5.2.8 2D SDS PAGE for differential proteomics

This technique separates the protein molecules based on both their net charge and their molecular weight.

Steps:

Rehydration buffer preparation stock: 42g urea, 15.2g thiourea, 4g Chaps, freshly add: 0.2% ampholytes + 15mMDTT. Vortex to mix buffer before use. For 7 cm IPG strip add 50ug sample protein in 150ul of rehydration buffer. Thoroughly mix the mixture and dispatch in the reswelling cassette track avoiding bubble formation in the track. Delaminate protective layer from IPG strip and place in the track so no bubble remains underside IPG gel. Cover IPG strip with 1ml mineral oil.

Protocol Carry out passive rehydration at room temperature. After rehydration Isoelectric focusing using Protean il2 IEF system with following programming: 500V for 1h, 1000V for 1h, 8000V for reaching 35000Vh (with 10,000V for 1 hr) and 50V pause step.

Equilibration before 2^{nd} dimension electrophoresis IPG strips are equilibrated to further degrade proteins and stabilize these degraded forms

Equilibration buffer Stock	100 ml
Urea	36g
SDS	2g
Glycerine	30mL
Tris Hcl (0.05M) pH 8.8	1.4mL

Equilibration buffer 1:	Add 2% DTT before use
Equilibration buffer 2:	2.5% Iodoacetamide and traces of bromophenol blue

Treat strip gels with equilibration buffer 1 for 25 minutes. Treat strip gels with equilibration buffer 2 for 25 minutes.

Second dimension gel electrophoresis

Cast 9mL of 12% resolving gel buffer between casting plates. Mount IPG on SDS-polyacrylamide gels: place the strip so the gel side faces the larger glass place, load marker on wick paper place wick paper in the well next to positive side of IPG strip. Electrophoresis at 100 V till dye reaches bottom of gel.

5.2.9 Total Protein Normalization:

MemCode reversible protein stain kit used to quantify the frequency and abundance of protein of interest without relying on the housekeeping proteins.

Material pre-preparation:

Destain/Methanol solution: mix reagent grade methanol 1:1 with MemCode destain.

Erase/Methanol solution: mix reagent grade methanol 1:1 with MemCode stain eraser.

Protocol: Pvdf membrane containing transferred proteins is placed in a clean container and rinsed with ultra-pure water 3 times manually. Memcode sensitizer solution poured on top of the membrane and counter was left on a shaker at room temperature for 2 minutes. Drain out sensitizer solution and add memcode reversible stain to the membrane leave it on the shaker for 1 minute. Rinsed the membrane three times manually with the memcode destain solution. Then pour the methanol destain solution onto the membrane and leave it on the shaker for 5 minutes. Rinse the membrane with ultra-pure water three times. Take the scanned image using the chemidoc and image lab software. In the end prior to saving the membrane at -20 wash it with eraser methanol solution (for 10 minutes) and rinse with ulta-pure water to remove erase methanol solution.

5.2.10 Cell Counting:

To continue counting first remove prior media from flask. Added 3ml PBS into the flask to wash off any remaining media. Then cells were incubated in 3ml trypsin for 2-5 minutes. Add 10ml media after trypsinization. Centrifuge the entire volume in a falcon at 128Xg for five minutes. Resuspend the pellet in 2-5ml of media (depending on the size of the pellet). Take 100ul from this solution in an Eppendorf tube and add 400ul of Tryphan blue (1:5). Load 10ul of this mixture between hemocytometer and its cover (both previously cleaned with ethanol). Count the number of viable cells in 4 boxes in the corners exclude the dead cells (blue spots). Calculate the number of cells/ml as follows:

a. Average number of cells in 4 boxes = 72.75
b. Correct the dilution: x X dilution (y) = 363.75×10^4
c. The volume: y X square area (z) = $2500 \times 10^4 / 364 \times 10^4 = 6.86$ cells per well

The experiment was conducted with three plates with same number of cells and samples mixture incubated for different time intervals. Total number of wells used were

51. So, 6.86 x 51 = 350 ul of stock will be used. When loading 100ul each well required total is 5100ul. 5100ul – 350 ul of stock = 4750ul of fresh medium is to be used. Mix the stock well with fresh medium and then load 100ul to each well leaving one well empty in each plate for MTS blank. Incubate plates at 37ºC and wait until cells are 100% confluent (2 days in this study).

5.2.11 MTS Assay:

8 ul of serum sample was taken in autoclaved Eppendorf's. MTS Reagent is ready to use as supplied before use equilibrate it to room temperature and briefly centrifuge. Removed previous medium from wells and added 8ul of sample + 92 ul of fresh media in each relevant well. Incubate cells for 3,6,9 hours in an incubator pre-set to 37ºC. MTS reagent (10ul) was added to each well and plates incubated for 0.5 - 4 hours at 37°C in standard culture conditions. Plates were shaken briefly, and absorbance was measured at 490 nm.

5.2.12 Alpha Synuclein ELISA:

Sample preparation: Serum samples of Parkinson's disease, Arthrogryposis Multiplex Congenita and Control were used (100 ug protein) in a ratio of 1:10 then total volume was raised to 40ul by adding PBS. For this study 11 PD samples, 10 AMC samples, and 3 control samples were used.

64 ng/mL	Standard 5	120ul original standard + 120ul standard diluent
32ng/mL	Standard 4	120ul standard 5 + 120ul standard diluent
16ng/mL	Standard 3	120ul standard 4 + 120ul standard diluent
8ng/mL	Standard 2	120ul standard 3 + 120ul standard diluent
4ng/mL	Standard 1	120ul standard 2 + 120ul standard diluent
0ng/mL	Standard Zero	Standard diluent only

Table 2: Number of standards prepared according to instructions in the kit

Antibody pre-coated strips were inserted in the frame. 50ul of standard was added to every well in duplicates. 40ul of sample was added to each sample well in duplicates. Then 10ul anti-alpha Synuclein (SNCA) antibody added to each sample well followed by 50ul streptavidin-HRP to sample wells and standard wells (Do not add in blank control well). Mix properly. Elisa plate is covered with a sealer and placed in an incubator pre-set to 37oC for 60 minutes.

Preparation of wash buffer: The provided 25x wash buffer concentrate is diluted in distilled water to yield 1x wash buffer. 20 ml of 25x wash buffer in 500ul distilled water.

Sealer is removed and wells are washed 5 times with wash buffer. Wash buffer (0.35mL) is added into each well for 30 seconds to 1 minute for each wash. Plate is blotted onto paper towels to remove any leftover wash buffer. After washes 50ul substrate solution A is added followed by 50ul of substrate solution B to each well. Plate is covered with a sealer again and placed in dark at 37oC for 10 minutes. In the end 50ul Stop solution is added to each well which changes the blue colour to yellow immediately. Then OD is determined for each well using a microplate reader set to 450nm. Calculation of result: Standard curve of OD on Y axis and concentration on X axis constructed using Prism GraphPad.

5.2.13 14-3-3 ELISA:

Sample preparation: Serum samples of PD, AMC and Control were used (100 ug protein) in a ratio of 1:10 final volume was raised to 40ul by adding PBS. The number of samples in this Elisa were as follows: 13 PD samples, 13 AMC, and 4 controls.

48 ng/mL	Standard 5	120ul original standard + 120ul standard diluent
24ng/mL	Standard 4	120ul standard 5 + 120ul standard diluent
12ng/mL	Standard 3	120ul standard 4 + 120ul standard diluent
6ng/mL	Standard 2	120ul standard 3 + 120ul standard diluent
3ng/mL	Standard 1	120ul standard 2 + 120ul standard diluent
0ng/mL	Standard Zero	Standard diluent only

Table 3: Number of standards prepared according to instructions in the kit

Standard solution (50ul) was added to each well in duplicates and 40ul of sample was added to each sample well in duplicates. All wells in the tray are pre-coated with

human YWHAH antibody. Then 10ul anti-14-3-3 protein eta was added to each sample well only. 50ul streptavidin-HRP then added to sample wells and standard wells (Do not add in control well). After thoroughly mixing the Elisa plate is covered with a sealer and placed in an incubator fixed in advance to 37oC for 60 minutes.

