COMPUTATIONAL INVESTIGATIONS AND EXPERIMENTAL VALIDATION OF ALZHEIMER'S DISEASE RELATED PATHWAYS WITH SPECIAL RELEVANCE TO DNA REPAIR AND AUTOPHAGY

Dissertation submitted in partial fulfilment of the requirement for the degree of

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IN

BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitle "**Computational investigations and experimental validation of Alzheimer's Disease related pathways with special relevance to DNA repair and autophagy"** submitted at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is an authentic record of my work carried out under the supervision of Dr. Tiratha Raj Singh (Supervisor) and Dr. Udaybanu M (Co-supervisor), Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation entitled "**Computational investigations and experimental validation of Alzheimer's Disease related pathways with special relevance to DNA repair and autophagy**" submitted by Tiyasha De (207815) at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is bonafide record of her original work and has not been submitted elsewhere for any other degree or diploma.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AD- Alzheimer's disease

Aβ- Amyloid beta

AMBRA1- Activated molecule in BECN1 regulated autophagy

APP- Amyloid precursor protein

APP-CFTs- Amyloid precursor protein-cleaved C- terminal fragment

APOE- Apolipoprotein E

ATG- Autophagy-related genes

AV- Autophagic vacuole

BACE1- β-site amyloid precursor protein cleaving enzyme 1

BER- Base excision repair

BECN1- Beclin-1

CDK5- Cyclin dependent kinase 5

CdS- Cadmium sulphide

CT- computerized tomography

CMA- Chaperone-mediated autophagy

CNS- Central nervous system

DDR- DNA damage and response

DMEM- Dulbecco's Modified Eagle's Media

DMSO- Dimethyl sulphoxide

DNA- Deoxyribonucleic acid

DNs- Dystrophic neuritis

DNA-PK- DNA-protein kinase

ESCRT- Endosomal sorting complexes for transport

EOAD- Early-onset autosomal dominant Alzheimer disease

FBS- Fetal Bovine Serum

Hcy- Homocysteine

HR- Homologous recombination

HSPA8- Heat shock proteins

HDAC1- Histone deacetylases

ICL- Inter-strand crosslink

KEGG- Kyoto Encyclopedia of Gene and Genomes

LAMP2A- lysosomal-associated membrane protein 2

LOAD- Late-onset Alzheimer's disease

MAPT- Microtubule Associated Protein Tau

MHB- Muller Hinton Broth

MMR- Mismatch repair

MRI- Magnetic resonance imaging

mTORC1- Mammalian target of rapamycin complex 1

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MRE1- meiotic recombination 11

mtDNA- mitochondrial DNA

mg- Milligram

ml- Millilitre

min- Minutes

NBS1- Nijmegen breakage syndrome 1

NER- Nucleotide excision repair

nDNA- Nuclear DNA

NFT- Neurofibrillary tangles

Nrf2- Nuclear factor erythroid-2 related factor-2

N2A- Neuro 2A

NHEJ- Non-homologous end joining

NPI- Neuropsychiatric inventory

NP- Nanoparticles

PANTHER- Protein Analysis Through Evolutionary Relationship

PBS- Phosphate saline Buffer

PSEN1- Presenilin-1

PSEN2- Presenilin-2

Pen Strep- Penicillin Streptomycin

PARP-1- Poly ADP-ribose polymerase-1

PET- Positron emission tomography

ROS- Reactive oxygen species

SBGN- System biology graphical notation

SBML- System biology Markup language

UVRAG- UV radiation resistance associated gene

ULK1- Unc-51 like autophagy activating kinase

UPS- Ubiquitin-proteosome system

VMP1- Vacuole membrane protein 1

8-OHG- 8-hydroxyguanine

5-OHC- 5-hydroxycytosine

8-OHA- 8-hydroxyadenine

5-0HU- 5-hydroxyuracil

°C- Degree centigrade

µg- Microgram

µL- Microlitre

µmol- Micromole

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Alzheimer's disease (AD), also known as senile dementia, is a neurodegenerative disease and the most common form of chronic dementia that shows progressive loss of memory, synaptic function, cognitive capacity and atrophy in different brain areas in the elderly. Epidemiological studies highlighted AD as a multifactorial disease i.e.; it occurs due to complex interactions between various intrinsic and extrinsic factors. The pathology of AD involves a combination of intrinsic genomic susceptibility and environmental factors. It entails a continual dynamic interplay between dysfunctional pathways and central homeostatic networks of nerve cells. The neuropathological changes of AD include Aβ plaques and neurofibrillary tangles (NFT). Innumerable evidences suggest that an autophagy-or DNA damage and repair (DDR) pathway-dysfunction further promote Aβ plaques and NFT.

Employing a systems biology approach to integrate protein alterations provides link between multiple molecular abnormalities leading to memory impairment revealing a broadly dispersed and multi-molecular targeting pathogenic process. Both DDR pathway and autophagy process hold constitutive role in AD. We have retrieved several pathways related to DNA damage, DNA repair and autophagy from the STRING, KEGG and other databases. All this information was compiled carefully to implement further analysis. We have created a Protein-protein interaction (PPI) network and then that network has been analyzed using Cytoscape to explore the role of these integrations. Further, a virtual electronic cell (E-cell) was built to study the interaction of these proteins and analyzed using Cytoscape. Topological features of the network analysis were used to interpret the model under study and determine hub proteins. Through in-vitro experimentation, the model was further validated by studying the role of the hub proteins on Neuro 2A cell line.

Through this analysis, we have identified important proteins that have key role in the regulation of all the three aspects i.e., autophagy, DDR and AD. This study will be further explored to to determine potential therapeutic targets or biomarkers for the AD.

Keywords: Alzheimer's disease, Systems Biology, DNA repair, Autophagy, Neuro 2A cells, Comet assay.

CHAPTER 1: INTRODUCTION

According to World Health Organisation (WHO) in 2021, roughly 55 million people worldwide are suffering from dementia with the addition of 10 million new cases each year. WHO claims that by 2030 and 2050, dementia is estimated to affect 78 million and 139 million people respectively worldwide. Dementia is a syndrome characterised by decline in cognitive ability of an individual beyond the usual expected consequences of biological ageing. Alzheimer's disease is one of the most common forms of dementia seen in the elderly.

1.1 ALZHEIMER'S DISEASE

In 1907, Dr. Alois Alzheimer studied the case of a 51 year old female patient Auguste D suffering from a relatively rapidly deteriorating memory along with other psychiatric disturbances [1]. This condition was later known as Alzheimer's disease (AD). AD is the most common form of dementia seen in patients over the age of 45 years. AD is a multifactorial neurodegenerative disease i.e., its aetiology is hallmarked by various intrinsic and extrinsic factors and their associated proteins [2].

Clinical features of AD [3]: -

- Impaired cognitive function, decision making & memory
- Behavioural disturbances
- Apraxia
- Visuo-spatial impairment
- Aphasia

These symptoms affect individuals severely hampering their daily functioning.

AD is broadly classified into two types [4]: -

1. Familial early onset autosomal dominant (EOAD) when it occurs in patients below 65 years age. This majorly occurs due to genetic abnormalities in chromosome 1, 14 and 21. Mutations in genes encoding amyloid precursor protein (APP), presenilin 1 (PSEN1), presenilin 2 (PSEN2) or apolipoprotein ε (APOE) leads to this AD type.

2. Non- familial late onset sporadic form (LOAD) which occurs mostly to patients over 65 years age. This polygenic condition prevails in 90-95% of the AD cases. Several factors such as aging, gender, lifestyle, environment and inheritance of one of the alleles of apolipoprotein ε (ε4) play a crucial role in this AD type.

Neuropathological changes involved in AD: -

Amyloid beta (Aβ) plaques: Unregulated production of Aβ peptides due to irregular cleavage of its precursor protein, APP, leads to the formation of neurotoxic amyloid plaques in the intraneuronal regions of the brain. These plaques render various detrimental effects on neuronal metabolism causing synaptic dysfunction and loss and imperilled cerebral blood flow. AD brains are marked with increased levels of Aβ42 peptide causing inflammatory responses and irreversible neuronal damage to the brain [5], [6].

Neurofibrillary tangles (NFT): These filamentous lesions develop due to the intraneuronal aggregation of hyperphosphorylated tau proteins. Microtubule-associated protein tau (MAPT) gene, encoding tau protein, undergoes alternative splicing to produce tau isoforms. Tau facilitates the assembly and stabilization of microtubules, which act as essential scaffolds for the cytoskeleton of the cell and promote vascular-mediated transport. Abnormal phosphorylation and truncation of tau play a major role in tauopathies [6].

1.2 AUTOPHAGY

In 1963, the term "autophagy" was coined by Christian de Duve from a Greek word meaning "self-eating". Autophagy is a highly catabolic process characterised by lysosomal degradation of toxic cytoplasmic content both endogenous and exogenous freely accessible in the cytoplasm [7]. In this process, cells self-digest their unnecessary cellular components to obtain nutrients for essential cellular functions. Autophagy is often triggered by various stimulations such as nutrient deprivation (fasting), reactive oxygen species, protein aggregation, subcellular damages and hypoxia impairment. This process is highly regulated process balanced by an autophagic flux i.e., the rate at which autophagic machinery identifies, segregates and brings in lysosomal degradation of its substrate [8]. Autophagy is broadly categorised into three types: macroautophagy, microautophagy and chaperone-mediated autophagy.

Macroautophagy	Microautophagy	Chaperone-mediated autophagy
•Autophagosomes act along with lysosome to sequester large molecules or entire organelle. •Autophagosome (double membraned vesicle) which fuses with the lysosome to form the autolysosome for lysosomal degradation. •Further subtyped on the basis of their dependence on specific proteins. •It leads to cell death/cellular atrophy & neurodegenerative disorders	•Target proteins directly taken up by lysosomes or vacuoles by membrane invagination. •It is independent of a specific splicing variant, LAMP2. •Drosophila and mammals endosomal microautophagy and relies on ESCRT system. •Yeast displays micropexophagy which involves degradation of peroxisomes	•Involves direct delivery of target cytoplasmic proteins to lysosome for degradation. •Can't degrade organelles. •Cytosolic chaperone (Hsc70) recognises proteins bearing KFERQ motifs. •Carries them to the lysosomal membrane. •It is then taken into the lysosomal lumen using LAMP-2A

Table 1: Comparative description of different types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy [8]

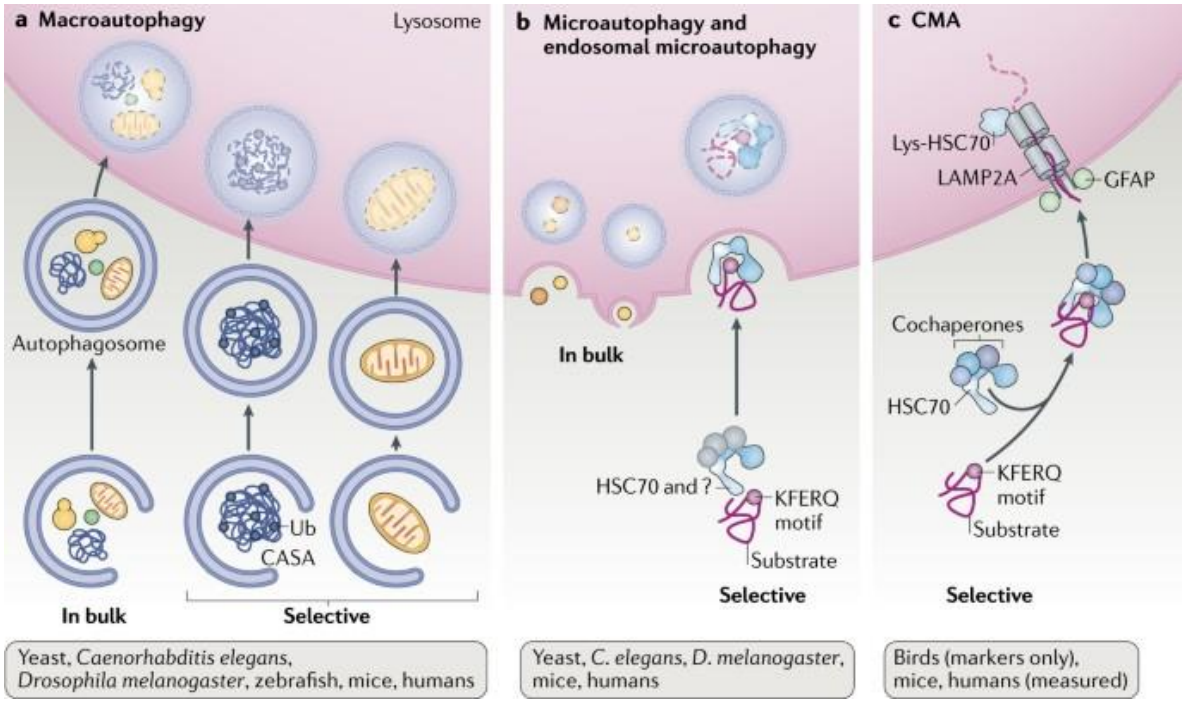

Figure 1: Descriptive representation of different types of autophagy: macroautophagy, microautophagy and chaperone mediated autophagy [9].

Physiological functions of autophagy include [8]:

- Helps survive metabolic stress
- Maintains cellular homeostasis
- Considered as a non-apoptotic programmed cell death
- Might be guardian of the genome

1.2.1 ROLE OF AUTOPHAGY IN AD

Autophagy is an essential homeostatic process in post mitotic cells like neurons to eliminate accumulated intracellular toxicants and damaged organelles. As mentioned earlier, AD patient's brain is marked by the presence of Aβ plaques and NFT. Autophagy is an Aβ clearance pathway that degrades and removes both Aβ peptides as well its precursor protein, APP [10]. Autophagy is also considered an effective tau degradation route and is involved in Tau turnover. Dysfunction of autophagy-lysosome degradation system leads to accumulation of hyperphosphorylated tau. On the other hand, tau is responsible for the assembly and stabilization of microtubules for the retrograde transport of autophagosomes along the axon. As the autophagosome moves along the axon, they bring in degradation of misfolded proteins and damaged organelles along with lysosome. However, in AD patients, hyperphosphorylated tau leads to the disassembly of microtubules causing impaired axonal transfer and accumulation of immature autophagic vacuoles in the axon tips [11]. Hence, autophagy shares a bidirectional role with AD.

1.3 DNA DAMAGE AND REPAIR

Over the years, we have realised the importance of preserving genomic sequence information for the perpetuation of life. Innumerable DNA damaging agents impact human health and wellness, and regulate disease states. DNA is an inherently reactive molecule and is particularly sensitive to chemical alterations via exogenous and endogenous factors. Hence, DNA damage is broadly classified into two major types on the basis of their origin: exogenous and endogenous. Endogenous factors include intracellular reactive oxygen species and hydrolytic reaction of DNA with water. On the other hand, exogenous factors include various physical, chemical and environmental agents such as UV and ionizing radiations, crosslinking and alkylating agents etc [12].

However, to combat such challenges, cells are well equipped with elaborate DNA repair mechanisms contributing towards the healthy functioning of the cell. Cells have various

arrangements to deal with DNA damage including DNA damage tolerance, cell cycle checkpoints, DNA repair and cell death pathway. DNA repair and damage tolerance mechanisms warrant the overall survival of the cell [13]. The five major DNA repair pathways that actively participate through different cell cycle stages to repair the DNA damage include base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ). Any disturbances in these pathways lead to an increase in mutation causing genomic instability and paving way for various lethal diseases [12].

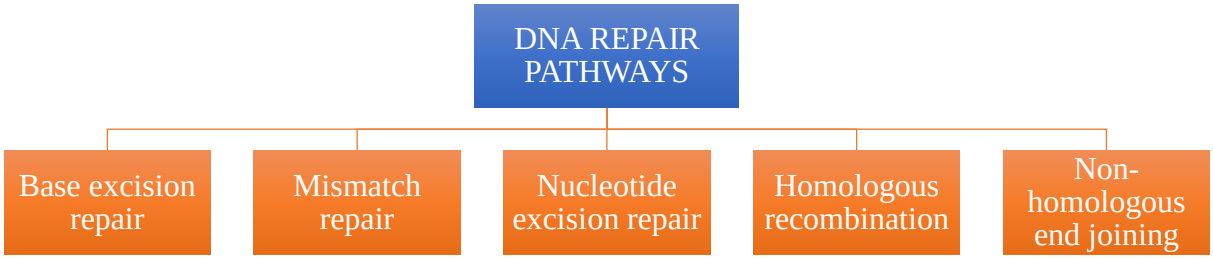

Figure 2: Various DNA repair pathways

1.3.1 DNA DAMAGE IN AD

Increased levels of oxidative DNA damage in the form of ROS have detrimental effect on neurons. Several regions of AD brain show accumulation of various oxidized base adducts, particularly 8-hydroxyguanine, due to oxidative attack of ROS. Neuronal DNA damage can be of two types: nuclear DNA (nDNA) damage and mitochondrial DNA (mtDNA) damage [14].

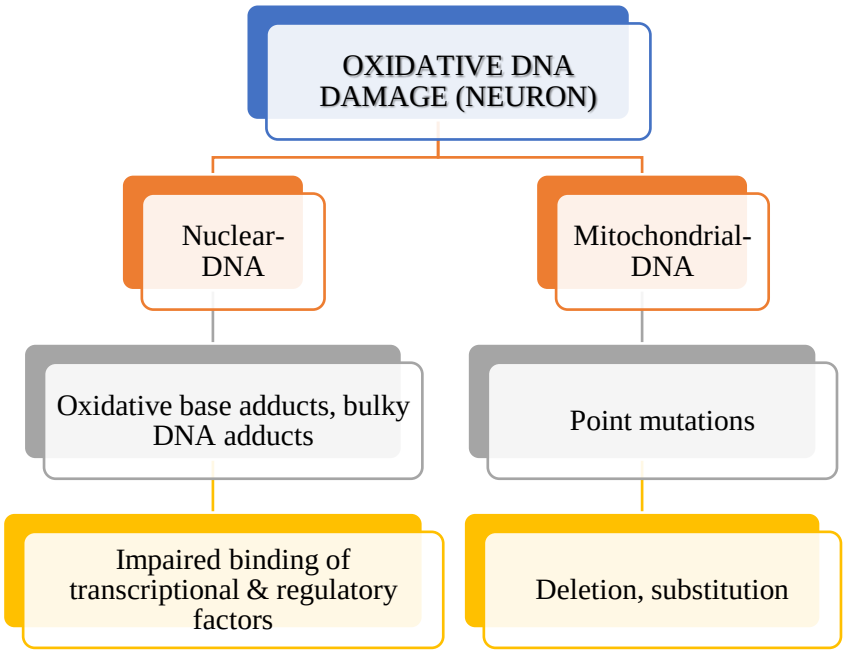

Figure 3: Representation of oxidative DNA damage in neurons.

Evidences from AD brain suggests a 10-fold elevation in the levels of oxidised bases in mtDNA as compared to nDNA suggesting increased oxidative stress in mitochondria. Such increased levels of 8-OHdG in the aging and AD brain supports the hypothesis that oxidative stress holds a crucial role in the high incidence of AD in old age subjects [15]. DNA lesions, both single strand breaks and double strand breaks, accumulate in AD brains. Comet assay is usually preferred to detect primary DNA damages (single strand breaks, alkali-labile sites, oxidative DNA adducts etc). Low folic acid intake and elevated homocysteine (Hcy) levels lead to the accumulation of DNA damage and Aβ toxicity increasing the risk of AD. Aβ42 peptide has demonstrated DNA nicking activities causing apoptotic death of neurons in the hippocampus. Other DNA adducts such as aldehydic DNA adducts, occurring due to peroxidation of membrane lipids by ROS and acrolein/guanosine adducts are also seen in the brain of AD patients [14].

1.3.2 DNA REPAIR IN AD

Adult human neurons are capable of re-entering the cell division cycle. However, in a normal human brain, the cell cycle undergoes cell cycle arrest in the G1 phase, and the cells re-differentiates into neurons. Meanwhile, in AD patients, the cell cycle progresses up to the G2 phase. At this stage, cell cycle arrest doesn't allow re-differentiation, and the neuron suffers one of the two fates: neuronal death via apoptosis or production of Alzheimer-type pathology [16]. Various DNA repair proteins and complexes are found to play a crucial role in AD etiology. One such protein is Poly ADP-ribose polymerase-1 (PARP-1) that is activated against single- and double-strand breaks. PARP-1 acts as a DNA damage sensor. However, extensive DNA damage in cases like AD activates an aggressive PARP-1 activity causing a depletion of NAD+ and eventually ATP, resulting in mitochondrial failure and cellular death due to energy deficiency [17]. Neurons handle double strand breaks with NHEJ mechanism which essentially involved DNA-protein kinase (DNA-PK) for its efficient activity. However, AD brains demonstrate decreased levels of DNA-PK enzyme and associated NHEJ activity establishing its direct link with the pathogenesis of AD [18].

1.4 INTERRELATION BETWEEN AUTOPHAGY, DNA REPAIR AND AD

Both autophagy and DDR play a key role in various neurodegenerative diseases including AD, however, their association hasn't been well established so far. They are correlated in various, non-exclusive pathways.

1.4.1 AUTOPHAGY DYSFUNCTION HINDERING DNA REPAIR IN AD

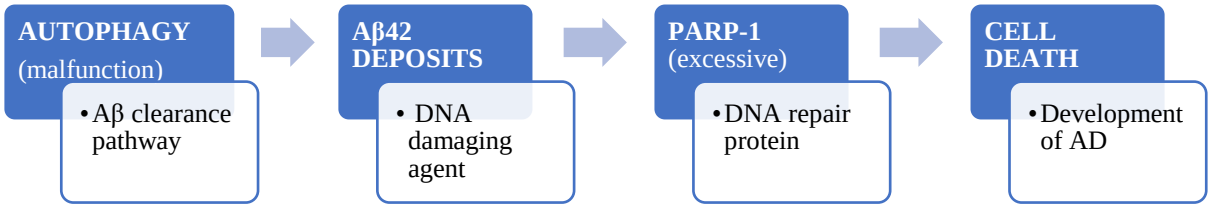

Figure 4: Autophagy is an Aβ clearance pathway and an autophagy malfunction leads to accumulation of Aβ42 peptide which is known to demonstrate DNA damaging activity. DNA damage leads to an excessive PARP-1 activity causing neuronal death due to energy depletion.

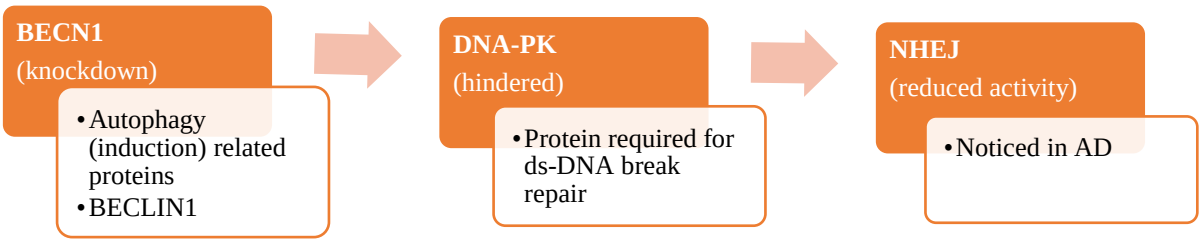

Figure 5: BECN1 gene, encoding for Beclin 1 (an autophagy induction protein), knockdown mice demonstrated hindered DNA-PK complex formation which is essential for efficient double strand break repairs through NHEJ.

1.4.2 DNA REPAIR DYSFUNCTION HINDERING AUTOPHAGY IN AD

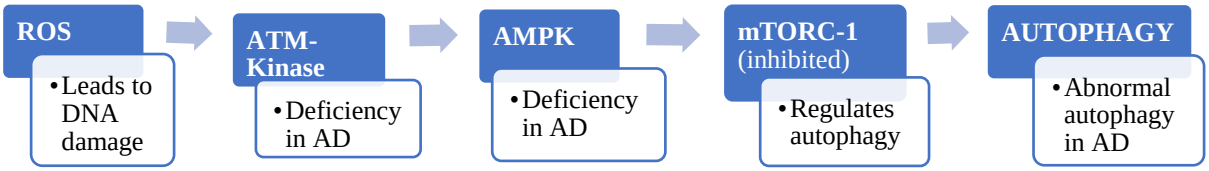

Figure 6: ROS lead to an excessive DNA damage triggering a cascade of reactions inhibiting mTORC1, a key regulatory protein complex of autophagy, leading to abnormal autophagy in AD.

Figure 7: mTORC1 phosphorylates UVRAG essential for binding DNA-PK in NHEJ and HR repair mechanisms and autophagy regulation. Phosphorylation of UVRAG hinders its activity.

CHAPTER 2: REVIEW OF LITERATURE

2.1 ALZHEIMER'S DISEASE

AD, also known as senile dementia, is a neurodegenerative disease and the most common form of chronic dementia that shows progressive memory decline, synaptic dysfunction, cognitive incapacity, and atrophy in different sections of the brain in the elderly. Epidemiological studies highlighted AD as a multifactorial disease i.e.; it occurs due to complex interactions between various intrinsic and extrinsic factors [19].

The common clinical symptoms include impaired cognitive function and decision making, gradual loss of memory, and behavioural disturbances. It is severe enough to hamper the daily functioning of the individual. No geographical or racial preference has been noticed for AD so far. However, it has been noticed that the incidence of the disease is higher in women as compared to men, especially among individuals over the age of 80 years old. Differences in brain morphology, higher cognitive reserve in men, and post-menopausal hormones in women were responsible for these gender-specific differences in the occurrence of AD [20], [21].

The onset of AD is marked by increased, uncontrolled neuronal death due to several reasons like synaptic disruption, reduced axonal transport, oxidative stress, mitochondrial dysfunction, increased Amyloid β (Aβ) peptide production, or Hyperphosphorylated Tau proteins which lead to the production of senile or neuritic plaques. Along with these cellular changes, mitochondrial dysfunction and synaptic disruption are the early noticeable changes in Ad pathogenesis [22]. In AD, mitochondrial dysfunction includes dysfunctional mtDNA expression, increased mtDNA damage, increased mtDNA mutation, reduced mtDNA copies, reduced mitochondrial axon transport, increased oxidative damage, and overall poor mitochondrial dynamics [23]. Histopathologically, an AD brain shows the presence of extracellular amyloid deposits as Aβ plaques and intraneuronal aggregates of hyperphosphorylated tau proteins in the shape of neurofibrillary tangles. Post-translational modifications in AD-related proteins like BACE1, tau, APP, and Aβ hold a crucial role in AD development and progression [24].

2.2 HALLMARKS OF AD

2.2.1 AMYLOID B (AB) PLAQUES: Aβ plaques originate from unregulated Aβ peptide production. Aβ peptides originate due to the cleavage of its precursor protein, amyloid

precursor protein (APP). Over the years, it was concluded that APP and its associated cleavage peptides own a crucial role in AD. APP, a member of the highly conserved APP family, is a type 1 transmembrane precursor protein with abundant expression in the central nervous system (CNS) and is largely responsible for brain homeostasis. The alternative splicing of the APP gene leads to the formation of different isoforms of APP mRNA [25].

Proteases cleave APP by different cleavage mechanisms to produce various unique short peptides, each exhibiting a specific function and independent physiological property. The initial cleavage of APP involves two pathways: the canonical and the non-canonical pathway. The APP canonical cleavage pathway mainly comprises three different secretases: α-secretase, β-secretase, and γ-secretase [26]. The γ- and α- secretases are involved in the non-amyloidogenic pathway giving rise to a soluble APPα (sAPPα) peptide. sAPPα, a neuroprotective peptide, is involved in the survival of neurons by protecting against cytotoxicity [5]. The γ- and β-secretases, related to the amyloidogenic pathway, are cleaved in endosomes by BACE1 (β-site cleaving enzyme-1) producing a soluble APPβ (sAPPβ) peptide with 99 amino acid residues. This peptide is further cleaved into two peptides: 40 amino acid residues long amyloid β 40 (Aβ40) and 42 amino acid residues long amyloid β 42 (Aβ42) [27], [28].

Healthy brains are characterized by well-maintained production and elimination of these peptides. Studies suggest that neuronal and synaptic activities are regulated by Aβ peptides. However, in AD brain, these Aβ peptides accumulate extracellularly to form neurotoxic amyloid plaques, rendering detrimental effects on neuronal metabolism, including loss of synapse, synaptic dysfunction, and compromised cerebral blood flow. This triggers a complex pathological cascade casing neuronal damage and initiating the disease [29], [30]. AD brains show increased levels of Aβ42 peptide due to its escalated hydrophobicity rendering it with better aggregation ability for plaque formation. These neuritic plaques initiate inflammatory responses causing irreversible neuronal damage in AD brains. sAPPβ can be cleaved to produce an N-terminally cleavage product of APP (N-APP) peptide. N-APP can bind to death receptor 6, initiating apoptosis, thus contributing to axonotmesis and neuronal death in AD brain [31].

2.2.2 NEUROFIBRILLARY TANGLES (NFTs): Intraneuronal aggregates of hyper-phosphorylated tau proteins usher the development of NFT. In the human brain, the 16 exons-comprising microtubule-associated protein tau (MAPT) gene encodes for tau protein which undergoes alternative splicing of several exons producing six different isoforms of tau. Tau

facilitates the assembly and stabilization of microtubules, which act as essential scaffolds for the cytoskeleton of the cell and promote vascular-mediated transport. In neurons, microtubules maintain neuronal structure and facilitate cellular trafficking, neuroplasticity, and axonal transport. In the normal brain, tau is found in the axon of the neuron. However, in neurodegenerative disorders related to tau proteins, these can also be translocated to the cell body and associated dendrites of the neuron.

Tau protein encounters multiple post-translational modifications including glycosylation, glycation, and phosphorylation. Among these, phosphorylation is the most crucial modification as tau protein is a phosphoprotein and its degree of phosphorylation modulates its biological activity i.e., its role in microtubule stability, and its pathological function i.e., its capability to self-assemble into neuro-filaments as observed in several neurodegenerative diseases [24], [32].

A fine-tuning between phosphorylation and dephosphorylation is essential for the normal functioning of tau protein. Abnormal phosphorylation and truncation of tau play a major role in tauopathies (i.e., neurogenerative disorders characterized by the accumulation and redistribution of hyperphosphorylated tau protein from its normal axonal position to the neuronal cell body and corresponding dendrites) including AD [33]. In AD, the abnormally hyperphosphorylated tau proteins aggregate as paired helical filaments and accumulate in the neurons in the form of NFT [34]. To study the human profile of tau protein, mice models were generated to express predominantly the isoforms of human tau (htau mice) and as they aged the neuronal cell bodies and dendrites exhibited the accumulation of hyperphosphorylated, conformationally altered tau proteins. Such redistribution of tau from the axons to the cell bodies started as early as the age of 3 months of htau mice [35] . In AD brains, tau loses its microtubule-binding ability, and hence, its role as a stabilizer of the cytoskeleton is no longer functional. The destabilization of microtubules hinders neuronal homeostasis and causes neuronal death [36].

AD is broadly classified into two types: Familial EOAD (Early-onset autosomal dominant) and non-familial LOAD (Late-onset sporadic forms). AD is said to familial EOAD type when the disease occurs due to utterly penetrant mutations in majorly three genes- PSEN1(Presenilin-1), PSEN2 (Presenilin-2), and APP (Amyloid Precursor Protein), mostly below the age of 65 years. All the three genes are directly associated with the increased production of Aβ42 [29], [37].

PSEN1 and PSEN2 are enzymes from the proteases A class, concerned with the cleavage of amyloid protein by regulating the function of the enzyme γ- secretase [38].

Non-familial LOAD type may occur due to both genetic and environmental factors, mostly in patients above 65 years of age. In the last few decades, interactions between various environmental factors and low penetrance polymorphisms were investigated to understand the possible genetic determinants of AD. After a vigorous systemic meta-analysis of more than 300 genes with over 1000 polymorphisms using the AlzGene database, it was concluded that polymorphism in the ApoE gene, encoding Apolipoprotein E (ApoE), a lipid metabolism protein, shows evident contribution in the non-familial sporadic form [39]. Studies from ApoE4 transgenic mice showed that overexpression of ApoE4 causes increased levels of Aβ42 inducing lysosomal activation leading to hippocampus neuronal death [40]. Another experiment conducted on 21 separate studies involving 1480 subjects with a total sample size of 6777 showed no heterogeneity in ApoE4. However, the allelic frequency of ApoE4 was remarkably higher in AD subjects supporting the increased risk of sporadic LOAD due to overexpression of ApoE4 [41]. Almost 90-95% of AD cases in the world are the non-familial sporadic form.

The diagnosis involves neurological investigation, neuronal imaging, the administration of the cognitive test, and cerebrospinal fluid test. Neurological investigation includes the use of neuropsychiatric inventory (NPI), involving a brief interview with a person who knows the patient well enough and can evaluate the patient on 12 behavioural areas commonly noticed in dementia. Neuronal imaging comprises CT (computerized tomography) and MRI (magnetic resonance imaging) scans. Rarely, PET (positron emission tomography) scans are also used for detecting amyloid bodies. The cerebrospinal fluid test helps measure amyloid and tau levels in the cerebrospinal fluid for the diagnosis of AD [42], [43].

2.3 AUTOPHAGY

In 1963, Christian de Duve coined the term "autophagy" at the CIBA Foundation Symposium on Lysosomes. The term “autophagy” is derived from a Greek word meaning “self-eating”. Autophagy is a highly conserved process in eukaryotes, both in terms of morphology and the protein components involved the core of autophagy machinery. Autophagy was first discovered in yeast by subjecting yeast to starvation and subsequent identification of autophagy-related (ATG) genes [44]. Autophagy is a highly degradative catabolic process that involves the lysosomal degradation of aged and toxic cytoplasmic material that is essential for survival,

differentiation, development, and homeostasis. The cytoplasmic substrate for autophagy is freely accessible cytoplasmic proteins can be either endogenous, such as nuclear fragments or damaged organelle, or exogenous, such as viruses or bacteria [45].

It is important to note that autophagy differs from endocytic processes (like phagocytosis, receptor-mediated endocytosis, and pinocytosis) [46] as well as ubiquitinated proteasomal degradation. Proteasomal degradation generates short peptides which can be used for antigen presentation while autophagy allows lysosomal proteases to degrade polypeptides to their amino acids which can be further used in other cellular processes [47]. Physiological functions of autophagy involve protection against metabolic stress, cellular homeostasis, guarding the genome, and non-apoptotic programmed cell death [48]. Fasting often triggers autophagy as it entails low amino acid or glucose concentration and autophagy tries to replenish these concentrations by catabolizing cellular proteins, lipids (lipophagy), carbohydrates (glycophagy), and iron (ferrtinophagy) to sustain major metabolic processes [7], [49]. Autophagy is also triggered by other stimulations like hypoxia impairments, reactive oxygen species (ROS), subcellular damage, and protein aggregation. However, self-cannibalistic and pro-survival processes of autophagy can be detrimental and instigate certain diseases including neurodegenerative disorders, infectious diseases, autoimmune diseases, cardiovascular diseases, aging, and, rheumatic and pulmonary diseases [8]. Autophagy is either selective or non-selective and is majorly of three kinds: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). While CMA (occurring only in mammalian cells) is highly selective, both microautophagy and macroautophagy can be either selective or non-selective in terms of disposing of the cytoplasmic cargo. In selective autophagy, damaged or redundant organelles such as mitochondria and peroxisomes, as well as pathogenic microorganisms are directly targeted whereas non-selective autophagy is employed for the turnover of bulky cytoplasm [50].

2.3.1 CHAPERONE-MEDIATED AUTOPHAGY: CMA was the 1st lysosomal process to be discovered which allows selective degradation of intracellular components. In CMA, the lysosome takes up cytosolic proteins for degradation directly past a protein translocation complex at the lysosomal membrane. This process can be either non-specific or specific to the cytosolic target protein. The requirements for cargo-specific CMA involve a degradation tag on the target, a chaperone, and a translocation complex across the lysosomal membrane. Substrates for CMA are translocated into the lysosomal lumen through committed molecular

machinery that essentially requires a specific splicing isoform of lysosomal-associated membrane protein 2 (LAMP2), namely, LAMP2A forming the cross-membrane LAMP2A translocation complex. CMA exclusively breaks down soluble proteins bearing an HSPA8 (Heat shock proteins) bound KFERQ motif but no other macromolecules (such as nucleic acids, lipids), integrated membrane proteins, or organelles [8], [51].

CMA regulates multiple cellular functions by facilitating DNA repair, glucose, and lipid metabolism, T-cell activation, and cellular response to stress by timely degradation of specific cellular proteins. Its role in different pathophysiological processes and genome integrity preservation suggest that CMA failure due to peroxide accumulation, aging, and/or other pathological signal interference may promote several diseases including age-related neurodegeneration and cancer, in addition to aggravating previously reported involvement in protein quality control [9].