Sealer is removed and wells are washed 5 times with 1x wash buffer. Let the wash buffer (0.35mL) rinse each well for 30 seconds to 1 minute for each wash. Plate is blotted onto paper towels to remove any leftover wash buffer. 50ul substrate solution A is added followed by 50ul of substrate solution B to each well. Plate is covered with a sealer again and placed in dark at 37oC for 10 minutes. 50ul Stop solution is added to each well the colour changes from blue to yellow instantly. The optical density (OD) is calculated using a microplate reader set to 450nm. Calculation of result: Standard curve of OD on Y axis and concentration on X axis constructed using Prism GraphPad.

5.2.14 Amyloid beta A4 ELISA:

Serum samples of PD, AMC and Control were used (100 ug protein) in a ratio of 1:10 final volume was raised to 40ul by adding PBS.

1600 ng/mL	Standard 5	120ul original standard + 120ul standard diluent
800 ng/mL	Standard 4	120ul standard 5 + 120ul standard diluent
400 ng/mL	Standard 3	120ul standard 4 + 120ul standard diluent
200 ng/mL	Standard 2	120ul standard 3 + 120ul standard diluent
100 ng/mL	Standard 1	120ul standard 2 + 120ul standard diluent
0ng/mL	Standard Zero	Standard diluent only

Table 4: Number of standards prepared according to instructions in the kit

Standard solution (50ul) was added to each well in duplicates (do not add Ab to standard well). 40ul of sample was added to each sample well in duplicates followed by addition of 10ul anti-Amyloid beta A4 (APP) antibody to each sample well only. In the sample and standard wells 50ul streptavidin-HRP was added (do not add in control well). Mix aptly then cover the Elisa plate with a sealer and placed in an incubator for 60 minutes at 37oC.

Sealer is removed and wells are washed 5 times with 1x wash buffer. Use minimum 0.35mL wash buffer to rinse each well for 30 seconds to 1 minute. Dry the wells completely by tapping the plate on paper towel. 50ul of substrate solution A substrate solution B were added to each well consecutively. Plate is covered with a sealer and placed in dark at 37oC for 10 minutes. 50ul Stop solution is added up to each well, the colour changes from blue to yellow instantly. The optical density (OD) is calculated using a microplate reader set to 450nm. Calculation of result: Standard curve of OD on Y axis and concentration on X axis constructed using Prism GraphPad.

5.2.15 Dot Blot Analysis of ABCF 1:

For Dot blot analysis grid for 16 samples is drawn on nitrocellulose membrane (0.45nm pore size). 10ul of each sample and 8 ul of each control is spotted on designated boxes and membrane is left to dry for 30 minutes. Blocked in blocking buffer (5% milk in PBST) for 1 hour. Incubated in ABCF 1 primary antibody (1:100) overnight at 4oC with gentle shaking. The next day the membrane is incubated in secondary antibody (Ms IGg 1:2000) for 1 hour after washes and prior to next washes with PBS-T. Membrane is then placed in ECL solution for 1 minute before scanning it in chemi-doc.

5.2.16 Dot Blot Analysis of ROBO1:

On a nitrocellulose membrane grid for 12 samples is drawn (0.45nm pore size). 10ul of each sample and 8 ul of each control is spotted on designated boxes and membrane

is left to dry for 30 minutes. Blocked in blocking buffer (5% milk in PBST) for 1 hour. Incubated in ROBO1 primary antibody (1:1000) overnight at 4oC with gentle shaking. The next day washes are given then incubation in secondary antibody (Rb IGg 1:2000) for 1 hour. Remaining washes are given according to protocol. Then membrane is incubated in the dark in ECL solution for 1 minute before scanning it in chemi-doc.

5.2.17 Dot Blot Analysis of Slit-2:

Primary antibody concentration: 1:500 in 5% milk in PBST. Membrane with samples blotted on it is incubated in SLIT2 primary antibody and left overnight on shaker at 4^{o}C. The next day following tasks are completed: washes, secondary antibody incubation (Rb IGg 1:2000), and image scanning.

6.Results and Discussion:

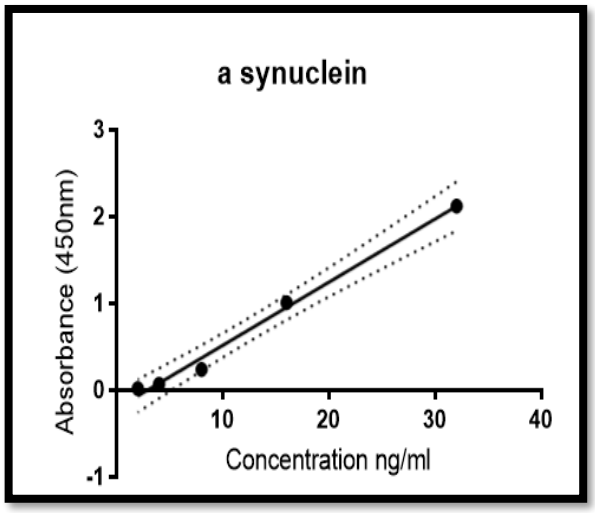
6.1 Expression Analysis through Elisa:

The expression analysis of a-synuclein, Tia 1, P-tau, 14-3-3 eta, and Amyloid beta 6E10 was also tried to be done through western blot analysis. Nevertheless, bands could not be detected for these antibodies on the blot. So, Elisa was performed for three proteins: a-synuclein, 14-3-3 and Amyloid beta A4.

6.1.1 Expression Analysis of Alpha Synuclein:

Enormous research studies are available that explore the role of α-synuclein as a central biomarker from csf and brain tissues. However, exploration of α-synuclein potential as a peripheral biomarker in PD diagnosis is relatively under-studied. For this reason, we examined alpha synuclein expression in serum samples of diseased and healthy individuals both.

A



B

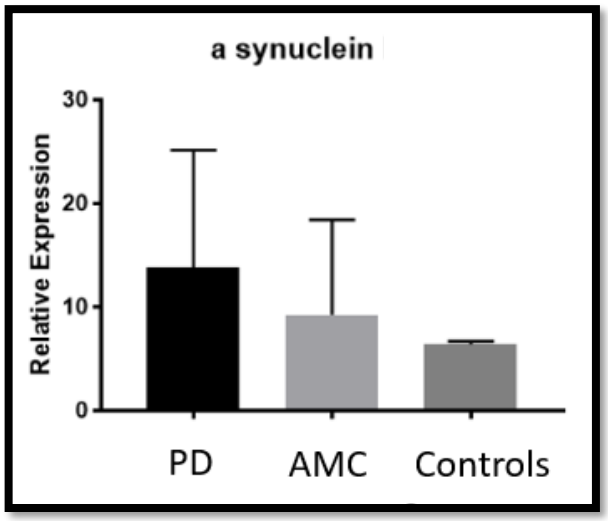


Figure 1: (A) Line graph with absorbance on y-axis and concentration on x-axis. R2 value: 0.9912. (B) Through ordinary one-way ANOVA test and multiple comparison analysis among PD, AMC and controls the p value: 0.4170 which means non-significant difference but visually it appears that expression is highest in PD compared to AMC and controls. Multiple comparison analysis showed non-significant difference for PD vs AMC p = 0.5554, insignificant difference for PD vs Control p = 0.5000, non-significant difference for AMC vs Control p = 0.9021.

6.1.2 Expression analysis of 14-3-3:

14-3-3 is a novel adapter protein that binds to phosphorene attached ends of other proteins. 14-3-3 has great significance in Lewy body associated diseases such as Parkinson's disease. 14-3-3 interacts with alpha-synuclein gene and LRRK2 gene. In familial PD cases an impaired phosphorylation dependent binding of 14-3-3 to LRRK2 is caused by genetic mutations. To analyse significance of 14-3-3 as a potential peripheral biomarker for PD Elisa was conducted. Following findings that came out are illustrated in the image below.