2.3.2 MICROAUTOPHAGY: Microautophagy, a form of autophagy, deals with lysosomal or vascular membrane dynamics as they directly invaginate cytosolic content into the lumen for degradation. Microautophagy is broadly categorized into two types based on the molecular uptake mechanism of autophagic cargo, namely, fission-type microautophagy dependent on Endosomal sorting complexes for transport (ESCRT) protein for their uptake, and fusion-type microautophagy, which simply involves the core autophagy mechanism along with SNARE (SNAP receptors) proteins [52].

Microautophagy, in plants and yeast, involves direct membrane invagination of cytoplasmic entities fated for degradation into the vacuole[53]. Selective degradation of cytosolic protein occurs by interaction of organelle proteins with surface proteins of the vacuole. However, in mammalian cells, microphagy involves late endosomes for complete or partial degradation [54], commonly known as “endosomal microautophagy” in yeast [53]. Cytosolic proteins are degraded by endosomal microautophagy, either in bulk or selectively. It depends on multiple ESCRT systems while core microautophagy is independent of the ESCRT system. It was also noticed that all proteins taken up by late endosomes are tagged by a KFERQ-like motif that is recognized by HSPA8 [55].In yeast, microautophagy is used to degrade multiple substrates, including damaged mitochondria, peroxisomes (micropexophagy), portions of the nucleus, and lipid droplets. In plants, it mediates the degradation of anthocyanin [8].

Selective endosomal microautophagy, although referred to as HSPA8-dependent autophagic response differs from CMA based on its independence from the LAMP2A translocation complex and dependence on ESCRT systems [8].

2.3.3 MACROAUTOPHAGY: Macroautophagy is a type of autophagic process which involves the degradation of the cytoplasmic substrate forming large, dedicated, cytosolic double-membraned vesicles called autophagosomes. Autophagosomes sequester large parts of the cytoplasm including unneeded and damaged organelles, cytoplasmic proteins, and invasive microorganisms. An efficient macroautophagy response involves the formation of a double-membraned compartment, phagophore, which matures into an autophagosome. This allows uptake of the subcellular constituents utilizing several autophagy receptors, their lysosomal fusion forming an autolysosome, and lysosomal degradation of molecules with two ubiquitin-like conjugation systems. After degradation, the resulting macromolecules are resuspended into the cytoplasm to generate energy for the maintenance of the cell under unfavourable stress conditions [56].

Macroautophagy is mostly studied in yeast, nematodes, flies, and mammals. ULK1, BECN1, ATG13, ATG5, ATG7, ATG9, ATG3, ATG16L1, and VPS34 are among the proteins that have been identified as key regulators of macroautophagy responses. These genes are involved in various steps including induction of the autophagosome to its maturation [8]. As these genes are necessarily involved in maintaining cellular homeostasis, macroautophagy deregulation is associated with numerous diseases, including neurodegenerative disorders like Alzheimer's, Huntington's, and Parkinson's diseases [57].

2.3.4 AUTOPHAGY IN AD

Autophagy is a vital homeostatic pathway in neurons. Neuronal cells, like other cells, acquire intracellular toxicants or damaged organelles as they age, which must be eliminated by autophagy to ensure intracellular homeostasis. However, unlike other cells, post-mitotic cells as neurons can't dilute their harmful toxicants through mitosis. As a result, in neurons, autophagy-dependent protein/organelle clearance would be more crucial [58]. Moreover, studies suggest that autophagy plays a crucial role in synapse development. They found that neuronal autophagy favourably regulates the development of synapses in the Drosophila neuromuscular junction [59]. In neurons, autophagosomes are mostly initiated at the axon tip and as they are transported retrogradely along the axon, they gradually undergo maturation,

become increasingly acidified with the formation of autolysosomal compartments for degradation of the misfolded proteins and damaged organelles like mitochondria [60].

A considerable number of evidence collected from AD animal models as well as AD patients suggest that autophagy deregulation is involved in its pathology. In 1967, Suzuki discovered that dystrophic neuritis (DNs) in AD patients' brains showed an accumulation of loads of sub-cellular vesicles and abnormal aggregated tau protein [61]. However, these findings remained unclear until, in 2005, Nixon's group used immunogold labelling and electron microscopy to visualize the abundant accumulation of pre-lysosomal autophagic vacuoles (AVs) in dystrophic neurites in AD brains [62]. According to loss-of-function studies in specific neurons of the brain, basal autophagy demonstrates a crucial role in the degradation of damaged organelles and misfolded proteins. Basal autophagy suppression in neural cells as in Atg5 deficient mice leads to accumulation of cytoplasmic inclusion bodies and progressive loss of motor functions [63]. Deregulation of autophagy hinders intracellular communication and eventually contributes to neurodegeneration. Normally, neurons exhibit low-basal autophagic activity due to the ensuing degradation of autophagosome by the lysosome. In AD, impaired autophagolysosome maturation due to disrupted retrograde transporting results in a massive accumulation of AVs, particularly, within dystrophic neurites. The hippocampal neuronal axons of AD mice demonstrated abnormal accumulation of immature AVs [64].

Innumerable shreds of evidence demonstrate the complex interactions among Aβ, tau, and autophagy contributing to the pathology of AD. Autophagy holds an essential role in Aβ metabolism and is known as one of the major Aβ clearance pathways. Autophagy promotes the degradation and removal of APP cleavage products like Aβ and APP-CFTs (amyloid precursor protein-cleaved C- terminal fragment), in addition to APP. Macroautophagy, involved in the lysosomal degradation of Aβ, demonstrates an exclusive pathway under pathological conditions or aging for Aβ production. In neuronal cells, inhibiting or inducing macroautophagy by regulating the Mammalian target of rapamycin kinase (mTORC) evokes simultaneous changes in AV proliferation and Aβ fabrication establishing a direct relationship with the beta-amyloidogenic pathway, activated abnormally in AD [65]. Thus, the accumulation of immature AV found in the AD brains of transgenic mice may be a source of Aβ production [62]. Recent studies also exhibit the involvement of autophagy in the extracellular secretion of Aβ. Autophagy-related protein 7(Atg7) knockdown in APP transgenic mice showed largely reduced extracellular secretion of Aβ in the mouse forebrain,

accompanied by a significant intracellular accumulation of Aβ [66]. On the other hand, pieces of evidence suggest that Aβ has a regulatory role in autophagy. Aβ40 inhibits human brain vascular endothelial cells by inducing autophagy through the intracellular regulation of phosphatidylinositol 3-kinase (PI3K) and Akt signaling [67].

In Tau turnover, although the role of the ubiquitin-proteosome system (UPS) is well established, in recent studies, autophagy has also been considered an effective degradation route for tau. Autophagy-lysosomal system dysfunction ushers accumulation of tau oligomers and insoluble clusters, while autophagy induction helps alleviate the abnormal accumulation of tau [68], [69]. Inhibition of autophagy in M1C cells (neuronal cellular model of tauopathy) by inducing autophagy inhibitors like chloroquine and 3-methyladenine demonstrated an increased tau accumulation [11]. Thus, disruption in the autophagy-lysosomal system disturbs the tau protein degradation via autophagy. Nuclear factor erythroid-2 related factor-2 (Nrf2) mediated activation of NDP52, an autophagy adaptor protein that modulates the autophagic degradation of phosphorylated tau [70], [71]. Moreover, macroautophagy shows an established role in the phosphor-tau pathway and the phosphorylation status of tau. Atg7-deficient mice model showed accumulation of hyper-phosphorylated tau in the forebrain [72]. On the other hand, tau, responsible for microtubule assembly and stabilization, is essential for the maturation of autophagosomes through its retrograde trafficking. Hyperphosphorylation of tau leads to instability and disassembly of microtubule cytoskeleton hindering autophagosome trafficking across the axon and causing accumulation of immature AVs in the axon tip [10].

Hence, we can conclude that Aβ and tau share a bi-directional role with autophagy as autophagy dysregulation regulate Aβ and tau abnormally and vice-versa.

2.4 DNA DAMAGE AND REPAIR

A small but influential group of physicists sparked early work on DNA damage and repair in the 1930s. Perpetual exposures of living organisms to innumerable DNA damaging agents impact their health, and wellness and regulate disease states. DNA is an inherently reactive molecule and is particularly sensitive to chemical alterations via exogenous and endogenous factors [12]. It is important to preserve genomic sequence information for the perpetuation of life.

DNA damage is broadly classified into two major classes based on its origin: exogenous and endogenous. Various exogenous and endogenous factors such as oxidative stress, metabolic

stress, genotoxic stress, telomere erosion, and oncogenic mutations lead to DNA damage. Endogenous DNA damage majorly arises from hydrolytic and oxidative reactions of the DNA with water and reactive oxygen species (ROS) present intracellularly [73]. On the contrary, exogenous DNA damage may arise due to various physical, environmental, and chemical agents such as UV and ionizing radiation, crosslinking agents, and alkylating agents. Activation of p53 plays a critical role in the intrinsic biological responses to DNA damage by regulating cell-cycle arrest, apoptosis, and senescence. Cellular senescence is described as an irreversible growth arrest followed by morphological changes and gene expression alterations [74]. Tissue regeneration and homeostasis are obstructed by the accumulation of senescent cells, especially senescent stem cells, resulting in metabolic dysfunction. Furthermore, senescent cell accumulation in tissues causes chronic inflammation, modulated by a variety of chemokines and pro-inflammatory cytokines [75].

However, cells are furnished with multiplex and sophisticated arrangements such as damage tolerance, cell cycle checkpoints, DNA repair, and cell death pathway. All of the mentioned processes contribute to reducing the deleterious consequences of DNA damage. DNA repair and damage tolerance mechanisms ensure the overall survival of the cell by either eliminating or bypassing the DNA damage. Any deviations in this highly regulated process impair cellular metabolic homeostasis, embodied by various types of cancer where DNA repair mechanism disruption or deregulation results in genome instability [76].

Cellular metabolism is directly linked to DNA damage and repair mechanisms. Three major linkages connect DNA damage and repair systems to cell metabolism: (i) different metabolic pathways regulating methyl- and acetyl-group donors can affect DNA folding and remodelling, which is an important aspect of efficient double-strand break (DSB) repair; (ii) aspartate, glutamine, and other nutrients essential for de-novo nucleotide synthesis, which regulates DNA repair and replication by determining the available nucleotide pool; (iii) different metabolic pathways regulate ROS, which may escalate oxidative DNA damage and therefore the constraints on the DNA-repair machinery [13].

In case of any DNA damage, the cell responds by initiating vigorous DNA damage response (DDR) pathways. Such DDR pathways are initiated until specific DNA repair pathways take over to eliminate the damage. Five major DNA repair pathways popularly known to actively participate through different cell cycle stages allowing the cells to repair the DNA damage include base excision repair (BER) for non-helix distorting base lesions; mismatch repair

(MMR) for the repair of erroneous insertion, deletion, or mis-incorporation of bases; nucleotide excision repair (NER) majorly for UV-induced DNA damage; homologous recombination (HR) for the repair of damaged chromosomes; and non-homologous end joining (NHEJ) for DSB repair. Direct chemical reversal or inter-strand crosslink (ICL) repair can be used for the removal of certain exclusive DNA lesions. These repair mechanisms exhibit an extraordinary role in maintaining the genetic stability of the cell [76].

Any disturbance or deregulation in DNA damage tolerance and repair pathway leads to increased mutation and genomic instability thereby assisting lethal diseases such as that of cancer. A conjugative failure of more than one of these processes may as well lead to neurodegenerative disorders like AD.

2.4.1 DNA DAMAGE IN AD

Accumulation of DNA damage is one of the well-known factors of aging. Innumerable exogenous and endogenous agents are constantly attacking our genetic material. In post-mitotic cells like neurons, DNA damage can be particularly deleterious as they do not undergo self-renewal through cell proliferation. In addition, aberrant re-entry in the cell cycle precedes various neurotoxic conditions causing neuronal loss and several neurodegenerative disorders such as AD [16]. Evidences suggest that tissue samples from mild cognitive impairment (MCI) and AD subjects demonstrate elevated levels of oxidative DNA damage.

The etiology of many neurodegenerative disorders, including AD, Amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), Huntington's disease, Multiple sclerosis, and Parkinson's disease, has been associated with oxidative stress. Oxidative stress involves the generation of excess ROS induced by an imbalance in the redox state in the aging brain. In neuronal cells, oxidative DNA damage and ROS contribute to a bulk of DNA damage. Evidences of oxidative DNA damage in MCI and pre-clinical AD further support the etiological role of ROS-induced-DNA damage in AD. Oxidative DNA damage occurs in two forms in neurons: nuclear DNA (nDNA) damage and mitochondrial DNA (mtDNA) damage. A slow build-up of DNA adducts in the genome leads to oxidative DNA damage accumulation in nDNA triggering neuronal death. On the other hand, base substitutions and deletions leads to DNA damage accumulation in mtDNA ushering erroneous gene transcription of associated subunits of the electron transport chain, with progressive mitochondrial dysfunction, increased oxidative damage, and neuronal death [77].

Oxidative attacks by ROS to DNA bases generate several oxidized base adducts such as 8-hydroxyguanine (8-OHG), 5-hydroxycytosine (5-OHC), 8-hydroxyadenine (8-OHA), and 5-hydroxyuracile (5-OHU, derives from cytosine degradation), in several regions of AD patient's brain [14]. One of the most prominent base adducts of ROS-induced DNA lesion is 8-hydroxyguanine (8-OHG). Hence, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is broadly used as a biomarker of DNA oxidation.

In 1993, Prof. M. Flint Beal at the Harvard Medical school measured 8-OHdG in nDNA and mtDNA isolated from the cerebellum and cerebral cortex of humans aged 42 to 97 years. These results demonstrated a progressive increase in the accumulation of oxidative DNA damage with age and particularly in mtDNA [78]. Evidences from AD brains demonstrated a 10-fold elevation in the levels of oxidized bases in mtDNA than nDNA proposing higher levels of oxidative stress in mitochondria [79]. AD patients' ventricular cerebrospinal fluid (CSF) is marked with increased levels of 8-OHdG suggesting the utility of 8-OHdG as a biomarker in AD diagnosis [15]. An increased presence of 8-OHdG in the aging and AD brain supports the hypothesis that oxidative stress holds a critical role in the high incidence of AD in old age subjects [80].

DNA damage brought about by ROS (produced during mitochondrial respiration) involves altered bases, apurinic sites, single-strand breaks (SSB), and DSB which are mostly avoided by the DNA BER pathway. Other repair pathways include direct reversal, MMR, NER, HR, and NHEJ pathways. In the human brain, oxidative stress progressively accumulates especially in the mtDNA, and plays a crucial role in aging and the pathogenesis of various neurological disorders including AD, ALS, and Parkinson's disease. Post-mortem patient brains with AD have higher DNA DSBs and lower DSB repair factors expression, and AD brain extracts have decreased in-vitro DSB repair capacity, indicating impaired NHEJ [81].

AD brains show elevated levels of activated cell cycle genes like Cyclin dependent kinase 5 (CDK5). The occurrence of NFT is promoted by intra-neuronal aggregation of hyperphosphorylated tau. Such irregular phosphorylation of tau is caused by a diverse range of kinases such as CDK5. Inappropriate activation of CDK5 due to irregulated proteolytic release of p25, the activator fragment, from the membrane leads to the formation of NFT and chronic neurodegenerative conditions [82]. Furthermore, Aberrant CDK5 activation attributes towards phosphorylation of APP which stimulates Aβ peptide accumulation. As mentioned earlier, Aβ42 accumulation leads to the formation of senile plaques found in abundance in AD brain.

CDK5 activities alter DNA damage response as well drawing a clear link between DNA damage and AD [83].

DNA lesions accumulate in AD brains. High incidences of DSBs and SSBs were also noticed at the DNA level in AD brain's hippocampus [84]. Senescing human cells and aging mice, exhibit increased genome rearrangement and DSBs. Elevated levels of γH2AX, a well-accepted marker of DSB, were detected in the cerebral cortex and astrocytes of the hippocampus in AD brains [85]. Such high incidences of DSBs and SSBs in AD brain strongly support that DNA damage is caused by endogenous ROS, considering the blood-brain barrier which protects the brain from external or environmental genotoxins. Post-mitotic neurons of the CNS exhibit a high metabolic rate [86].

2.4.2 DNA REPAIR IN AD

Repetitive inefficacious cell-cycle events lead to neuronal loss in AD. In the adult human brain, neurons are capable of re-entering the cell division cycle. However, in a normal human brain, the cell cycle fails to surpass the G1 phase, and the cells re-differentiate into neurons. Meanwhile, in AD patients, the cell cycle progresses up to the G2 phase. At this stage, cell cycle arrest doesn't allow re-differentiation, and the neuron suffers one of the two fates: neuronal death via apoptosis or production of Alzheimer-type pathology [87]. Further observation of the degenerative neurons of AD subjects marked the increased production of cyclins and cyclin-dependent kinases (involved in the transitional phases of G1-S and G2-M of the cell cycle) indicating the progressive loss of neurons through programmed cell death [88].

In humans and rodents, DNA repair genes mutagenesis and poor DNA repair mechanism are consistently linked to pre-mature aging and neuropathological symptoms, whereas long-lived mice display upregulated DNA repair pathways, and lowering DNA damage improved results in model systems. Greater knowledge of the machinery governing DDR in the brain could aid the development of new therapeutics for aging and disease [89].

Shreds of evidence suggest the involvement of many DNA repair proteins or complexes in mediating cellular death in AD. One such DNA repair protein is poly-ADP-ribose polymerase-1 (PARP-1), a zing-finger-associated DNA binding protein that is activated by SSB or DSB in DNA. Primarily, PARP-1 acts as a DNA damage sensor for repair processes and the prevention of chromatid exchanges. PARP-1 utilizes NAD+ as a substrate and catalyzes the transfer of

mono-ADP-ribose or PAR to a variety of acceptor proteins, including PARP-1. This is classified as one of the first responses of the cell against DNA damage. As a result of this occurrence, DNA repair proteins and nucleases are recruited to damage sites, aiding DNA damage repair. However, excessive PARP-1 activation may lead to PARP-1-dependent cell death, commonly known as parthanatos. Prolonged activation of PARP-1 activation in cells with significant DNA damage leads to exhaustion of its substrate NAD+ and eventually ATP, resulting in mitochondrial dysfunction and cellular death due to energy failure [90]. AD brains studied through in-situ labelling methods provide evidences of widespread SSB and DSB triggering an increased PARP-1 activity, leading to cell death [91]. Moreover, NAD+ depletion contributes to dysfunctional mitochondria (a hallmark of AD) as NAD+ is a cellular metabolite crucial for mitochondrial health and biogenesis, and neuronal stress resistance [92]. Experimental evaluation of AD brains using double immunolabelling techniques suggests increased expression of both PARP-1 and poly-ADP-ribose (its end product) in AD brain in comparison to control brains [93].

Similarly, the Mre11 protein complex consisting of Rad50, meiotic recombination 11 (Mre11), and Nijmegen breakage syndrome 1 (Nbs1) is another protein complex involved in DNA repair. This protein complex is a key regulator of cellular responses against DSBs, including HR, NHEJ, telomere maintenance, and activation of DNA damage checkpoint [94]. Any defective element of this protein complex hampers the survival of the cell. In the adult human brain, neurons of the cerebellum and cortex exhibit the presence of Mre11 complex protein. Careful observation provides evidence of reduced levels of this protein complex in the neuron of AD cortex compared to that of healthy control brains suggesting an association of Mre11 complex loss to the pathogenesis of AD [95].

HDAC1 (Histone deacetylases), a class I HDAC, have been found to play a crucial role in maintaining the genomic integrity of cultured neurons and the mouse brains [96]. HDACs are enzymes that catalyze the deacetylation of lysine residues in histone and non-histone molecules. HDACs regulate many cellular activities, including transcription, chromatin remodelling, and DNA repair [97]. DSBs trigger the regulation of HDAC1 to the break sites promoting DSB repair via NHEJ, the prime DSB repair mechanism in neurons. HDAC1 is also known to modulate OGG1-initiated 8-oxoguanine (8-oxoG) repair in the brain. OGG1, 8-oxoguanine DNA glycosylase, is a DNA glycosylase that plays an essential role in transcriptional regulation and maintenance of metabolic homeostasis. It is known to remove 8-

oxoG lesions that otherwise act as transcriptional repressors. OGG1 non-catalytically binds to oxidatively-induced DNA damage in promoter regions and serves as a site for nucleation [98]. In case of deficiency of HDAC1 in the brain, OGG1 activity is impaired causing accumulation of 8-oxoG at the promoter regions advocating transcriptional repression of genes essential for brain functioning. When studied in 5XFAD mouse model of AD, elevated levels of 8-oxoG DNA damage were found in addition to reduced HDAC1 activity and downregulation of associated gene expression. Hence, HDAC1 plays a crucial role in 8-oxoG repair and its deficiency in AD brains leads to DNA damage accumulation [99].

Another such DNA repair protein found to be missing in AD patients is MTH1, an oxidized purine nucleoside tri-phosphatase, that averts neurotoxicity caused by oxidized purine nucleotides in the nucleotide pool such as 2'-deoxy-2-hydroxyadenosine triphosphate (2-OH-dATP) and 2'-deoxy-8-oxoguanosine triphosphate (8-oxo-dGTP), thus preventing their amalgamation in the DNA or RNA. MTH1 is expressed in neurons to efficiently minimize 8-oxoG build-up in both nDNA and mtDNA in the brain, thus protecting the brain against oxidative stress [100]. Induced MTH1- and/or OGG1- deficiency in six month old AD mice showed accelerated 8-oxoG amassing and microgliosis in the amygdala and brain [101]. MTH1 and OGG1 levels are remarkably lessened in the brains of sporadic AD patients. According to gene expression profiling, Aβ/Tau accumulation triggers MTH1 and OGG1 to enhance the expression of numerous protective genes against AD pathogenesis, thus effectively decreasing AD progression in 3xTg-AD brain [102].

In neurons, the principal mechanism to repair DSBs is NHEJ which essentially requires DNA-dependent protein kinase (DNA-PK) activity. DNA-PK, a holoenzyme, consisting of the p460 kDa DNA-PK catalytic subunit (DNA-PKcs) and its activator Ku, a heterodimer of p86 and p70 subunits. To process NHEJ, Ku employs DNA-PKcs upon binding to DNA ends. AD brains are marked with reduced DNA-PKcs and Ku protein activity, hence reduced NHEJ activity. Aging human brains show a deficiency of both DNA-PKcs and Ku levels establishing the chances of a direct relation between NHEJ reduction and pathogenesis of AD. Ku is a somatostatin receptor and its deficiency can disrupt somatostatin signaling triggering Aβ plaques generation which can additionally affect DSB repair negatively by degenerating DNA-PKcs and consequently hinder NHEJ activity [18], [103]. Experiments were conducted on PC12 cells with sub-lethal concentrations of aggregated Aβ (25-35) showing the inhibitory role of Aβ on DNA-PK activity, consisting of DSB repair and sensitizing cells with non-lethal

oxidative damage. The oxidative stress induced by Aβ leads to the down-regulation of DNA-PKcs, hence inhibiting DNA-PK activity and the NHEJ pathway. Accumulation of DSBs may lead to neuronal loss if not efficiently repaired [104].

2.5 DDR, AUTOPHAGY, AND AD

Both autophagy and DDR play a key role in various neurodegenerative diseases including AD, however, their association hasn't been well established so far. They are correlated in various, non-exclusive pathways.

Elevated levels of ROS, one of the established causes of nDNA and mtDNA damage in AD, lead to the induction of autophagy. Cytoplasmic ATM(Ataxia-telangiectasia mutated) acts as a ROS sensor and activates TSC2 tumor suppressor by signaling the LKB1/AMPK metabolic pathway in the cytoplasm to relieve mTORC1 repression of autophagy and induce autophagy in response to the elevated levels of ROS[105], [106]. ATM-deficient neurons showed both abnormal autophagy and lysosomal trafficking and hampered cellular functions while ATM itself is degraded through the autophagy pathway [107].

Similarly, PARP-1 signaling pathway is a key regulator of starvation-induced autophagy as researchers found that PARP-1 knockout mice subjected to acute starvation displayed autophagy deficiency. PARP-1 deficiency weakened AMPK activation and hindered AMPK-induced mTORC1 repression leading to delayed autophagy. PARP-1 deficient cells are also credited with the prevention of ATP and NAD+ depletion and hence lack of cellular starvation keeps the autophagy negative regulator mTORC1 activated [108], [109]. On the other hand, autophagy dysfunction may have regulatory activity towards PARP-1. Autophagy is a well-established Aβ clearance pathway and dysfunction in autophagy leads to Aβ peptide accumulation in the form of Aβ plaques as seen in AD. Aβ enhances PARP-1 activity especially in astrocytes [110], [111] and prolonged PARP1 activity leads to ATP and NAD+ depletion leading to cellular starvation and subsequent cell death, parthanatos in neurodegenerative diseases including AD [17].

BECN1 encodes for a core mammalian autophagy inducer protein, In-vitro deletion of BECN1 in human cells attenuated the expression of several DSB repair proteins and the association of repair complexes hindering the DNA repair mechanism. BECN1 knockout attenuates DNA-PK complex formation, essential for the initiation of NHEJ [112]. Studies have revealed reduced DNA-PK level and NHEJ activity in AD brain referring to the plausible role of

unrepaired DSB in the development and progression of AD [18], [113]. MRN (Mre11-Rad50-Nbs1) complex plays a crucial role in DNA damage recognition, DNA repair, and initiation of cellular checkpoints [94]. CMA deficiency leads to hyperphosphorylation and destabilization of the MRN complex obstructing the recruitment of ATM at the damage site for DNA repair [114].

Ultraviolet irradiation resistance-associated (UVRAG) is an essential promoter of the autophagy pathway and mediates DNA DSB repair. UVRAG helps in the assembly and activation of DNA-PK complex modulating NHEJ repair of DNA [115], [116]. However, mTORC1-mediated phosphorylation of UVRAG negatively regulates the formation and maturation of autophagosomes [117]. Interestingly, UVRAG overexpression inhibits neuronal necroptosis in cell and mouse models of AD [118]. Both mitochondrial dysfunctions as in AD and DNA damage can invoke mitophagy to maintain mitochondrial function, which may have a protective role against DNA damage-induced cellular death [119], [120].

HDAC is a key regulator of DNA repair, especially NHEJ. However, researchers have found that HDAC inhibition triggered unprogrammed autophagy-mediated turnover of key DNA repair proteins, thus contributing to DNA damaging sensitivity [121]. Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, induces autophagy by inactivating mTOR and transcriptionally up-regulating LC3 expression, essential for prolonged autophagy [122]. In neurodegenerative diseases like AD, histone acetylation homeostasis is largely disabled leading to a state of hypo-acetylation. Inhibition of HDAC is recommended as a potential novel therapeutic approach for AD [123]. HDAC inhibitors have been proposed to act as neuroprotectors by promoting neuroplasticity and cognitive ability in AD. HDACs regulates histone acetylation levels and HDAC inhibitors are capable of upregulating histone acetylation levels crucial for improving cognitive ability [124], [125].

Nrf2 is a key transcription factor involved in protection against oxidative stress, one of the key sources of DNA damage in AD. The Nrf2 deals with oxidative stress by binding to the consensus antioxidant response element (ARE) sequence followed by the expression of a cascade of genes involved in protein degradation and cytoprotection [126]. Nrf2 also exhibits a regulatory role towards autophagy by inducing an autophagy adaptor protein, NDP52, containing LC3-interacting regions in neurons [127]. In AD, NDP52 acts as a crucial downstream facilitator of Nrf2-mediated degradation of phosphorylated tau via autophagy.

Nrf2 knockout mice showed increased levels of phosphorylated tau and sarkosyl-insoluble tau in the hippocampal tissues [128].

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 COMPUTATIONAL INVESTIGATION

3.1.1 COMPUTATIONAL TOOLS AND DATABASES

STRING (version 11.5), Cytoscape (version 3.9.1), KEGG, PANTHER, CellDesigner (version 4.4.2).

3.1.2 DETERMINATION OF ESSENTIAL PROTEINS OR PROTEIN COMPLEXES FOR NETWORK CONSTRUCTION

An intensive literature survey of numerous research and review articles related to autophagy, DNA damage and repair mechanisms, and AD was done with a comprehensive approach to identify simple proteins and protein complexes associated to all three of them. This helped us obtain a list of gene/protein which plays a crucial role in AD with relevance to autophagy and DNA repair.

The key proteins selected were: BECN1, PARP1, UVRAG, mTORC1 and PIK3C3. For each protein, the available network on STRING (Search Tool for Retrieval of Interacting Genes/proteins) database, version 11.5, was carefully studied and analysed. STRING (version 11.5), a biological database, integrates all the known and predicted interactions between proteins, achieved from various sources such as text mining of scientific literature, interaction experiment databases and elucidated pathways, and co-expression and conserved gene region predictions. It includes both physical interaction and functional associations of proteins from 14,000 different organisms [129]. After studying the available networks for the selected proteins, a list of associated proteins with relevant role in either of the three criteria i.e., AD, autophagy, and DNA repair was documented. This list comprised of the following proteins: PARP1, POLB, XRCC1, XRCC5, XRCC6, PRKDC, PARG, AMBRA1, PIK3C3, PIK3R4, VMP1, ULK1, KIAA0226, ATG14, UVRAG, DNA-PK, BCL2, RB1CC1, ATG13, BECN1, RAB5A, MTOR, RPTOR, DEPTOR, TSC1, MAPILC3A, APP, BACE1, APOE, APBB1, CDK5, MAPT, GFAP, PSEN1, GABARAPL2.

3.1.3 ANALYSIS OF SHORTLISTED PROTEINS

The STRING database was further used to study the interaction between the shortlisted proteins and to curate a protein-protein interaction network of the same. This network was further saved in TSV (Tab-Separated Values) file format and imported to Cytoscape (version 3.9.1) to study

and analyse the various network topology parameters with the NetworkAnalyzer plugin. Cytoscape (version 3.9.1) is a freely available software tool that allows visual investigation of complex biological networks comprising proteins, genes, or other interactions. It provides various plugins to load, visualize, filter and analyse networks to fulfil specific research requirements on the basis of their file format [130]. The NetworkAnalyzer plugin allows analysis and calculation of network topology parameters of loaded biological networks. Such network topology parameters including average number of neighbours, neighbourhood coefficient, degree of the network (both indegree and outdegree), average clustering coefficient, shortest pathlength etc. allow quantitative analysis of the network.

Using the NetworkAnalyzer plugin, various topological parameters of the obtained network was analysed for the shortlisted proteins. This provided an insight to the key regulatory proteins of the obtained network. Simultaneously, the individual role of each protein in autophagy, DNA repair and AD was studied from the available scientific literature.

3.1.4 MODEL CONSTRUCTION

To construct a detailed cell model for AD with relevance to autophagy and DNA repair, various databases and literature manuscripts from the scientific community was referred. One such database referred was KEGG (Kyoto Encyclopedia of Genes and Genomes) which is a freely available encyclopedia of databases dealing with genomes, biological pathways, and ligands (drugs and chemical substances) [131]. Similarly, PANTHER (protein Analysis Through Evolutionary Relationships) database helps in high-throughput analysis of gene/protein families on the basis of their molecular function, biological processes, and associated pathways [132].

All the collected information from the databases and literature review was used to roughly manuscript a pathway. A System Biology Graphical Notation (SBGN) allows building of biological networks for visualization of the interactions and analysis of their overall effect on the system as a whole. Hence, the AD pathway with relevance to autophagy and DNA repair was further computationally designed using CellDesigner (version 4.4.2) and stored in System Biology Markup Language (SBML), a machine-readable standard format used for the representation of biological networks. CellDesigner is a well organised diagram editor tool for constructing gene regulatory pathways and biochemical networks using symbols of SBGN (Figure 8). This tool allows simulation and other analysis of the network through the Systems Biology Workbench (SBW). It provides a user-friendly graphical interface with various

symbols like protein, gene, RNA, ions, simple molecules, catalyst, phenotype, etc. as nodes and state transition, inhibition, catalysis, transport, modulation, physical simulation, etc. as edges.

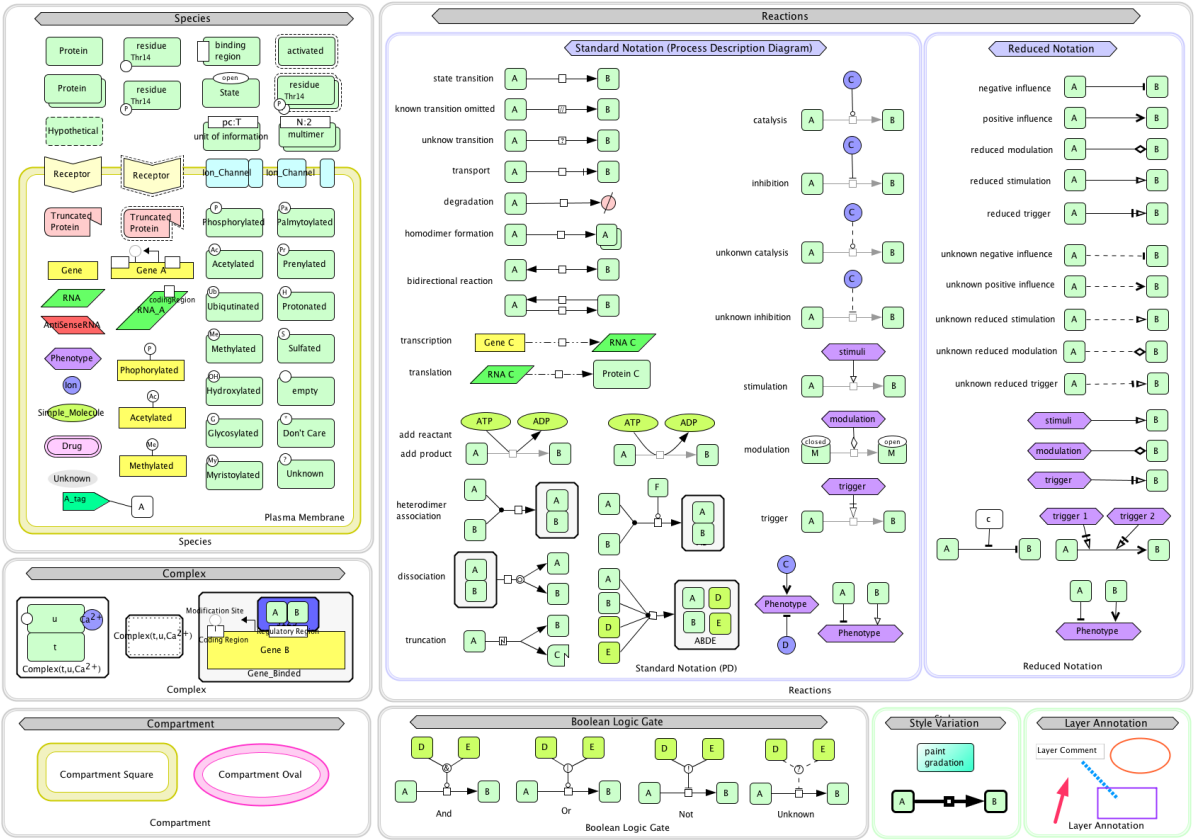

Figure 8: SBGN symbols provided in CellDesigner 4.4.2 for modelling biological networks.

3.2 EXPERIMENTAL VALIDATION

3.2.1 MATERIALS AND REAGENTS

Standard laboratory grade reagents/chemicals were utilized for the study. Dulbecco's Modified Eagle's Media (DMEM), 0.05% Trypsin-EDTA and Pen Strep (Penicillin Streptomycin) were obtained from Gibco, Pune, India. Some other chemicals such as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), Phosphate saline Buffer (PBS), Fetal Bovine Serum (FBS), Muller Hinton Broth (MHB), Tris base, Sodium phosphate dibasic, Aluminium chloride, Quercetin, Gallic acid, Ampicillin sodium (91%), and Trypan blue were obtained from HiMedia, Mumbai, India. Sodium chloride, Potassium chloride, Potassium phosphate monobasic, Disodium EDTA, SYBR green dye, Resazurin dye, ammonium acetate, sodium lauryl sarcosinate, and 1% Triton X-100 were obtained from Merck Ltd., Mumbai, India. Cadmium sulphide (CdS) nanoparticles (NP) was obtained from

Department of Physics and Material science, Jaypee University of Information Technology, Himachal Pradesh, India.

3.2.2 CELL CULTURE MAINTENANCE AND VIABILITY

Neuro 2A (N2A) cell line was procured from National Centre of Cell Sciences, Pune, India. N2A cell line, derived from the neural crest cells of mouse, has neuronal and amoeboid stem cell morphology that has been extensively used to study neuronal differentiation, axonal growth and signaling pathways. Cell lines were maintained in DMEM with 10% (v/v) FBS and 1% Pen strep (Penicillin Streptomycin) antibiotic at 5% CO_2 concentration and 37℃ temperature in an incubator.