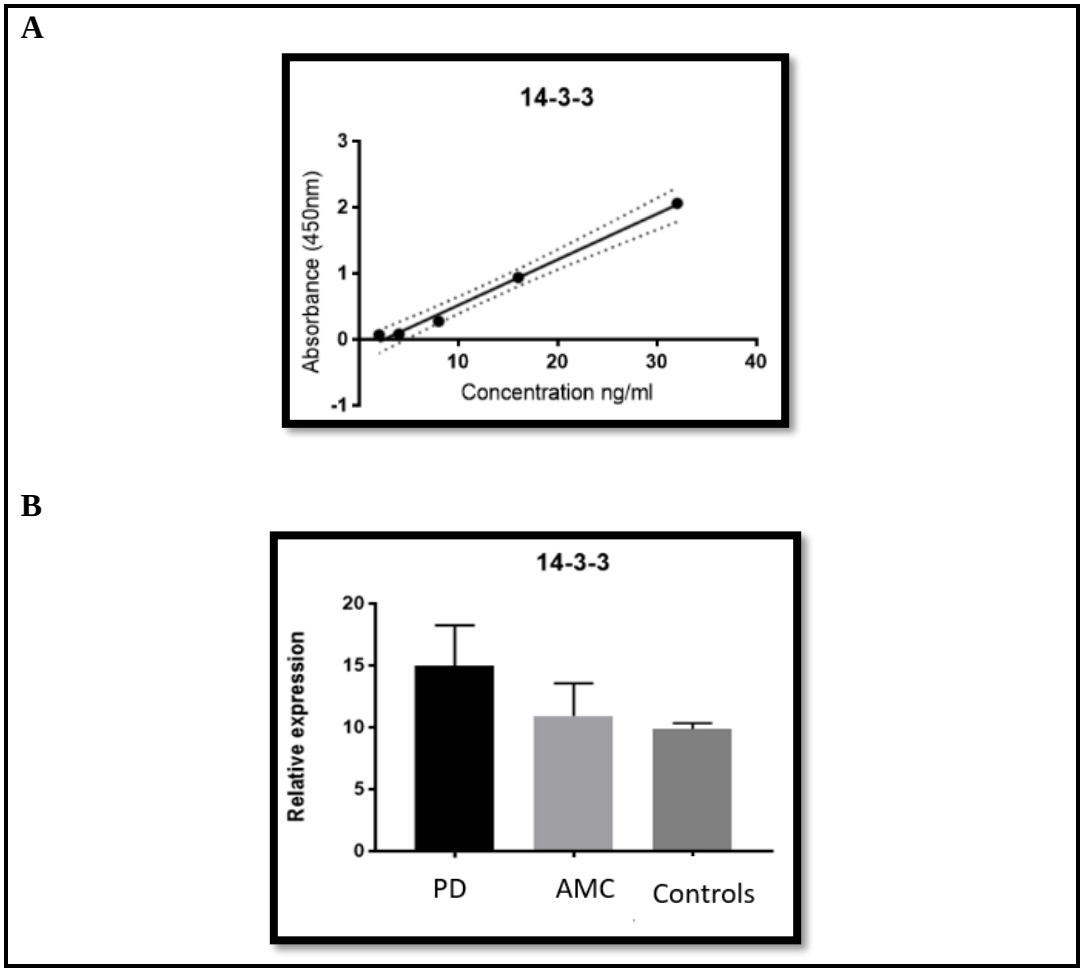


Figure 2: (A) Line graph of absorbance values on y-axis and concentration on x-axis. R2 value: 0.9919. (B) Relative expression of 14-3-3 among PD, AMC, and controls. p value

of 0.5108 means difference is non-significant. However, the expression is highest in PD samples as it visually appears.

6.1.3 Expression Analysis of Amyloid beta A-4:

Amyloid beta accumulation is a commonly shared feature of neurodegenerative disorders. Amyloid beta A-4 is a precursor amyloid beta protein, it has a role to play in cognitive processes in brain. Amyloid beta A-4 levels are important players thus to be determined for diagnostic and therapeutic purposes.

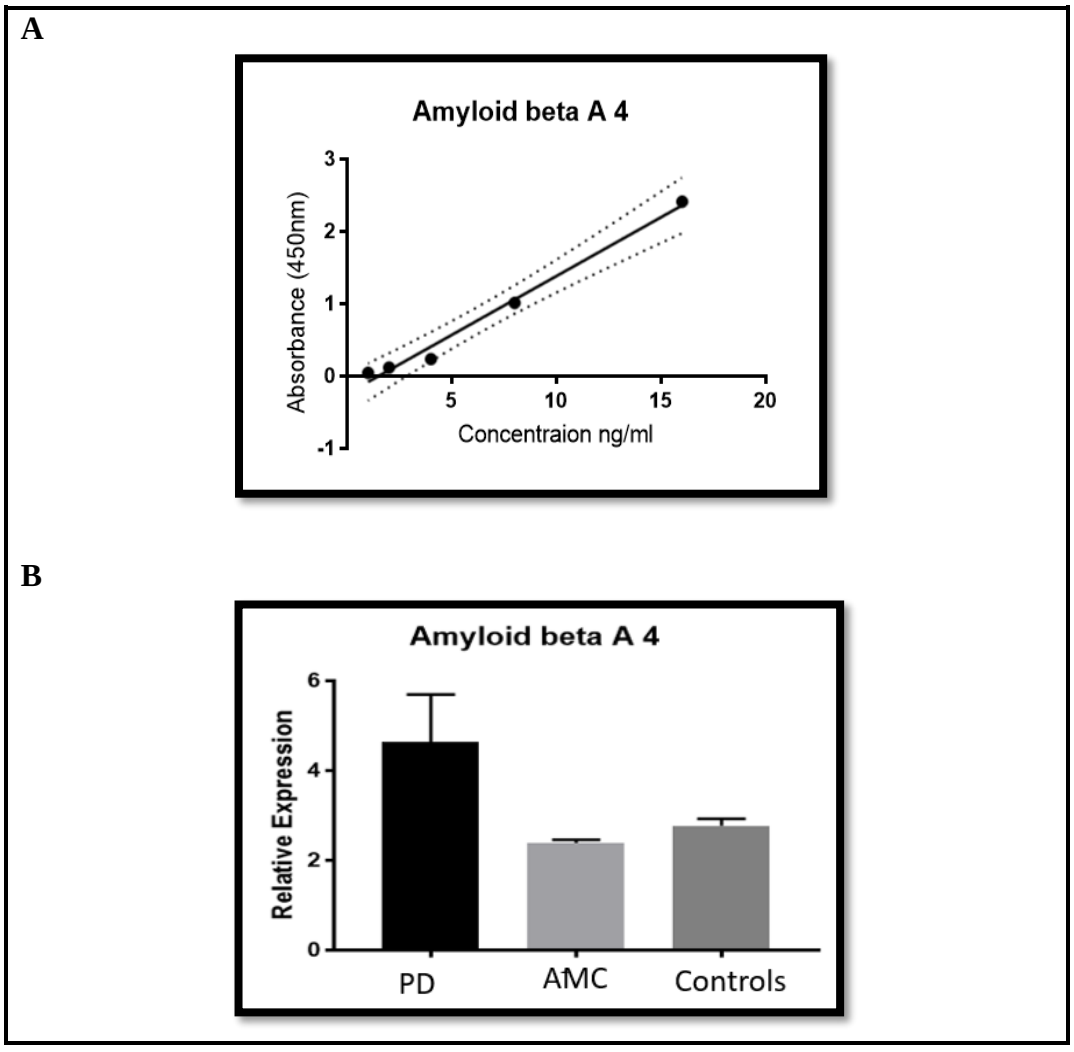


Figure 3: (A) Line graph of absorbance values on y-axis and concentration on x-axis. R2 value: 0.987. (B) Amyloid beta A-4 Elisa and analysis through ANOVA shows that

although p value turns out to be 0.1869 still a greater expression of protein can be observed in PD as compared to protocol.

6.2 Expression Analysis using WB:

As augmented levels of leucocytes are a hallmark of most infectious diseases, in case of neurodegenerative disorder composition of different brain areas and csf changes quickly. Imaging or scanning shows neuronal loss and cell death in brain region in case of PD, AD, ALS, and other neurodegenerative disorders however this approach is not cost effective. Protein upregulation or downregulation and accumulation or aggregation is a hallmark of nearly all neurodegenerative diseases. Protein expression upregulation and downregulation can be observed from following biofluids: csf, blood serum, blood plasma, urine, and saliva.

6.2.1 Expression Analysis of CST3:

CST 3 regulates the enzymatic activity of cysteine proteinases as an inhibitor of cysteine proteinase. Cysteine proteases enzyme functions to degrade proteins. In literature CST 3 is mentioned as a possible cerebrospinal fluid biomarker for Creutzfeldt-Jakob disease. Significance of Cst3 in neurodegenerative disorders is of great value, this protein is referred as established histological marker of amyotrophic lateral sclerosis (ALS) in literature. Abnormal aggregation of certain proteins is a clinical feature of many neuropathies including Parkinson's disorder, so serum samples of PD patients were analysed for this protein as well. The results from western blot analysis indicated increased expression of CST 3 in diseased as compared to healthy controls.

For CST 3 expression analysis following number of samples from each cohort were used: n = 9 PD patient samples, n = 7 AMC patient samples, n = 4 controls.

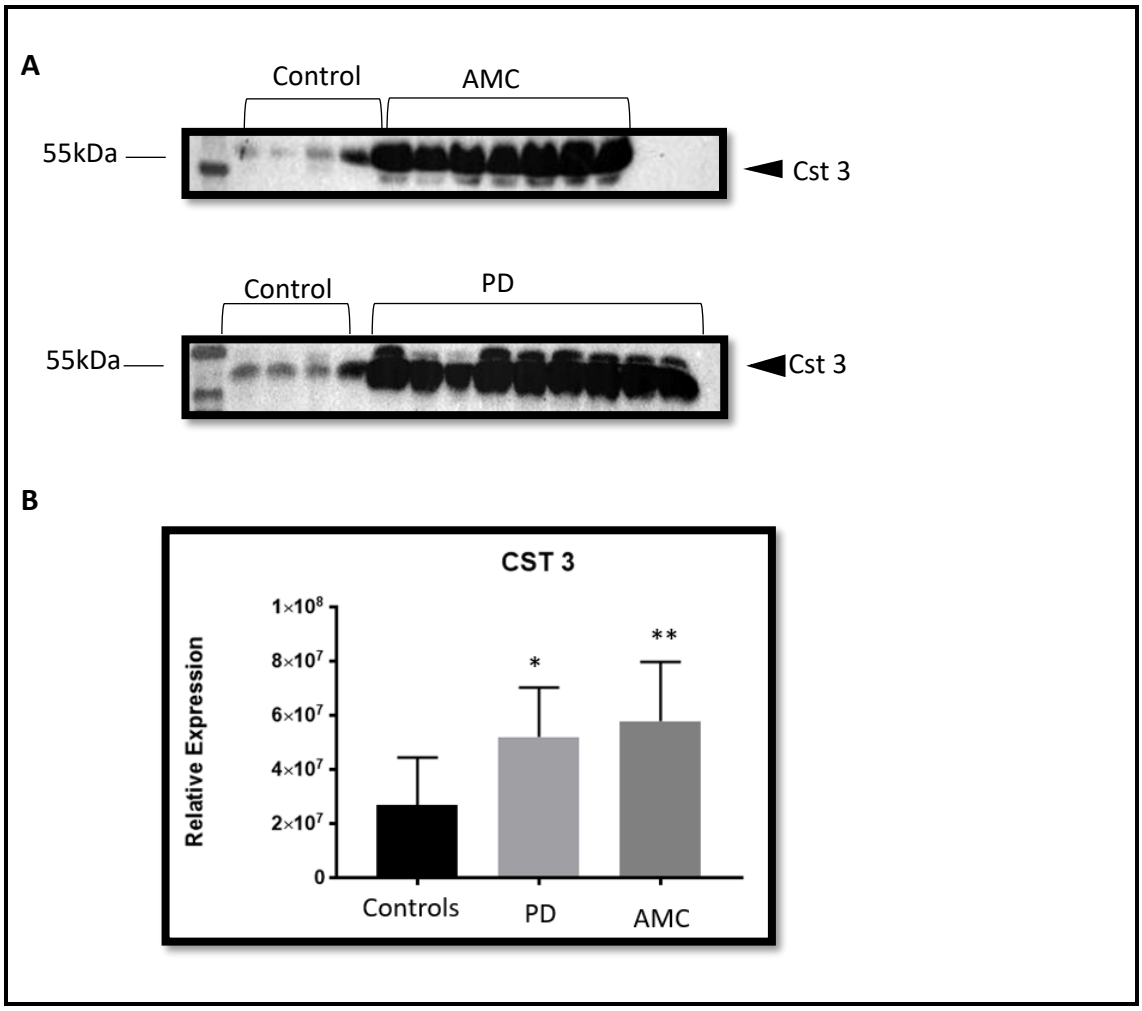


Figure 4: (A) Cst 3 expression on immunoblot of PD and control group serum samples. (B) Cst 3 expression analysed on immunoblotted serum proteins from AMC and control. (C) Quantification of Cst 3 levels using ANOVA test and multiple comparison using Tukey indicated a p value < 0.05 (p= 0.0046) which shows for this number of samples from all three groups the difference in expression levels is significant. The increased expression in patient samples indicates Cst 3 protein has potential to aid in Parkinson's diagnosis.

6.2.2 Expression Analysis of SHBG:

SHBG is a protein that binds to steroids and androgens and manages the concentration of steroids in plasma by regulating metabolic removal of plasma steroids. The blood levels of hormones effect patient's mood, cognition, and quality of life. The

neuroprotective role of oestrogens and lower ratio of PD occurrence in females points towards the important role of sex hormones and their transporters in PD pathology. Following number of samples from each cohort were used: n = 10 PD samples, n = 10 AMC samples, n = 4 controls.