Viability of the cells was confirmed by Hemocytometry. Cell sample and trypan blue were mixed in 1:1 ratio and then loaded in hemocytometer for visualization under Olympus™ inverted microscope. Number of live and dead cells were counted under 40x magnification.

$$Live\ cells\ \% = \frac{No.of\ live\ cells}{Total\ no.of\ cells} \times 100$$

$$Total\ number\ of\ viable\ cells = No.of\ live\ cells \times Dilution\ factor \times 10{,}000/ml$$

3.2.3 CYTOTOXICITY AND CELL PROLIFERATION ASSAY

3.2.3.1 SAMPLE PREPARATION

Stock solution of our samples were prepared for respective samples. The samples used are CdS NPs, Quercetin, and Gallic acid. For CdS NPs, a stock solution of 0.2 mg/ml and further working solutions of 0.1, 1.0, 10 and 100 µg/ml was prepared. For both Quercetin and Gallic acid, a stock solution of 2 mg/ml and working solution of 50, 100, 150 and 200 µg/ml was prepared.

3.2.3.2 MTT ASSAY

MTT assay is a colorimetric assay to measure cellular metabolic activity indicative of cell viability, proliferation, and cytotoxicity. The assay is based on the reduction of a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. To prepare the cells for MTT assay, the media was discarded from the T-flask containing the cells and the cells were dislodged with Trypsin-EDTA. The T-flask was then placed in the incubator for 2 minutes with 37℃ temperature for better activity of the enzyme. The cells were then washed with excess PBS to stop the activity of Trypsin-EDTA and briefly centrifuged for 4-5 minutes at 300 x g to form a pellet. The pellet was resuspended in growth media (DMEM and 10% (v/v)

FBS) and the cells (1×10^5 cells/ml) were seeded in a 96-well plate. The cells were treated with increasing concentration of the samples i.e., CdS NP (0.1-100 µg/ml), Quercetin (50-200 µg/ml), and Gallic acid (50-200 µg/ml). the cells were incubated for 24 hours at 5% CO_2 concentration and 37℃ temperature in an incubator. As a control, wells seeded with cells containing the media were left unaltered. Following 24-hour incubation, cell viability was determined using the commercially available MTT dye. 5mg/ml MTT (light sensitive) solution was prepared and 10 µl was added to each well and incubated for 4 hours at 5% CO_2 concentration and 37℃ temperature. After incubation, the media was removed and 100 µl of DMSO was added to each well followed by incubation at 5% CO_2 concentration and 37℃ temperature for 30 minutes. The optical density (OD) was determined at 570 nm using Multiskan™ Thermo fisher Microplate Spectrophotometer reader and cell viability was calculated. The assay was evaluated spectrophotometrically as the colour was directly proportional to the number of metabolically active cells in the assay.

$$Cytotoxicity\ \% = \frac{OD\ control - OD\ sample}{OD\ contol} \times 100$$

$$Proliferation\ \% = \frac{OD\ sample - OD\ control}{OD\ contol} \times 100$$

3.2.4 ANTI-MICROBIAL ACTIVITY TEST

3.2.4.1 SAMPLE PREPARATION

Stock solutions were prepared by suspending the following samples in distilled water to yield a final concentration of 50 mg/ml. The samples used were CdS NPs, Quercetin and Gallic acid. These stock solutions were then sonicated for 40 minutes at 30℃ temperature. Every assay was performed within 1 hour of sonication. 2mg/ml solution of Ampicillin sodium in DMSO was used was a positive control.

3.2.4.2 INNOCULUM PREPARATION

Bacterial inoculums were prepared from well-maintained cultures grown on nutrient agar plates and maintained at 4℃. A well isolated colony was selected from agar plate and transferred in 10 ml of MHB broth medium. The broth culture was incubated at 37℃ for 24 hours. Bacterial cultures of *Escherichia coli* (E. coli), *Salmonella typhi* (S. typhi), *Staphylococcus aureus* (S. aureus), and *Bacillus subtilis* (B. subtilis) were used for this study.

3.2.4.3 WELL DIFFUSION ASSAY

The antimicrobial activity for the samples were evaluated by agar well diffusion assay. MHA plates were prepared for testing antibacterial activity. 100 µl of the prepared inoculum of each bacterial culture was spread over 4 different plates. 4 wells were made in each plate and 40 µl of the prepared samples were added to each well in a concentration of 50 mg/ml. MHA plates were incubated at 37℃ for 24 hours. After incubation, the zone of inhibition was measured using a scale.

3.2.4.2 MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC is defined as the lowest concentration of any anti-microbial agent that potentially inhibits the growth of the test organism under study. MIC of the three samples were performed using broth micro-dilution method. 96-well plates for 4 different bacterial culture were filled with 100 µl of MHB. 100 µl of each sample and the positive control was added to the first well of each row respectively and serially diluted throughout the row. A serial dilution in the concentration range of 25 - 0.19 mg/ml was made for each sample and the positive control. To this, 30 µl of the bacterial inoculum was added to each well and incubated at 37℃ for 24 hours. The final volume in each well was 230 µl. After incubation, 10 µl of Resazurin dye was added to each well and incubated at 37℃ for 3 hours. After incubation, the colour change of the dye was observed to determine the anti-microbial activity of the samples.

3.2.5 COMET ASSAY

Alkaline comet assay allows us to study smaller amount of DNA damage, including ssDNA and dsDNA breaks, alkali-labile sites, DNA-protein or DNA-DNA crosslinking, and ssDNA breaks due to incomplete excision repair sites. This assay allows the visualization of the fragmented DNA as well as quantitative evaluation of the DNA damage. The underlying principle of the assay involves the migration of the fragmented DNA out of the nucleoid body (known as “comet head”) leaving a DNA stain in the agarose gel (known as “comet tail”) under an electric field.

3.2.5.1 PREPARATION OF REAGENTS

1X PBS: To make 1X PBS, 0.137M sodium chloride, 0.0027M potassium chloride, 0.01M sodium phosphate dibasic, and 0.0018M potassium phosphate monobasic was dissolved in 900mL distilled water and the pH was adjusted to 7.4 using a pH meter. The final volume was made up to 1000 mL.

Lysis solution (LS): 2.5M sodium chloride, 100mM disodium EDTA, 200mM sodium hydroxide, and 10 mM Tris base was dissolved in 900 mL distilled water. The pH was adjusted to 10 using a pH meter. Then, 1% sodium lauryl sarcosinate and 1% Triton X-100 was added and the final volume was adjusted to 1,000 mL. It was stored at 4 °C for at least 30 minutes prior to use.

Alkaline electrophoresis solution (AES): 200 mM sodium hydroxide and 1 mM disodium EDTA was dissolved in 800 mL of distilled water. The pH was adjusted to pH >13 using a pH meter. The final volume was adjusted to 1,000 mL. It was stored at 4 °C for at least 30 min prior to use.

DNA precipitation solution (DPS): To prepare 50 mL of DNA precipitation solution, 6.7 mL 7.5 M ammonium acetate was mixed with 43.3 mL 95% ethanol and stored at room temperature.

Staining solution: 1 µL 10,000x SYBR Green was added in 30 mL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.4) and stored at 4 °C under dark conditions.

1% low melting agarose: 1 g of low melting point agarose was melted in 100 mL distilled water in a microwave.

3.2.5.2 PREPARATION OF N2A CELLS

The N2A cells were subjected to Aluminium chloride to induce DNA damage and Alzheimer's disease like condition. The cells were then incubated for 24 hours at 5% CO_2 concentration and 37℃ temperature in an incubator. The cells were then divided into three separate T-flask and treated with Quercetin and Gallic acid respectively while the third flask was kept as a control. After incubation, the cells were then digested using 1mL trypsin-EDTA for 3 min at 5% CO_2 concentration and 37℃ temperature. The cells were then washed with excess PBS to stop the activity of Trypsin-EDTA and briefly centrifuged for 4-5 minutes at 300 x g to form a pellet. The pellet was resuspended at 1×10^5 cells/ml in 1X PBS. The sample was prepared in dim light to avoid DNA damage from light.

3.2.5.3 PREPARE COMET SLIDES

The glass microscope slides were dipped into the 1% low melting point agarose and wiped from one side of the slide using a lint-free wipe. The slides were then laid on a flat surface to air-dry until a transparent agarose film formed after drying. The coated slides were placed at

37 °C before use. The cell suspension was combined with 1% molten low melting point agarose (at 37 °C) at a ratio 1:10 (v/v), and 30 µL was immediately pipetted onto a slide. Using the side of the pipette tip, the agarose/cell mixture was spread on the slide to ensure the formation of a thin layer. The slides were placed at 4 °C in the dark for 10 min. The slides were then immersed at 4 °C LS in the dark for 1 hour.

3.2.5.4 SINGLE CELL ELECTROPHORESIS

Slides were placed in the electrophoresis slide tray and pre-chilled AES was added (not exceeding 0.5 cm above the slides). The power supply voltage was set to 50V and run for 30 min. After electrophoresis, the slides were gently immersed twice in distilled water for 5 min each at room temperature. The slides were then gently immersed in 70% ethanol for 5 min at room temperature.

3.2.5.4 STAIN COMET SLIDES

The slides were dried at 37 °C for 10 - 15 min in the dark. 50 µL of staining solution was added onto each dried agarose and stained for 15 min at room temperature in the dark.

3.2.5.5 IMAGE ACQUISITION AND ANALYSIS

The gel was then visualised in Alpha Imager Gel Doc as well as under the microscope in 10X magnification. The length of the comet tail was measured.

CHAPTER 4: RESULTS

4.1 COMPUTATIONAL INVESTIGATION

4.1.1 STRING DATABASE RESULTS FOR OUR QUERY PROTEINS

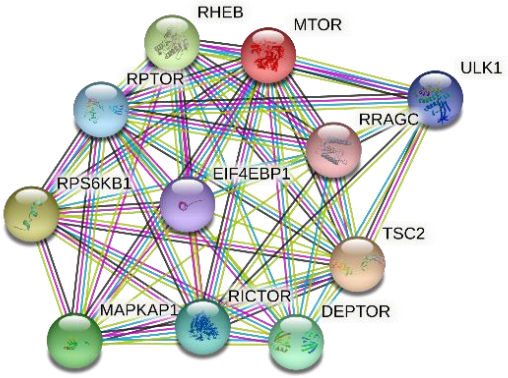

Figure 9: STRING network for MTOR. The associated proteins of the network were further analysed on the basis of their role in AD, autophagy and DNA repair. The selected proteins include RPTOR, DEPTOR, TSC1, ULK1.

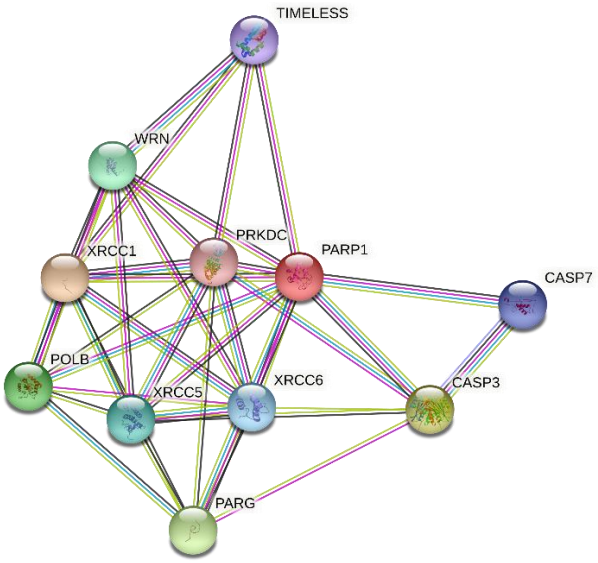

Figure 10: STRING network for PARP1. The associated proteins of the network were further analysed on the basis of their role in AD, autophagy and DNA repair. The selected proteins include POLB, XRCC-1, XRCC-5, XRCC-6, PRKDC, PARG.

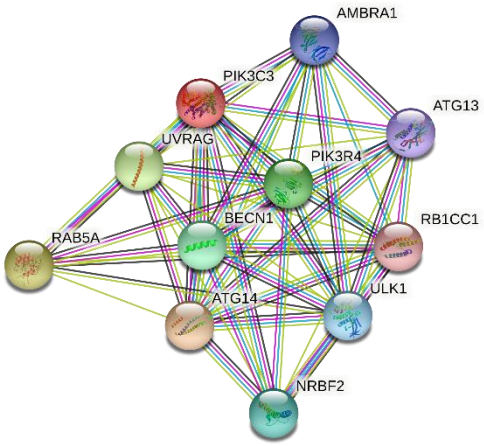

Figure 11: STRING network for UVRAG. The associated proteins of the network were further analysed on the basis of their role in AD, autophagy and DNA repair. The selected proteins include PIK3-C3, -R4, MAPILC3A, ATG14, BEECN1, DNA-PK.

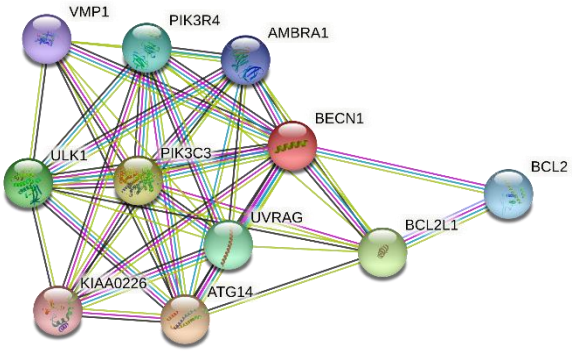

Figure 12: STRING network for BECN1. The associated proteins of the network were further analysed on the basis of their role in AD, autophagy and DNA repair. The selected proteins include PIK3-C3, -R4, AMBRA1, VMP1, ULK1, KIAA0226, ATG14, UVRAG.

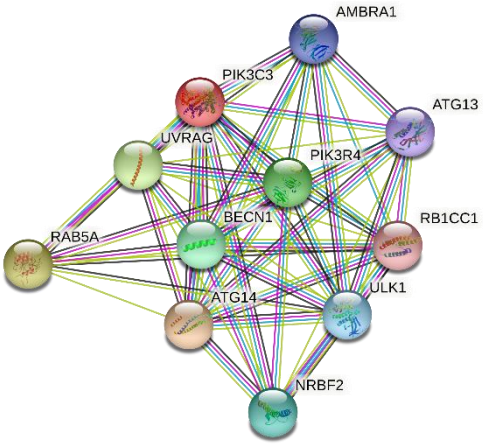

Figure 13: STRING network for PIK3C3. The associated proteins of the network were further analysed on the basis of their role in AD, autophagy and DNA repair. The selected proteins include RB1CC1, ATG13. AMBRA1, PIK3R4, UVRAG, BECN1, RAB5A.

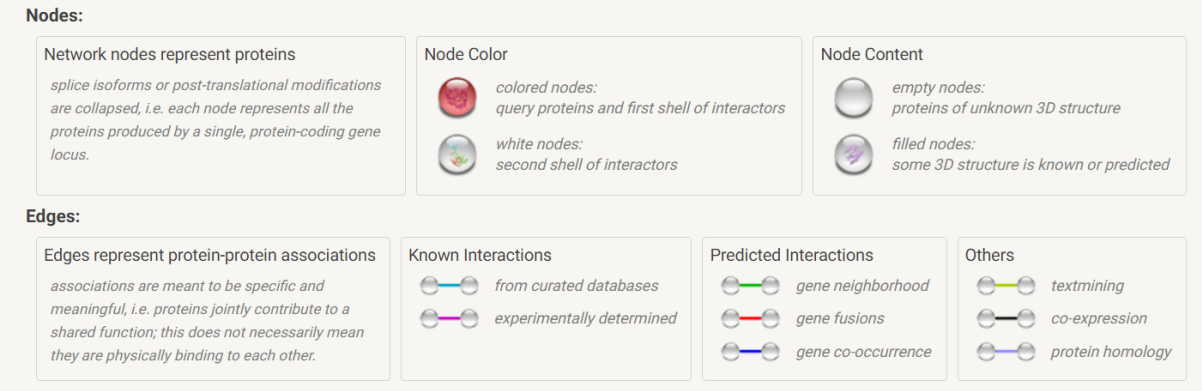

Figure 14: Representation of STRING annotations for nodes and their various interactions.

	ASSOCIATED PROTEINS				
PROTEIN	**PARP1**	**BECN1**	**PIK3C3**	**MTOR**	**UVRAG**
AUTOPHAGY		AMBRA1, PIK3-C3, R4, VMP1, ULK1, KIAA0226, ATG14, UVRAG	RB1CC1, ATG13, AMBRA1, PIK3R4, UVRAG, BECN1, RAB5A, ULK1	RPTOR, DEPTOR, TSC1, ULK1	PIK3C3, PIK3R4 MAPILC3A, ATG14, BECN1, KIAA0226
DNA REPAIR	POLB, XRCC1, XRCC5, XRCC6, PRKDC	DNA-PK	RB1CC1		DNA-PK
ALZHEIMER'S DISEASE	PARG	AMBRA1, BCL2, PIK3C3, UVRAG	AMBRA1, PIK3R4, UVRAG	DEPTOR, TSC1	PIK3C3, PIK3R4, KIAA0226

Table 2: Comprehensive representation of various associated proteins of BECN1, PARP1, PIK3C3. MTOR and UVRAG w.r.t autophagy, DNA repair and AD.