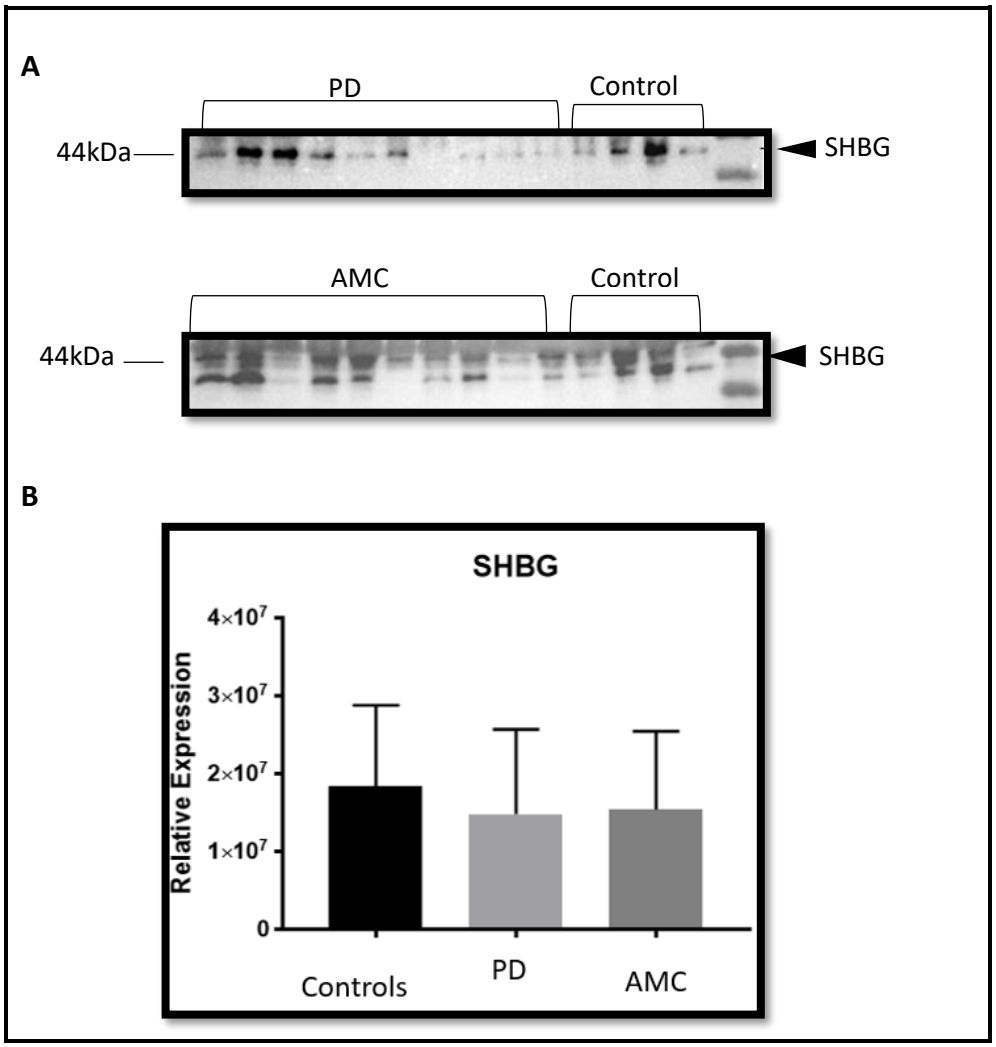


Figure 5: Western blot analysis of serum from clinically diagnosed PD, AMC patients and age-matched healthy controls. (A) Serum samples of PD patients and controls run on gel electrophoresis followed by western blot with SHBG. (B) The vertical bars indicate relative expression of SHBG slightly decreased in PD samples compared to AMC samples and control group. The ANOVA test followed by multiple comparison analysis using Tukey indicated ($p = 0.5886$) $p>0.05$ which means the difference of SHBG expression among three groups is non-significant.

6.2.3 Expression Analysis of Rab 9:

Rab 9 GTPase plays a key role in release of vesicle from late endosome and in formation of mitochondria derived vesicle (MDV). Rab 9 and Rab 7 are known stress markers. Stress markers lie at the core of a good PD biomarker search. For stress is attributed to enhanced and speedy neurodegeneration. Mitochondrial dysfunction is key event in PD pathology. Disproportionate phosphorylation of Rab 9 GTPase (as a stress biomarker) leads to disruption in mitochondrial functioning which causes oxidative stress and finally mitochondrial damage. Rab 9 expression analysis on PD serum samples showed slight but not significant increase in case of PD compared to control and AMC samples. Following number of samples from each cohort were used: n = 10 PD samples, n = 10 AMC samples, n = 4 controls.

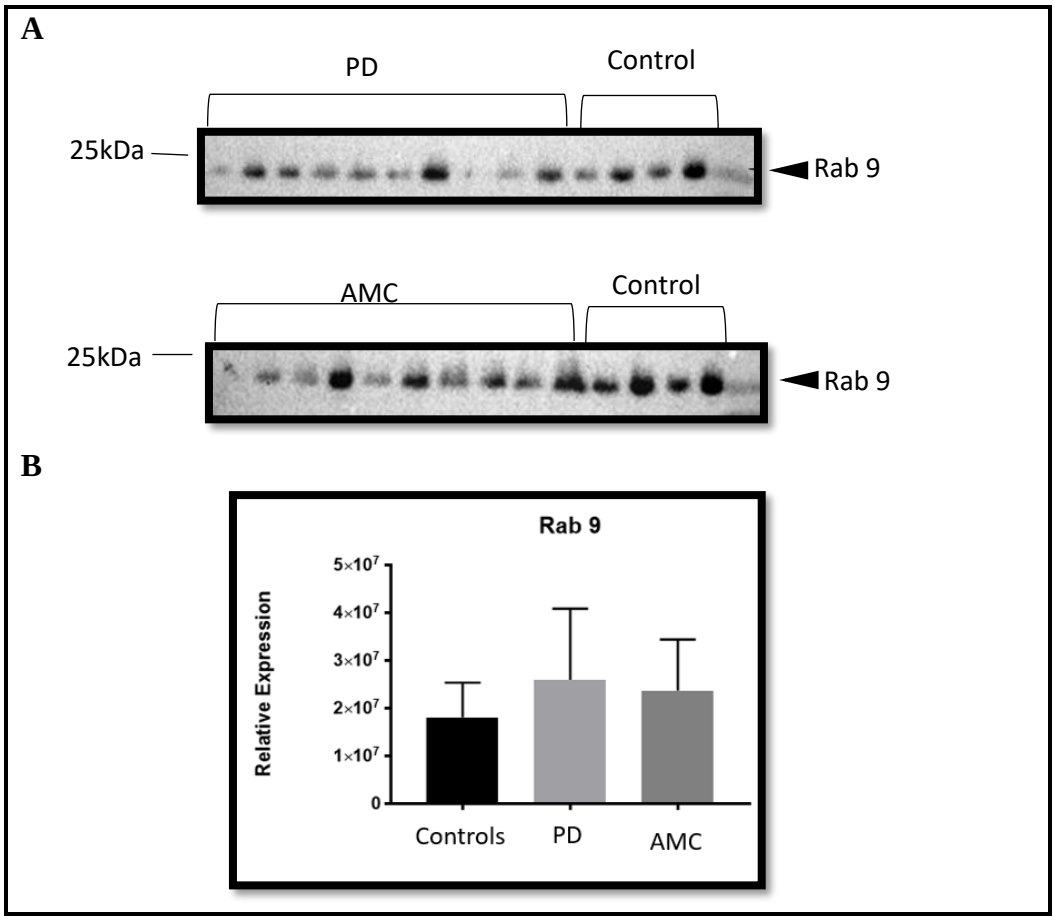


Figure 6: (A) Rab 9 expression on a immunoblot of PD and control group serum samples. (B) Rab 9 expression analysed on immunoblotted serum proteins from AMC and control. (C) Quantification of Rab 9 levels using ANOVA test and multiple comparison using Tukey indicated a p value > 0.05 (p= 0.173) which shows for this number of samples from all three groups the difference in expression levels is insignificant. However, visually it can be seen expression is a bit higher in case of PD which suggest Rab 9 has the potential as a specific biomarker to be studied using further proteomic techniques.

6.2.4 Expression Analysis of Saf 32:

Scrapie associated fibril 32 (Saf 32) is a prion protein. Prion proteins (PrP^c) are located ubiquitously but dominantly in neurons, on the cell membranes. PrP^c a glycosylphosphatidylinositol (GPI) attached protease sensitive protein, plays role in copper/zinc homeostasis, signal transduction and as a receptor. Although, the exact biological roles of PrP^c are yet unknown. (Tamgüney, 2018) The role of copper binding sites in the maintenance of neuritogenesis in PrP^c has been proven crucial. (Nguyen, 2019) Expression evaluation of PrP^c Saf 32 in PD serum samples projected significant rise in the protein's expression in PD patients as compared to healthy control. Emergent research underlines those various neurodegenerative disorders including PD share similarities with prion diseases in the prion like progression. Self-aggregation, templated misfolding of proteins (especially alpha synuclein) and transmission between the cells are key similarities of PD with prion diseases. (Ma, 2019) Following were the number of Samples n = 10 PD samples, n = 10 AMC samples, n = 4 controls

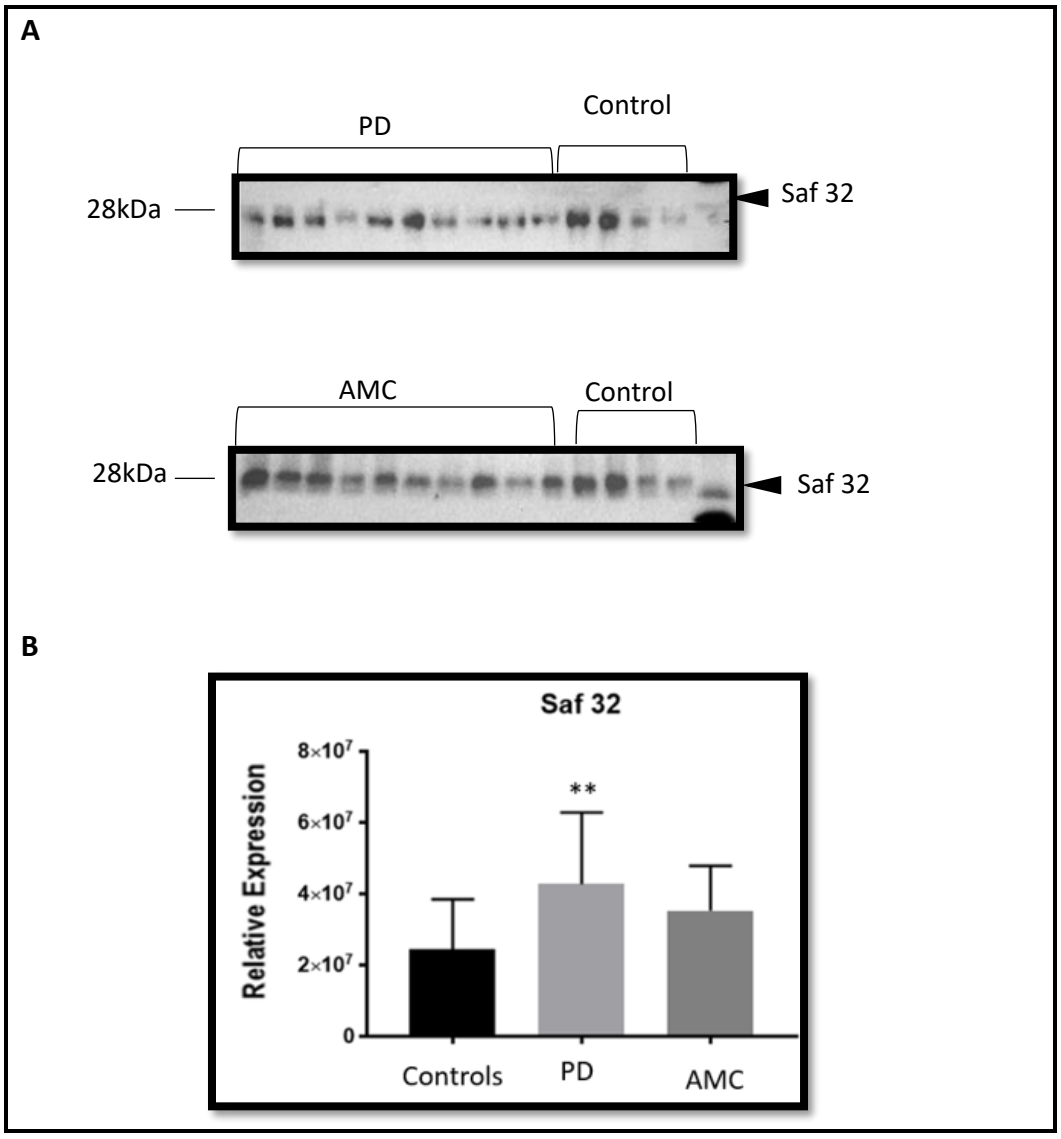


Figure 7: (A) Gel electrophoresis and immunoblotting of proteins from PD samples and controls on a PVD membrane with application of Saf32 antibody. (B) Gel electrophoresis and immunoblotting of proteins from AMC samples and controls on a PVD membrane with application of Saf32 antibody. (C) Relative expression analysis using standard deviation (SD) indicates here an increased levels of Saf 32 in PD patients as compared to AMC and control group ($p = 0.0058$)

6.3 Differential Expression Analysis of αβ synuclein:

Molecular weight and charge wise separation of αβ synuclein on a PD sample blotted membrane showed expression at the desired band size and pH. 2D gel examination

clearly differentiated the expression analysis of same protein on healthy individual's and patient's sample. This contributes to the vast amount of research currently going on about the role of synucleins in the pathological events of PD.

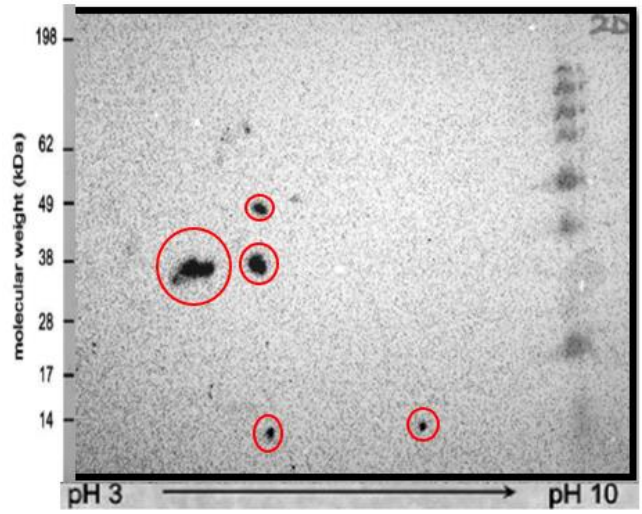


Figure 8: 2 DGE of PD patient serum sample followed by western blot and application of a, B synuclein. Monomeric forms of alpha synuclein observed around pH 5 and 8 and dimers of alpha synuclein and beta synuclein observed above 38kDa.

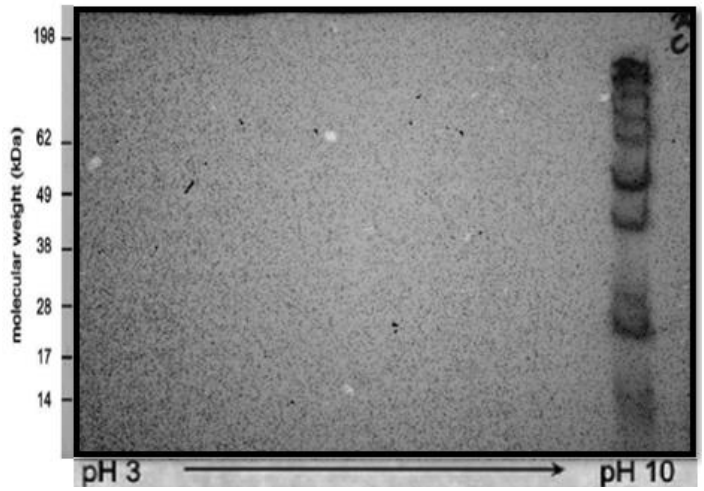


Figure 9: 2 DGE of control serum sample followed by western blot and application of α, β synuclein. On an age-matched healthy individual's sample the α, β synuclein protein expression was not detectable. Alpha-synuclein ever since its relationship with PD has

been discovered it remains an important protein related to PD pathology. This opens new prospects for further expression quantification and comparison of healthy individual's samples to the patient's sample.

6.4 Viability Analysis using SH cell line:

SY5Y cells cultures were exposed to diseased serum proteins and that of controls for 3 hours, 6 hours and 9 hours' time intervals. The viability of cells under the serum proteins was analysed using MTS assay and following results were obtained:

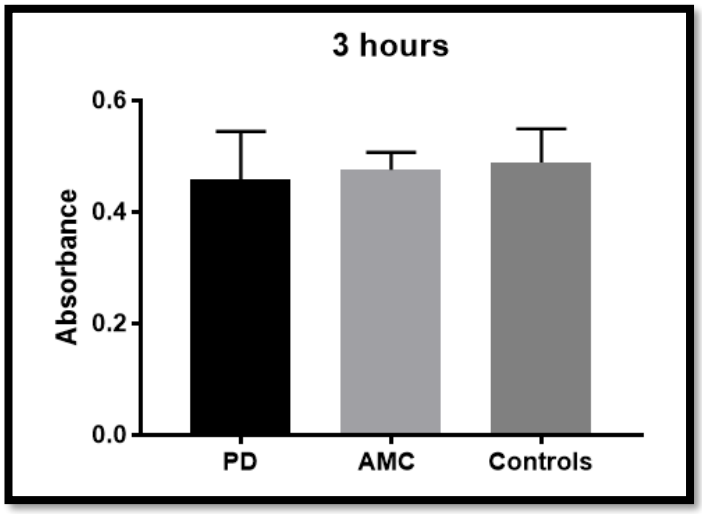


Figure 10: MTS assay value taken after 3 hours. p value: 0.7600 non-significant difference observed between PD, AMC, and controls.