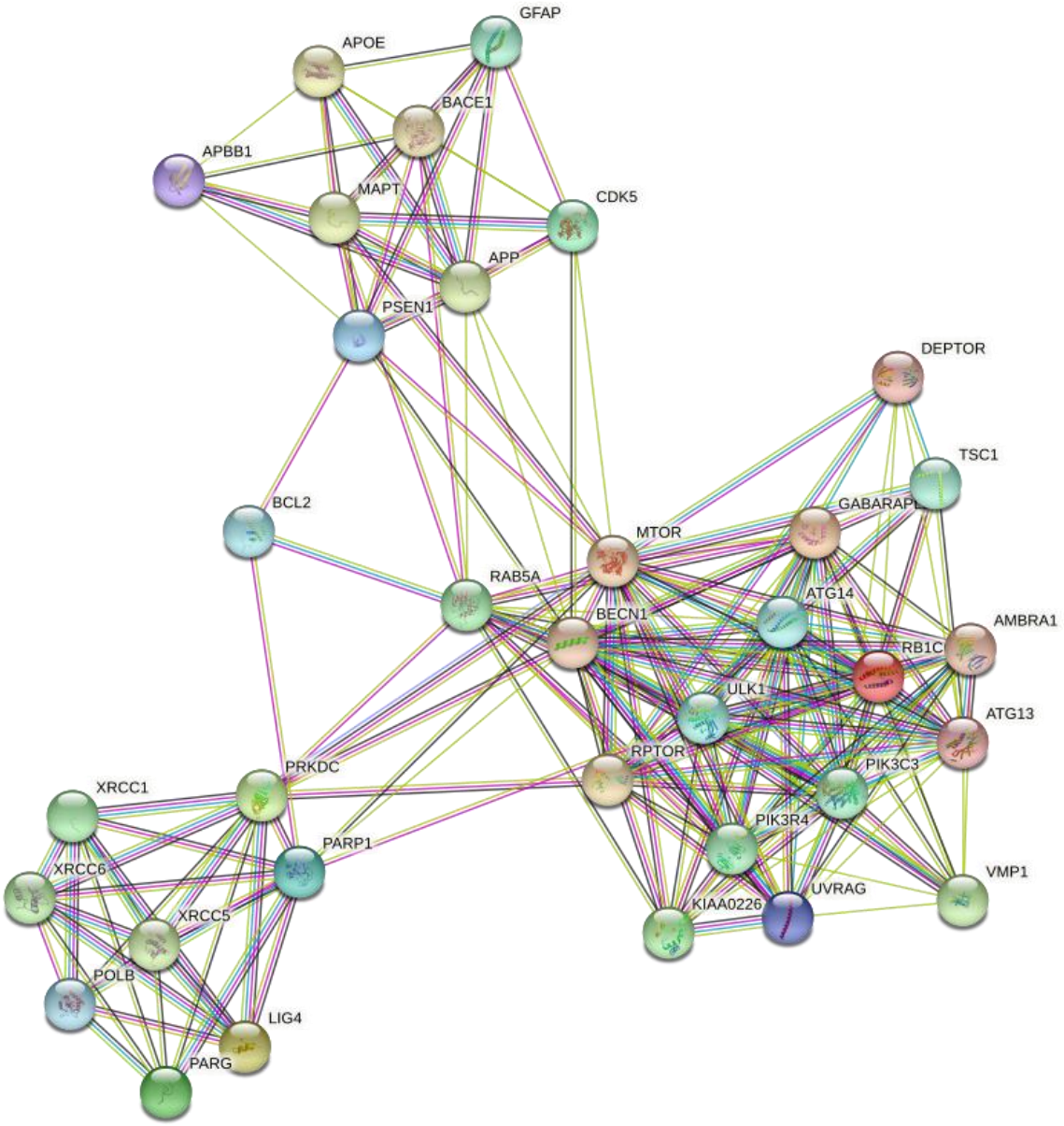

Figure 15: Self-curated STRING network for all the 34 shortlisted proteins as mentioned in Table 2 which show significant association with at least either of three criteria i.e., autophagy, DNA repair and AD. The interaction between these proteins in the network are closely linked through various parameters represented in the form of colourful interactions such as protein homology (blue), protein co-expression (black), gene fusion (red) etc. The network nodes are coloured differently based on their degree of interaction and availability of 3D structure of the protein. This network was downloaded in TSV file format for further analysis. In this network, the 3D structure of all the involved proteins is known or predicted by the scientific community.

4.1.2 DETAILED STUDY OF INDIVIDUAL PROTEINS

GENE NAME	PROTEIN NAME	LENGTH	ACCESSION NO.	FUNCTIONAL ROLE
parp1	Poly (ADP-ribose) (PAR) polymerase-1	3978 bp	NM_001618	DNA damage sensor and repair facilitator
Polb	DNA polymerase beta	1005 bp	CR541802.1	Base excision repair (BER)
xrcc1	X-ray repair cross complementary protein	1902 bp	CR456728.1	single strand break repair
xrcc5		3978 bp	NM_021141.1	Non homologous end joining (NHEJ)
xrcc6		1005 bp	CU012089.1	
Prkdc	DNA dependent protein kinase catalytic subunit	99443 bp	U63630.4	Molecular sensor for DNA damage and NHEJ
Parg	Poly (ADP-ribose) glycohydrolase	3978 bp	3978 bp	DNA replication and repair
becn1	Beclin1	1005 bp	1005 bp	Autophagosome nucleation & DNA DSB repair
ambra1	Activated molecule in BECN1 regulated autophagy protein	99443 bp	99443 bp	Protein turnover during neuronal development
bcl2	B-cell lymphoma 2	870 bp	KY098799.1	Apoptosis regulator
Rptor	Regulatory associated protein of mTOR	1269 bp	KJ902963.1	Controls mTOR activity
Deptor	DEP domain-containing mTOR-interacting proteins	1359 bp	KJ906303.1	Negative regulation of mTORC1 and mTORC2 signalling pathways
tsc2	Tuberous sclerosis complex 2	5487 bp	KJ897707.1	Negatively regulate mTORC1
atg14	Beclin-1 associated autophagy related key regulator	4742 bp	BR000826.1	Enhances PI3KC3-C1 activity

ulk1	Unc-51 like autophagy activating kinase 1	5322 bp	NM_003565.4	Downstream effector & negatively regulate mTORC1 via RPTOR
kiaa0226	Rubicon	2980 bp	BC160011.1	Impair autophagosome maturation & sequester UVRAG
pik3r4	Phosphoinositide-3-kinase regulatory subunit	4209 bp	KJ898776.1	Membrane trafficking & regulatory subunit of PI3K complex
vmp1	Vacuole membrane protein 1	1301 bp	CR533521.1	Initial stages of autophagy (with BECN1)
pik3c3	Phosphatidylinositol-3-kinase catalytic subunit	9226 bp	NM_001308020.1	Initiation & maturation of autophagosome
Uvrag	UV radiation resistance-associated gene protein	3515 bp	AB012958.1	Membrane trafficking & retrograde transport from Golgi & ER
Gabarap	Gamma aminobutyric acid receptor	351 bp	CR542235.1s	Late stage in autophagosome maturation

Table 3: Proteins with their associated gene, gene length, accession number and summarized functional role.

4.1.3 CYTOSCAPE ANALYSIS OF THE CURATED NETWORK

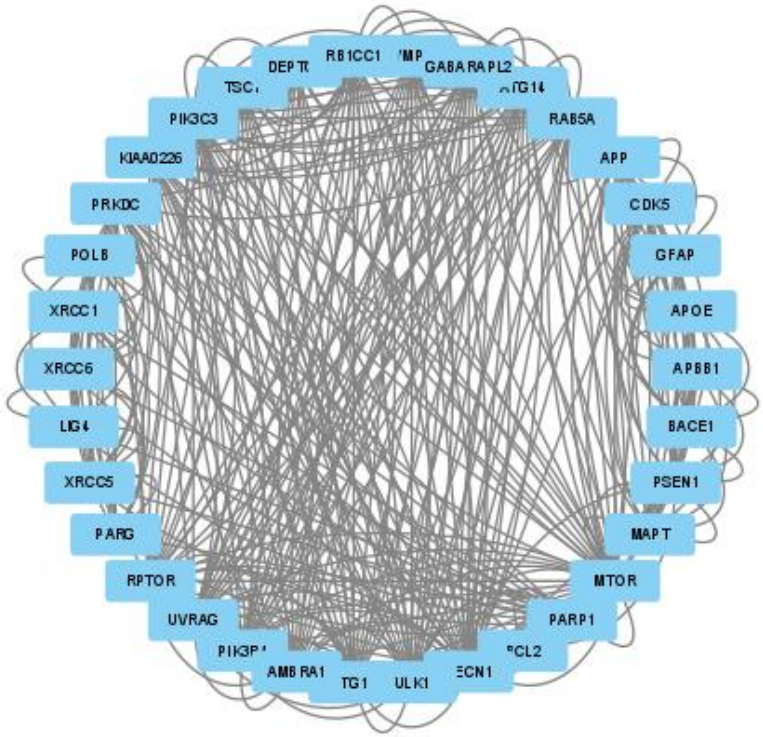

Figure 16: Cytoscape representation of the curated network in circular layout for clear visualization. This network includes 34 nodes and 368 edges. The average number of neighbours is 10.824 and the characteristic pathlength is 2.020. The clustering coefficient of the network is 0.816 with a network density of 0.398, network heterogeneity of 0.409, and network centralization of 0.328. the diameter of the network is 4.

Name	Degree	Eccentricity	Partner Of Multi-edged node Pairs	Average Shortest Path Length	Neighbour-hood Connectivity	Clustering Coefficient
MTOR	42	2	21	1.36	12.76	0.49
BECN1	42	2	21	1.36	12.61	0.49
ULK1	34	3	17	1.57	13.76	0.71
RB1CC1	32	3	16	1.78	14	0.79
PIK3C3	32	3	16	1.78	14	0.791
ATG13	30	3	15	1.84	14.06	0.81
GABARAPL2	28	3	14	1.84	15.07	0.91
ATG14	28	3	14	1.84	15.07	0.91
UVRAG	28	3	14	1.84	15.071	0.91

Table 4: The various topological parameters of the network was studied using the NetworkAnalyzer plugin available in Cytoscape. The data was sorted on the basis of degree (highest to lowest). The first ten proteins with the highest degree distribution were further analysed on the basis of other network parameters such as eccentricity, average shortest path length, neighbourhood connectivity and clustering coefficient.

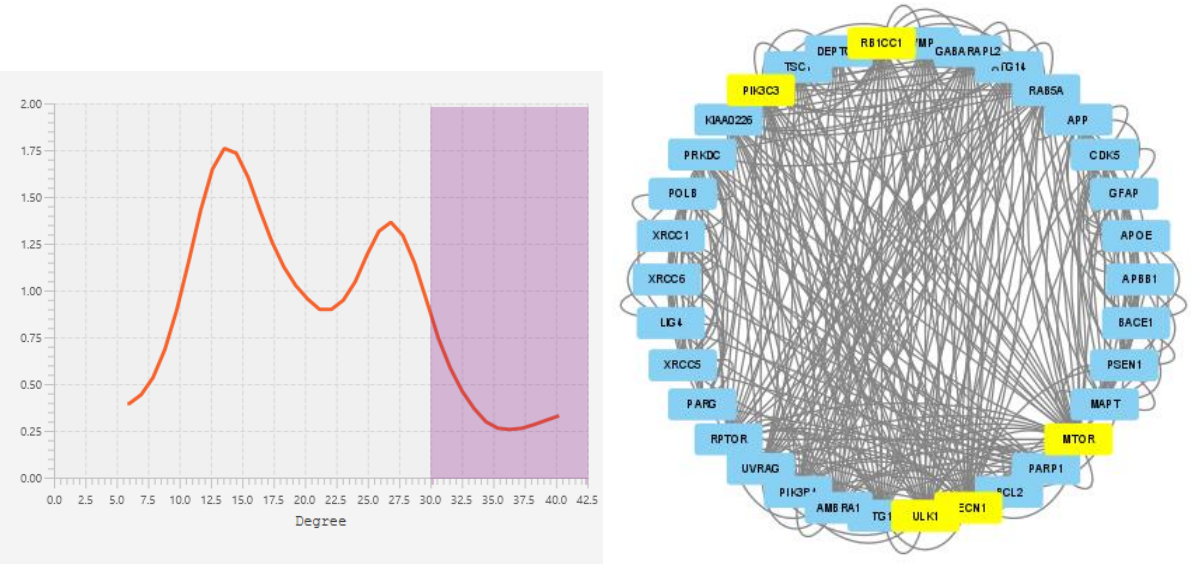

Figure 17: Cytoscape histogram for degree of nodes with a selected range of 30.8 and above highlighted 5 nodes in the network including PIK3C3, RB1CC1, ULK1, BECN1, and MTOR.

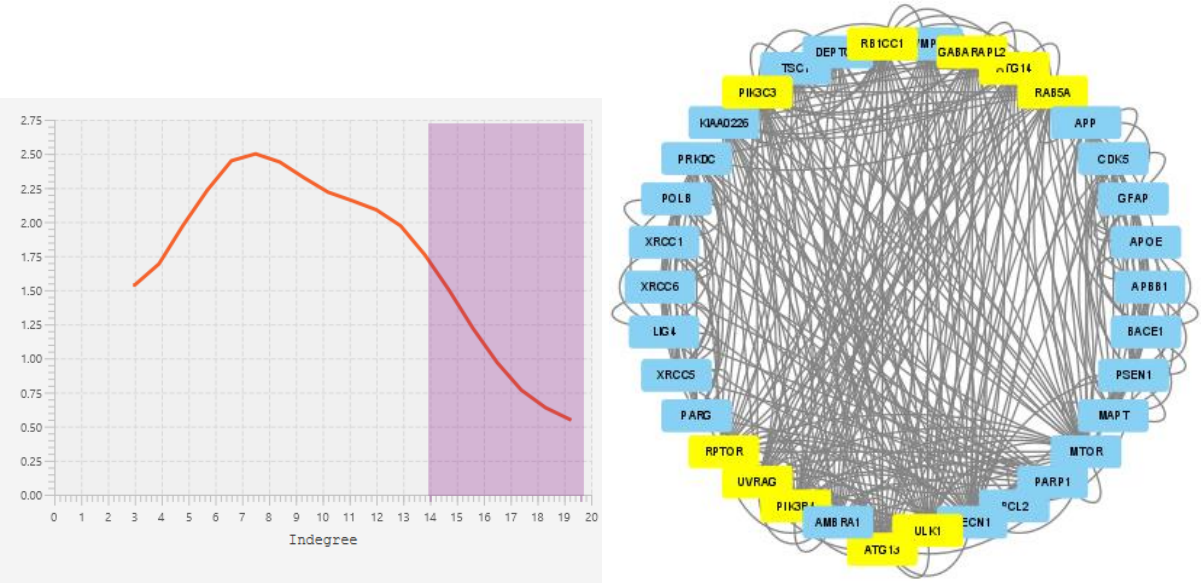

Figure 18: Cytoscape histogram for indegree of nodes with a selected range of 14 and above highlighted 10 nodes in the network including PIK3C3, RB1CC1, GABARAPL2, ATG14, RAB5A, ULK1, ATG13, PIK3R4, UVRAG and RPTOR.

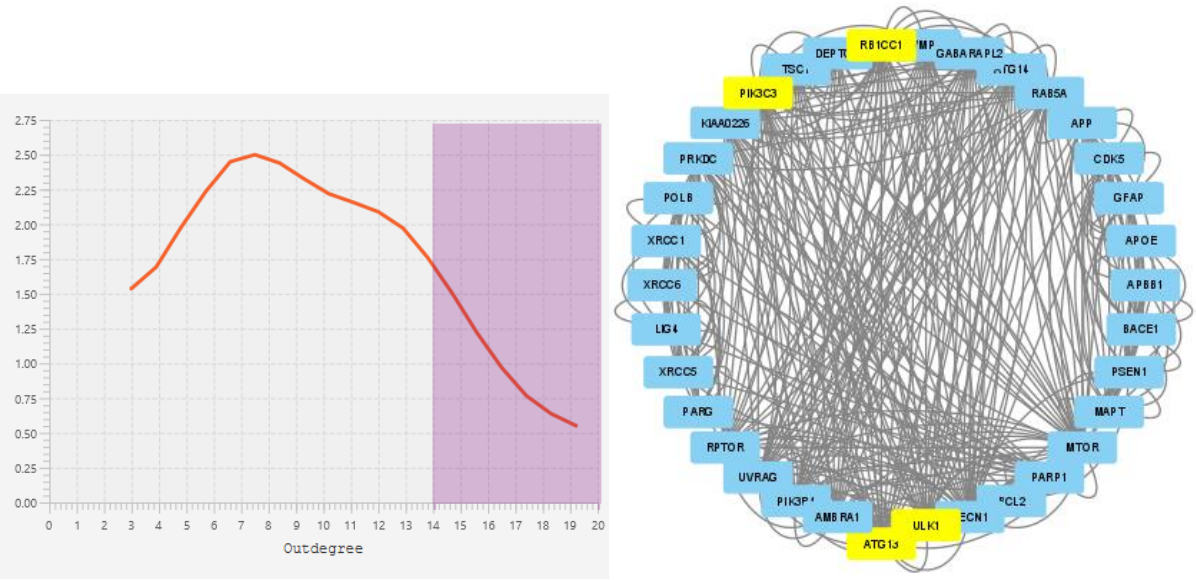

Figure 19: Cytoscape histogram for outdegree of nodes with a selected range of 14 and above highlighted 4 nodes in the network including PIK3C3, RB1CC1, ULK1, and ATG14.

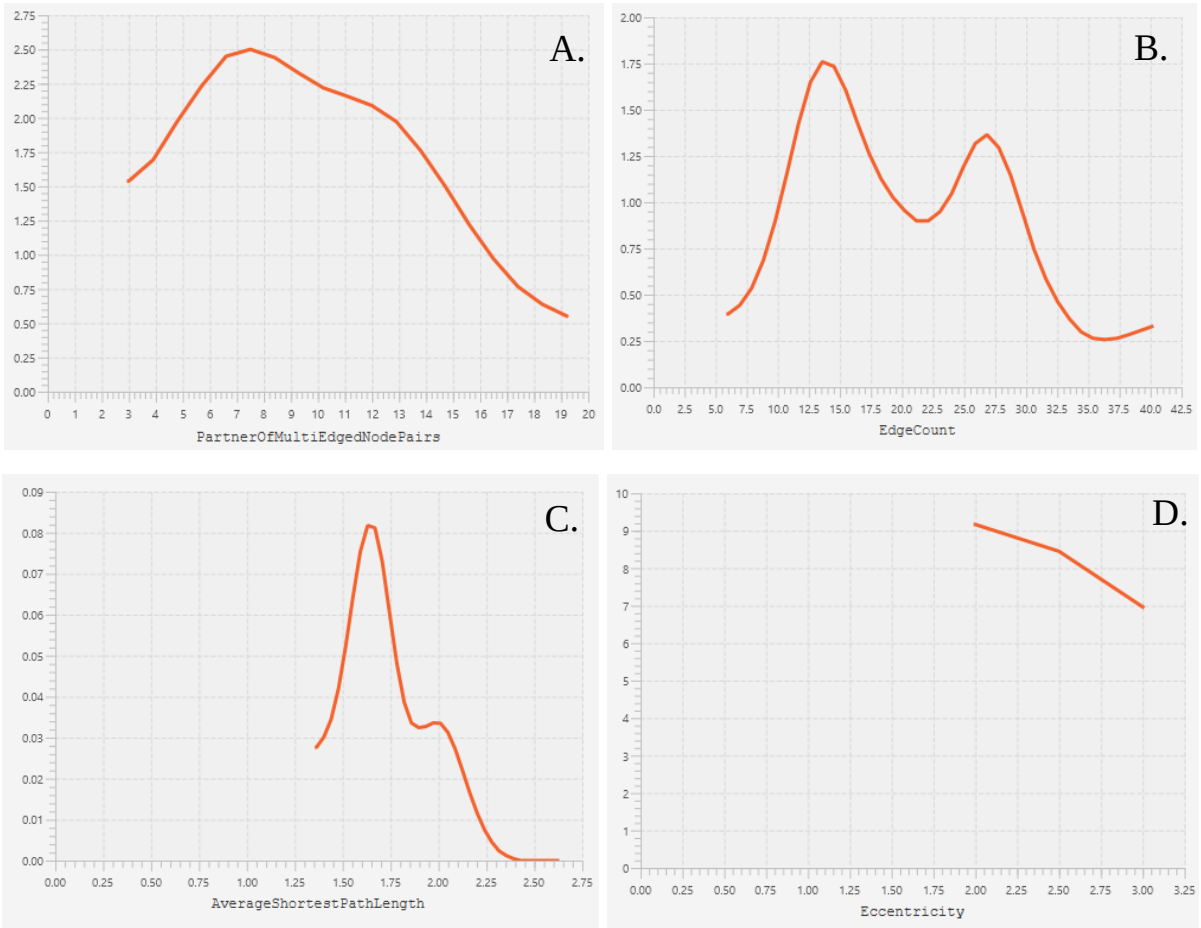

Figure 20: Various network topology parameters were analysed through individual histograms plotted with respect to the average nodes involved respectively. Several parameters such as

partner of multi-edged nodes, average shortest path length, eccentricity, and edge count was analysed through these histograms.