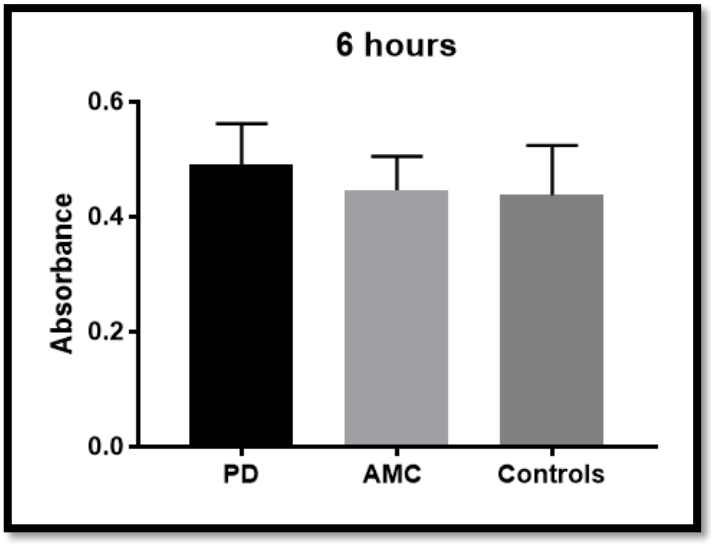


Figure 11: MTS assay value taken after 3 hours. p value: 0.4467 non-significant difference observed between PD, AMC, and controls.

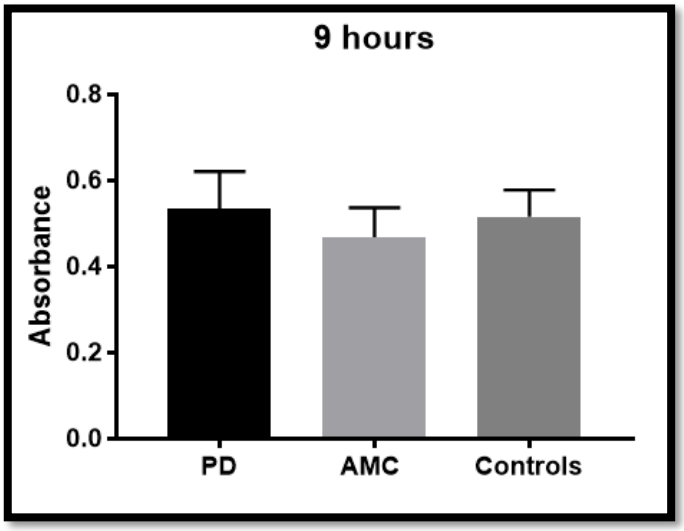


Figure 12: MTS assay value taken after 3 hours. p value: 0.3228 non-significant difference observed between PD, AMC, and controls.

6.5 Expression Analysis through Dot Blot:

Dot blot is conducted on nitrocellulose membrane. To detect the quantity of a target protein in the sample dot blot is a less time-consuming approach. Dot blot is quicker and less expensive than Elisa, western blot, and other expression analysis approaches.

6.5.1 Expression Analysis of ABCF1:

ABCF 1 could not be detected appropriately in western blot, so its dot blot was performed with greater protein concentrations. In this case analysis of ABCF1 antibody affinity required larger protein concentration of sample to be available.

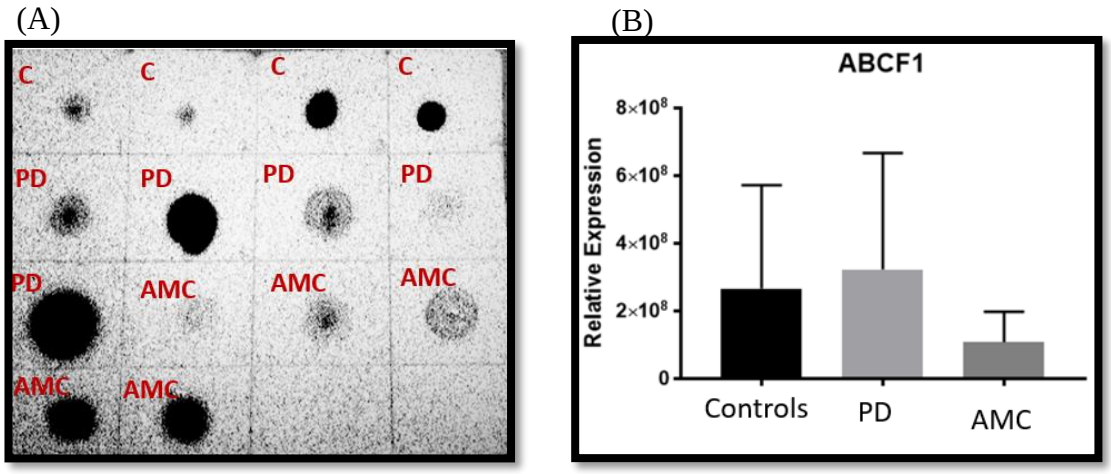
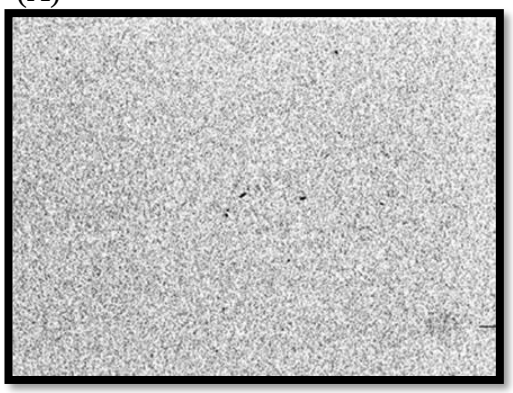


Figure 13: (A) ATP-binding cassette sub-family F member 1 (ABCF 1). Dot blot analysis shows expression of ABCF 1 in all the samples. (B) Quantification and comparison using ANOVA test detected a p value of 0.458 which means non-significant expression difference between three samples. However, expression levels in PD are closer to controls and minimum in AMC.

6.5.2 Expression Analysis of Robo 1 and Slit 2:

Robo 1 and Slit 2 are reported to be uniquely expressed in PD patients according to comparative proteomics. These two proteins interact with each other and play role in neuronal communication. In this research study attempts were made to quantify these proteins' expression in serum sample through western blot and dot blot however, expression could not be detected.

(A)



(B)

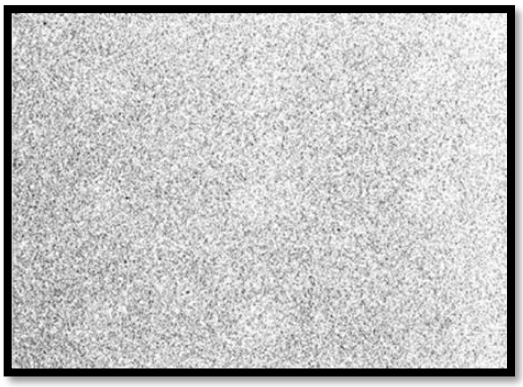


Figure 14: (A) Dot blot of Robo 1 conducted on 8 PD samples (6ul, 600ug protein) and 1 control used from each sample showed no expression for the protein. (B) Dot blot of slit 2 performed using nitrocellulose membrane and 8 PD samples (6ul, 600ug protein) and 1 control revealed no expression.

7. Discussion:

Parkinson's is a well-established neuronal proteinopathy, abnormal accumulation of certain proteins is observed in patient's central nervous system and peripheral body fluids. PD is clinically diagnosed through cardinal symptoms like walking difficulty, muscle rigidity, tremors, drooping posture, and autonomic symptoms, however by the time clinical features of the disease begin a major chunk of dopaminergic neurons is already lost. Hence for differential diagnosis, prognosis, and therapy of PD we need early, specific, sensitive, and cost-effective peripheral and/or central biomarker(s).