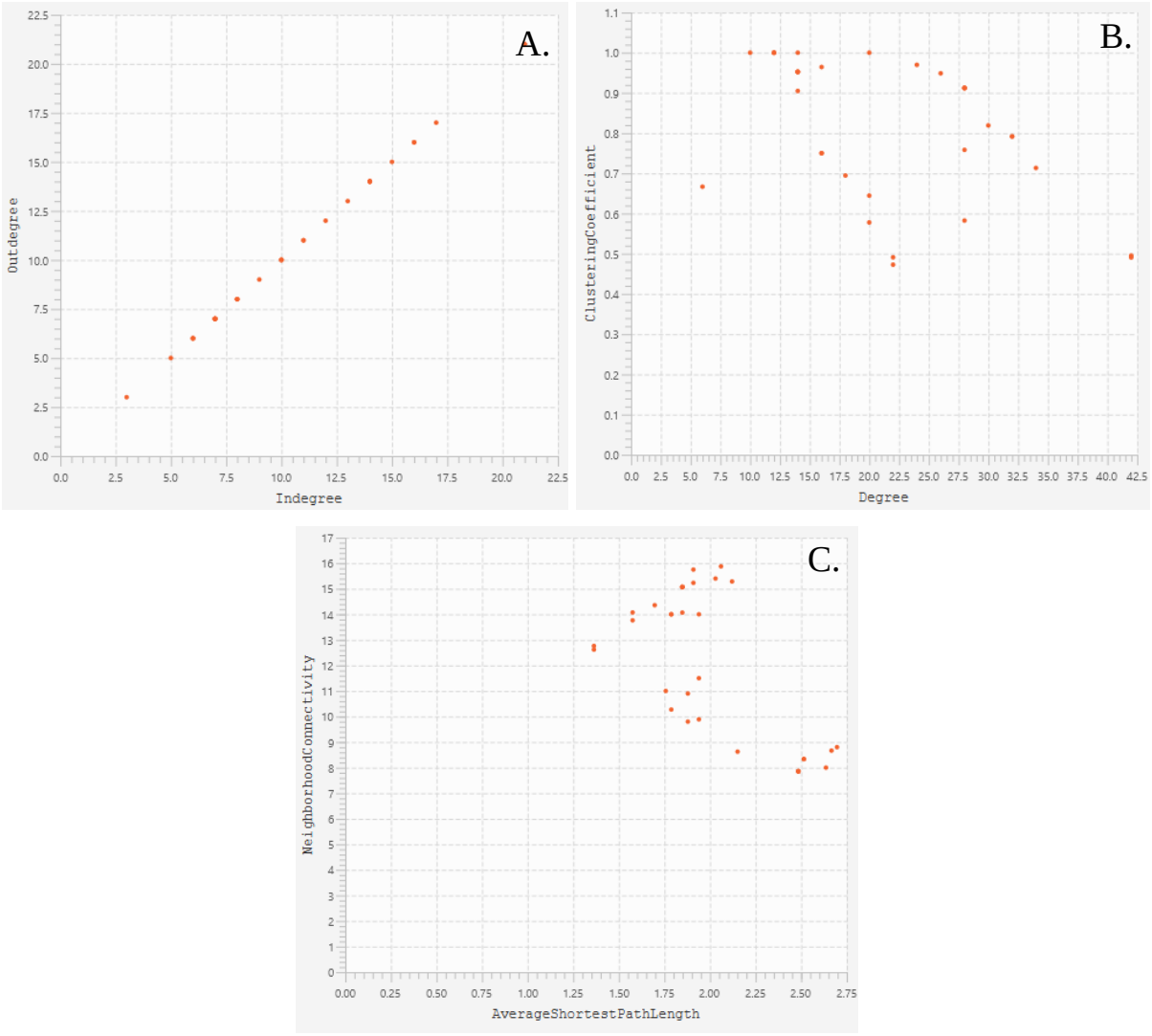

Figure 21: Scattered graphs were plotted relating two different network parameters to locate the distribution of nodes in the plot. The first plot, indegree v/s outdegree represents a linear distribution of edges throughout the network. The second plot, degree v/s clustering coefficient represents the distribution of edges among the various clusters or subnets in the network. The third plot, average shortest path length v/s neighbourhood connectivity represents the connectivity of the network by relating it with the shortest distance between the nodes.

4.1.4 DATA COLLECTION FOR MODEL CONSTRUCTION

PROTEIN	ROLE	
	AUTOPHAGY	ALZHEIMER'S DISEASE
AMBRA1	Positive regulator of the Becn1-dependent programme of autophagy	Protein turnover during neuronal development; regulates normal cell survival and proliferation [133]
PIK3R4	Regulatory subunit of PI3K complex involved in initiation and maturation of autophagosome	Involved in the PIK3C3 complex to maintain the brain homeostasis
ULK1	Triggers autophagosome formation in response to starvation Acts upstream on PIK3C3 (regulates formation of autophagophore)	Inhibition of the autophagic protein ULK1 attenuates axonal degeneration in vitro and in vivo, enhances translation and modulates splicing [134]
PIK3C3	PIK3C3-C1is responsible for initiation of autophagosome and PIK3C3-C2 for maturation of autophagosome and endocytosis	Loss causes neuronal degradation
PARP1	Positive regulator of autophagy induced by DNA damage, ROS, and starvation	In neurons, PARP1 can induce a special type of cell death, 'parthanatos', and impedes autophagy, leading to the accumulation of toxic proteins [109]
BECN1	Acts as a core subunit of the PIKC3 complex and regulates autophagosome formation	Heterozygous deletion of BECN1 in mice decreased neuronal autophagy and resulted in neurodegeneration and disruption of lysosomes [112]
UVRAG	Promotes autophagosome formation and maturation along with BECN1 and PIK3C3	Regulation of neuronal necroptosis along with TNF-α signaling and RIPK1/3 activity [15]
RB1CC1	Early and late events of autophagosome	RB1CC1 insufficiency causes neuronal atrophy through mTOR

		signaling alteration and involved in the pathology of AD [16]
BCL2	BCL2 is an anti-apoptotic factor that interacts with BECN1 to regulate autophagy	Apoptosis in neural cells
RAB5A	Vacuolin-1 can block the autophagosome-lysosome fusion by activating Rab5a; inactivation of Rab5a results in reduced mTOR activity and disordered intracellular localization of mTOR [21]	RAB5A is critical for the complete endocytosis of exogenous α-synuclein; excess causes neuronal cell death
CDK5	CDK5 is an autophagy-regulating kinase; enriched expression in CNS	Influences the metabolism and effects of Aβ; involved in Tau metabolism
VMP1	Initial stages of autophagic process through BECN1 interaction	Overexpression of VMP1 can trigger autophagy, which could lead to neuronal clearance of accumulated proteins in AD
mTORC	Regulates the formation of phagophore and autophagosome, the degradation of autolysosomes, and the reformation of autophagic lysosomes	Activation of mTOR induces Aβ production and aggregation by direct inhibiting autophagy/lysosome system [17]
GABARAP	Covalent attachment to lipid membrane is essential for growth and closure of auto phagophore[24]	Essential for engulfing damaged mitochondria into phagophores and vesicle formation in mitophagy
DNA-PK	Regulates lysosomal AMP-dependent protein kinase activation and autophagy [4]	Essential role in repairing double-strand DNA breaks [3]
GFAP	Binds withLAMP2A	Increased levels of GFAP in tissues of AD patients

Table 5: Bidirectional role of individual protein/protein complexes in autophagy and AD

PROTEIN	ROLE	
	AUTOPHAGY	**DNA DAMAGE AND REPAIR**
mTORC	Regulates formation of autophagosome, the degradation of autolysosomes, and the reformation of autophagic lysosomes	Binds to DNA-PK and hinders its activity in NHEJ and HR [5]
PARG	Causes PARP1-induced loss of ATP. Excessive activity causes energy depletion in cell triggering autophagy	PARG is involved in DNA replication and repair and PARG depleted/inhibited cells show increased sensitivity to DNA damaging agents
CDK5	CDK5 is an autophagy-regulating kinase; enriched expression in CNS	Cdk5 attenuates DNA repair pathway through phosphorylation of Ape1 in neuronal death in vitro and in vivo [82]
GABARAP	Covalent attachment to lipid membrane is essential for growth and closure of auto phagophore	Prevents excess ROS production
PARP1	PARP1 in mediating various aspects of DNA metabolism, such as SSB repair, nucleotide excision repair, DSB repair	In neurons, PARP1 can induce a special type of cell death, 'parthanatos', and impedes autophagy, leading to the accumulation of toxic proteins [90]
BECN1	Attenuates the formation of DNA-PK complexes	Heterozygous deletion of BECN1 in mice decreased neuronal autophagy and resulted in neurodegeneration and disruption of lysosomes [90]
DNA-PK	Plays a critical role in DSB repair and in V(D)J recombination; triggers apoptosis in response to severe DNA damage or critically shortened telomeres	Reduced activity in AD brains leading to reduced NHEJ activity in response to DSB repair [18]

AMBRA1	Downregulation of AMBRA1 enhanced DNA replication stress; associated with Cyclin D	Protein turnover during neuronal development; regulates normal cell survival and proliferation [133]
ULK1	In response to DNA damage, p53 activates ULK1 to initiate autophagy	Inhibition of the autophagic protein ULK1 attenuates axonal degeneration in vitro and in vivo, enhances translation and modulates splicing [103]
RB1CC1	Early and late events of autophagosome	Repair of DNA damage caused by ionizing radiation

Table 6: Bidirectional role of individual protein/protein complexes in autophagy and DNA repair.

PROTEIN	**ROLE**	
	DNA DAMAGE AND REPAIR	**ALZHEIMER'S DISEASE**
RB1CC1	Repair of DNA damage caused by ionizing radiation	RB1CC1 insufficiency causes neuronal atrophy through mTOR signaling alteration and involved in the pathology of AD
BCL2	Bcl2 negatively regulates DNA double-strand break repair through a NHEJ pathway [133]	Apoptosis in neural cells
MTORC	Binds to DNA-PK and hinders its activity in NHEJ and HR	Activation of mTOR induces Aβ production and aggregation by direct inhibiting autophagy/lysosome system [105]
GABARAP	Prevents excess ROS production	Essential for engulfing damaged mitochondria into phagophores and vesicle formation in mitophagy
CDK5	Cdk5 attenuates DNA repair pathway through phosphorylation of Ape1 in neuronal death in vitro and in vivo	Influences the metabolism and effects of Aβ; involved in Tau metabolism [82]

UVRAG	Helps in the assembly of DNA-PK and activates DNA-PK to maintain the stability of chromosomes through modulation of the NHEJ repair	Regulation of neuronal necroptosis along with TNF-α signaling and RIPK1/3 activity [116]
PARP1	PARP1 in mediating various aspects of DNA metabolism, such as SSB repair, nucleotide excision repair, DSB repair [90]	In neurons, PARP1 can induce a special type of cell death, 'parthanatos', and impedes autophagy, leading to the accumulation of toxic proteins [111]
BECN1	Attenuates the formation of DNA-PK complexes	Heterozygous deletion of BECN1 in mice decreased neuronal autophagy and resulted in neurodegeneration and disruption of lysosomes [112]
DNA-PK	Plays a critical role in DSB repair and in V(D)J recombination; triggers apoptosis in response to severe DNA damage or critically shortened telomeres	Reduced activity in AD brains leading to reduced NHEJ activity in response to DSB repair [18]
AMBRA1	Downregulation of AMBRA1 enhanced DNA replication stress; associated with Cyclin D	Protein turnover during neuronal development; regulates normal cell survival and proliferation [133]
ULK1	In response to DNA damage, p53 activates ULK1 to initiate autophagy	Inhibition of the autophagic protein ULK1 attenuates axonal degeneration in vitro and in vivo, enhances translation and modulates splicing [134]

Table 7: Bidirectional role of individual protein/protein complexes in DNA repair and AD

PROTEIN	AUTOPHAGY	DNA DAMAGE AND REPAIR	ALZHEIMER'S DISEASE
PARP1	YES	YES	YES
PIK3R4	YES		YES
BCL2	YES	YES	YES
ULK1	YES	YES	YES
PIK3C3	YES		YES
PARG		YES	YES
AMBRA1	YES	YES	YES
DNA-PK	YES	YES	YES
VMP1	YES		YES
GFAP		YES	YES
BECN1	YES	YES	YES
RB1CC1	YES	YES	YES
BCL2	YES	YES	YES
RAB5A	YES		YES
UVRAG	YES	YES	YES
GABARAP	YES	YES	YES
CDK5	YES	YES	YES
MTORC	YES	YES	YES

Table 8: Summarization of the role of individual protein/protein complexes in AD with relevance to autophagy and DNA repair.

4.1.5 E-CELL MODEL AND ITS ANALYSIS

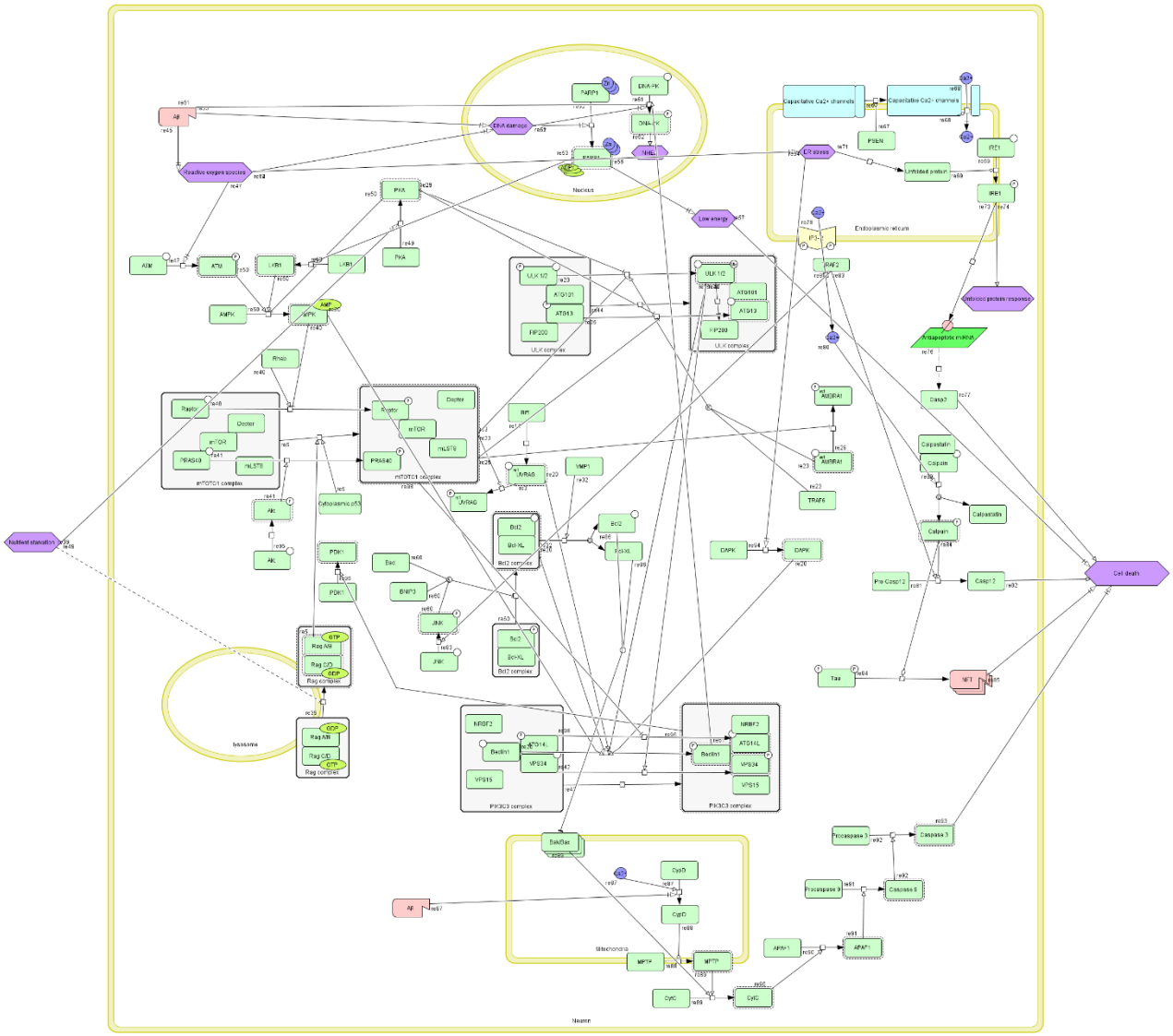

Figure 22: Exclusive E-cell model of AD with relevance to autophagy and DNA repair mechanisms. This model includes various protein-protein interactions in the form of state transition, inhibition, physical stimulation, modulation, trigger, phosphorylation, ubiquitination, catalysis etc. The model also incorporates the effect of these interactions on various organelles of the E-cell such as mitochondria, nucleus, endoplasmic reticulum and lysosome. Various triggering signals and cellular stresses such as ROS, nutrient starvation, DNA damage etc. were also included in the form phenotypes which finally contributed towards neuronal death. According to Cytoscape NetworkAnalyzer plugin, this model includes 133 nodes and 168 edges with 2.33 average number of neighbours. The model incorporates 13 multi-edged node pairs signifying the high connectivity of the network.