Preclinical biomarkers are used for PD diagnosis however determination of biomarkers specific and sensitive to PD is required. Neuroimaging biomarkers (APECT, MRI, PET) are being used for PD differential diagnosis but are expensive. Other promising biomarkers of PD reported in literature are neuromelanin antibodies, bio-fluid markers, pathological forms of a-Synuclein, Amyloid Beta, and tau in csf. (Sharma, 2013) Researchers are interested in finding a way in which genetic as well as non-genetic biomarkers of PD can be used for personalized treatment of PD during preclinical and clinical stages of PD. Therefore, protein profiling in the csf and in the blood are of great interest for PD researchers to identify central and peripheral biomarkers specific for PD.

In this study protein aggregations, protein interactions, protein regulations have been analysed in serum samples preserved at -80^{o}C. Parkinson's is a recognized neuronal proteino-pathy and alpha-synuclein is the well-known protein related to PD pathology. Results of Elisa showed enhanced serum α-synuclein levels in PD patients compared to healthy individuals and other disorders. (Shi, 2011)and other research studies have revealed that a decline in a-Syn levels. In literature there is a debate regarding alpha-synuclein upregulation or downregulation, and mixed findings on the subject exist. Elisa

results in this study indicate that a-synuclein levels are greater in serum samples of PD patients as compared to healthy controls.

In a search for peripheral protein biomarkers specific to PD it was observed that cystatin c (Cst 3), sex hormone binding globulin (SHBG), Rab 9 and Saf 32 expression levels in PD patients varied from that in normal controls. Cst 3 was upregulated with a significant p-value: 0.0046 in PD and AMC samples (even more expression in AMC than PD). Cst3 is a protein of great significance in neurodegenerative disorders. Interestingly Cystatin C is stated to be an internal protease inhibitor protein with significant neuroprotective roles in diseases such as AD, ALS and SAH. (Liu, 2014). Cystatin C potential as a biomarker for neurodegenerative disorders has been studied and it was found that this protein can be good histopathological marker of ALS. However, protein profiling of blood from ALS patients showed Cst3 levels did not differ much in ALS patients from healthy controls. This indicates need for careful comparative analysis of each protein in multiple biofluids to find an accurate biomarker specific to relevant disease.

SHBG appeared downregulated in PD patients as compared to controls and AMC samples although the difference was not significant with p-value: 0.5886. It is suggested in literature decreased levels of sex steroids increase susceptibility to PD. The blood levels of hormones effect patient's mood, cognition, and quality of life. The neuroprotective role of oestrogens and lower ratio of PD occurrence in females points towards the important role of sex hormones and their transporters in PD pathology. Nonetheless these findings suggest more research needs to be done on the role of SHBG in PD pathology and prognosis analysis. Maybe with increased sample size significant downregulation can be observed and thus this biomarker has the potential to be a specific and sensitive PD progression biomarker.

Rab 9 is a member of Rab GTPases protein family which plays the role of carriers between organelles. In this study western blot analysis of PD, AMC, controls serum samples indicated a relatively increased expression of Rab9 in PD patients compared to controls and AMC. Although in this number of samples p-value is 0.1773, maybe upon increased number of samples significant conclusion can be devised. The cytoplasmic protein Rab 9 has a role to play in mitochondrial antigen presentation (MitAP) and lipopolysaccharide induced mitochondrial derived vesicle formation (MDV). Mutation in Parkin and PINK1 gene along with increased phosphorylation by Rab Gtpases is reported to have role in PD onset. The autoimmune mechanisms involved in PD are being studied extensively where role of these proteins is critical in understanding mitochondrial dynamics associated with autoimmune mechanisms. (Kucera, 2016)

PrP saf32 expression via immunoblotting presented a p-value of 0.0058 indicating higher expression of PrP saf 32 protein in PD samples as compared to AMC and controls. Prion like properties of PD is now at the core of PD related research projects. It is mentioned in different studies that upon grafting of affected tissue in mice PD onset and spread began in the animal model. Apart from this accumulation and spreading pattern of a-synuclein resembles very much with the patterns of prion proteins. In PD research study of a-synuclein interaction with various other proteins is of great significance. An insight of the protein-protein interaction has established the diagnostic markers for neurodegenerative disorders including PD. More research on role of prion proteins and prion like mechanisms of PD can be beneficial for specific and sensitive biomarker discovery.

Other proteins like 14-3-3 eta, which is an established biomarker for rheumatoid arthritis (RA) for its role in joint damage (Kadavath, 2014), was analysed in PD serum samples. The Elisa results suggested in-significant expression difference for 14-3-3 eta in

PD, AMC, and controls with a p-value: 0.5108. 14-3-3 proteins have role in various brain functions such as neural signalling, neuroprotection, and development of neurons thus it is an important protein to study in neurological disorders. 14-3-3 proteins consist of seven isoforms and present ubiquitously with the highest expression in brain and comprises to almost 1% of total soluble proteins in brain. 14-3-3 protein shares enough sequence homology with α-synuclein and likely to have fair amount of interaction with the protein. (Shirakashi, 2006) Tyrosine hydroxylase enzyme (TH) controls the synthesis rate of dopamine and 14-3-3zeta influences functioning of this enzyme as an activator and binding affiliate of the enzyme. It is notable that α-syn not only binds to 14-3-3 but also mitigates the activity of TH enzyme. Which means a complex interaction of these three proteins has a role in dopamine depletion in PD patients. (Wang J. L., 2009) This indicates that a crux of knowledge from past and present studies reveal the significant role 14-3-3 has to offer in PD pathogenesis. More and advanced research is due on this protein's role in PD to find out better diagnostic and therapeutic approaches for PD.

Amyloid Beta depositions are associated with cognitive decline in PD. Topical studies illustrate that CSF Aβ is connected to the postural instability and gait difficulties (PIGD) or the recently proposed cholinergic subtype of PD, a conceivable risk factor for cognitive deterioration in PD. (Lim, 2019) AB A 4 Elisa of PD, AMC, and control serum samples indicated p value: 0.1869 however levels of AB A4 visibly appear quite higher in PD as compared to AMC and controls. An attempt to determine peripheral value of Aβ proteins in PD diagnosis has been made in this study. The results though insignificant for the allotted number of samples still points at the prospect of this protein in PD diagnosis.

Protein profiling of serum samples can establish good diagnostic and prognostic methods for Parkinson's disease. The successful discovery of sensitive and specific biomarkers by a common and less expensive laboratory protein expression technique like

western blot can be a breakthrough in the diagnosis of Parkinson's disease. There remains lot of room for research and an ample scope for the discovery of early, sensitive, specific, non-invasive, economical diagnostic biomarker(s) for the treatment and diagnosis of PD. This research study considerably contributes to the proteomics and biomarker discovery in PD and lays a solid foundation for future research in this field.

7.1 Conclusion

Alpha-synuclein is the key functional protein responsible for PD onset. The genetic mutations or environmental factors leading to misfolding of α-synuclein are the reasons for dopaminergic neuronal loss in Substantia Nigra of brain. The findings of this study indicated visible trend of alpha synuclein overexpression in PD compared to normal controls. 14-3-3 is another significant protein known for its role in neurodegenerative disorders and for its interactive functioning with alpha synuclein. Observation that 14-3-3 accumulates are highly expressed in PD samples compared to healthy controls is a signature finding of this study. Observation of PrP saf-32 as significantly highly expressed in PD compared to healthy controls is a novel finding of this research study. Significantly increased expression of Cst-3 in blood samples suggests that the normal function, to degrade proteins, of cysteine protease enzyme is disrupted by excessive presence of its inhibitor Cst-3 protein. This leads to abnormal accumulation of misfolded proteins in PD pathology. Rab-9 levels were moderately increased however for this data number of samples results were not significant. SHBG levels were relatively lowered, though not statistically significant, in PD samples compared to healthy controls. The proteins α-synuclein, 14-3-3, amyloid beta A-4, Saf 32, Cst3, SHBG, ABCF1, Rab9 can be potential peripheral biomarkers for PD diagnosis but require further thorough investigation determining each one's potential. This study concluded that Cst3 and Saf 32 expression is significantly greater in PD patient samples compared to healthy controls.

α-synuclein and 14-3-3 expression showed visible trend of increase. Identification and characterization of these potential biomarkers has been successfully conducted in this study and the findings contribute significantly to the ongoing PD diagnosis related research.

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