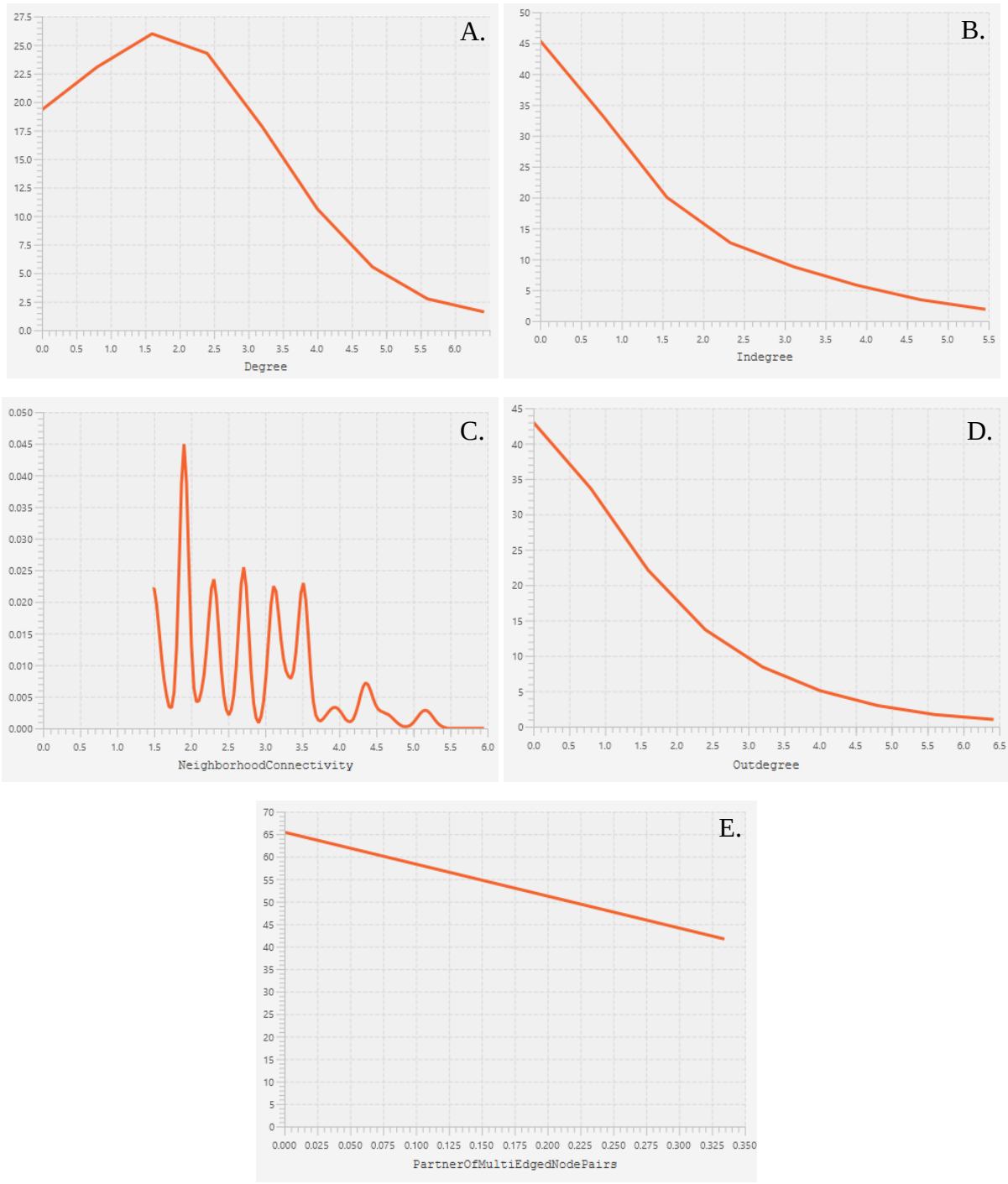

Figure 23: Various network topology parameter such as degree, indegree, outdegree, neighbourhood connectivity, and partner of multi-edged nodes was analysed through individual histograms plotted with respect to the average nodes involved respectively through Cytoscape NetworkAnalyzer plugin.

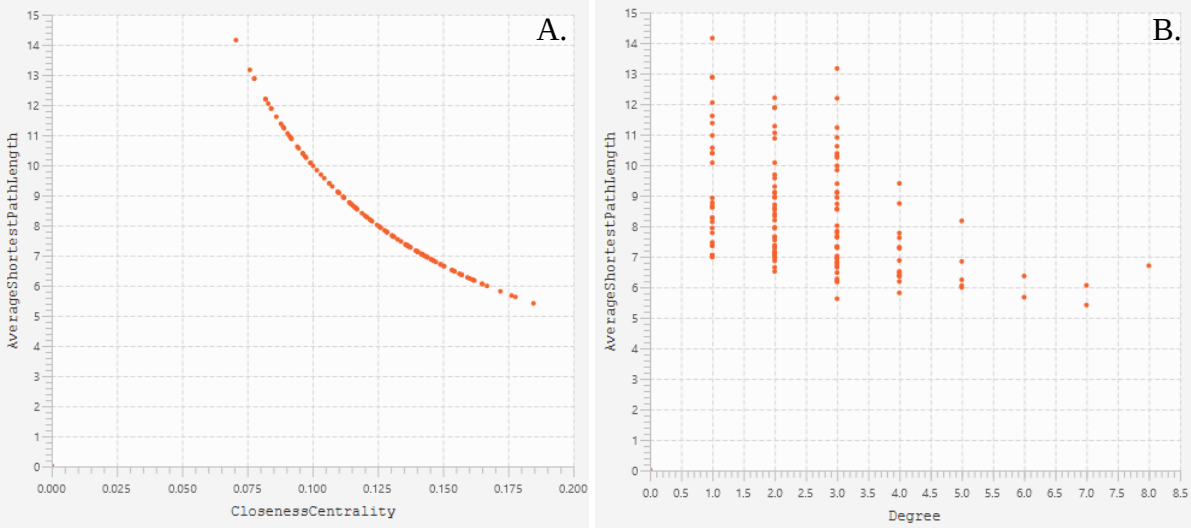

Figure 24: Scattered graphs were plotted relating two different network parameters to locate the distribution of nodes in the plot. The first plot A, average shortest path length v/s closeness centrality represents the connectivity of the network by relating it with the shortest distance between individual nodes. The second plot B, degree v/s average shortest pathlength represents the number of edges and their lengths w.r.t each node.

4.2 EXPERIMENTAL VALIDATION

4.2.1 HEMOCYTOMETRY

The percentage of live N2A cells came up to 94.56% indicating that most cells are in log phase of growth. The total number of cells came up to 3.202×10^7 cells/ml.

4.2.2 CELL PROLIFERATION AND CYTOTOXICITY ASSAY

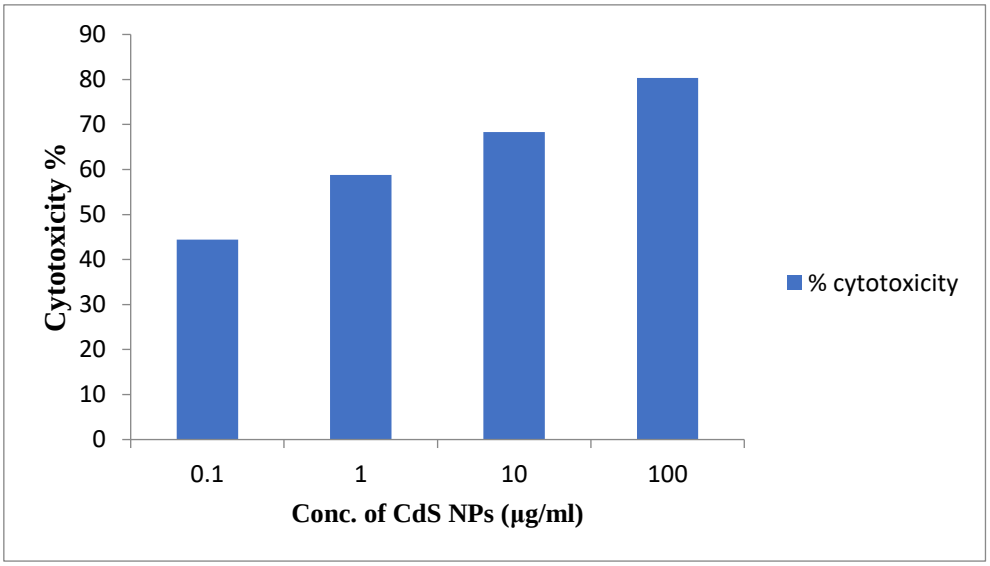

Figure 25: Concentration of Cadmium sulphide nanoparticles (µg/ml) v/s percentage cytotoxicity on N2A cell line. This graph demonstrates that with the increase in concentration of CdS NPs, the percentage cytotoxicity on N2A cell line also increases.

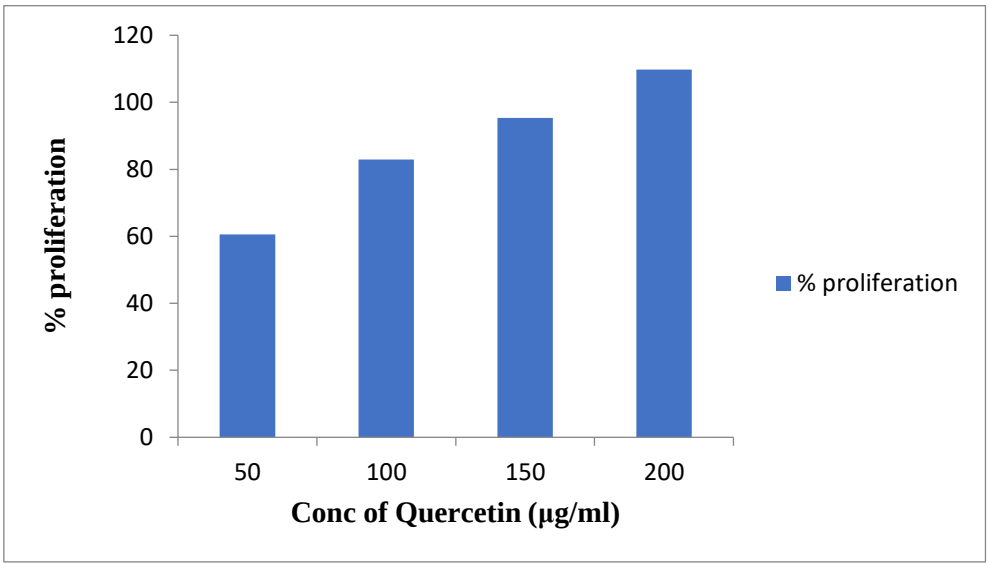

Figure 26: Concentration of Quercetin (µg/ml) v/s percentage proliferation activity on N2A cell line. This graph demonstrates that with the increase in concentration of Quercetin, the percentage proliferation of N2A cell line also increases.

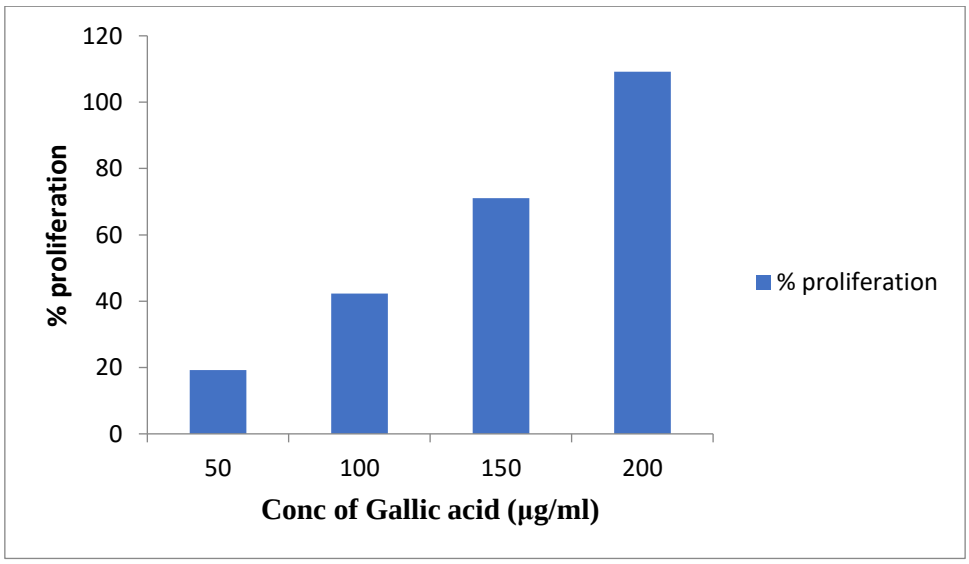

Figure 27: Concentration of Gallic acid (µg/ml) v/s percentage proliferation activity on N2A cell line. This graph demonstrates that with the increase in concentration of gallic acid, the percentage proliferation of N2A cell line also increases.

4.2.3 ANTI-MICROBIAL ACTIVITY TEST

4.2.3.1 WELL DIFFUSION ASSAY

BACTERIAL STRAINS	ZONE OF INHIBITION (mm)			
	Cadmium Sulphide Nanoparticles	Quercetin	Gallic acid	Positive control (Ampicillin)
Escherichia coli (E. coli)	Not detected	12	14	16
Salmonella typhi (S. typhi)	Not detected	16	10	13
Staphylococcus aureus (S. aureus)	Not detected	18	13	30
Bacillus subtilis (B. subtilis)	Not detected	20	15	15

Table 9: Summarization of sample's zone of Inhibition (in mm) for different bacterial strains.

4.2.3.2 MINIMUM INHIBITORY CONCENTRATION

BACTERIAL STRAINS	MIC (μg/ml)			
	Cadmium Sulphide Nanoparticles	Quercetin	Gallic acid	Positive control (Ampicillin)
Escherichia coli (E. coli)	Not detected	6.25	3.13	0.39
Salmonella typhi (S. typhi)	Not detected	3.13	3.13	0.39
Staphylococcus aureus (S. aureus)	Not detected	1.56	6.25	1.56
Bacillus subtilis (B. subtilis)	Not detected	1.56	3.13	0.78

Table 10: Summarization of sample's minimum inhibitory concentration (in μg/ml) for different bacterial strains.

4.2.4 COMET ASSAY

TREATMENT OF N2A CELLS	TAIL LENGTH (mm)
Quercetin	23
Gallic acid	21
Control	32

Table 11: Tail length (mm) of comet in comet assay with relevance to the treatment provided to N2A cells. The longer the tail of the comet, the greater is the DNA damage.

CHAPTER 5: DISCUSSION

AD is one of the most common forms of dementia seen in the elderly with increasing number of cases each year. The multifactorial nature of the disease makes it an interesting subject to investigate the various factors that contribute towards the etiology and pathogenesis of the disease. In this study, we have tried to investigate the role of autophagy and DNA repair combinatorically towards the pathogenesis of AD. For this analysis, various autophagy and DNA repair related proteins in relation to AD were selected and analysed through various databases and tools.

After a comprehensive literature survey, a set of proteins were selected which demonstrated exclusive contribution towards AD with relevance to autophagy and DNA repair. The STRING (version 11.5) database was further used to probe associated proteins to expand the spectrum of study as mentioned in Table 2. These proteins were used to build a network exclusively associated to our study using the STRING database as mentioned in Figure 15. This network helped in conforming the existing protein-protein interaction supporting the objective of the study. Before proceeding, the individual role of each protein was confirmed with the support of scientific literature as mentioned in Table 3. For better visualization and analysis of this network (Figure 15), it was imported in Cytoscape (version 3.9.1) and analysed using the NetworkAnalyzer plugin as observed in Figure 16. Table 4 gives us an overview of the various network topology parameters w.r.t to the key regulatory nodes of the network with the maximum degree distribution. The top three protein/ protein complexes that stood out in this analysis were mTORC1 (mammalian target of Rapamycin complex 1), BECN1 (Beclin 1), and ULK1 (Unc-51 like autophagy activating kinase 1) complex as they demonstrated maximum connectivity throughout the network regulating several other proteins. The network was exploited further for better understanding of the network parameters such as degree, indegree, outdegree, eccentricity, average shortest path length, partner of multi-edged nodes etc. by plotting quantitative histograms as mentioned in Figure -17, -18, -19, -20, and -21.

Several scientific literature and databases such as PANTHER, KEGG etc. were explored to collect appropriate data for model construction. The goal of this extensive study was to collect all the essential information to construct a virtual cell model with the entire AD pathogenesis pathway keeping autophagy and DNA repair as the key regulatory mechanisms (Table -5, -6,

and -7). This helped prioritize proteins which showed indispensable role in all the three criteria of the study i.e., autophagy, DNA damage and repair, and AD. The goal was achieved employing CellDesigner (version 4.4.2) which allows the construction of E-cells displaying protein-protein interactions, protein-DNA interactions, gene expression regulation, and other metabolic activity of an actual biological cell with a set of reaction rule. An exclusive model was constructed using the essential SBGN annotations (Figure 8) in CellDesigner as mentioned in Figure 22.

The constructed model (Figure 22) includes various protein-protein interactions in the form of state transition, inhibition, physical stimulation, modulation, trigger, phosphorylation, ubiquitination, catalysis etc. The model also includes the effect of these interactions on various organelles of the E-cell such as mitochondria, nucleus, endoplasmic reticulum and lysosome. This was an essential part of the study as mitochondrial dysfunction; endoplasmic reticulum stress and lysosomal dysfunction are essential hallmarks of the etiology of AD. Various triggering signals and cellular stresses such as ROS, nutrient starvation, DNA damage etc. were also included in the form phenotypes which finally contributed towards neuronal death. The hallmarks of AD i.e., Aβ plaques and NFTs were also included in the model for better analysis of the model. This model also comprises of ion channels involved in Ca^{2+} ion influx imbalance in neuronal cells as commonly observed in AD brains.

The model comprises of three major protein complexes i.e., mTORC1 complex, ULK1 complex and PIK3C3 complex. Each complex is intensely regulated by other protein/protein complexes. The mTORC1 complex comprises of five proteins: Raptor, Deptor, mTOR, PRAS40, and mLST8. These proteins are regulated by each other as well as other proteins. Association of PRAS40 and Deptor with mTOR keeps the complex in inactive state. However, dissociation of PRAS40 due to its phosphorylation by Akt initiates the activation of the complex. Following association of Raptor and mLST8 with mTOR leads to the formation of the active complex. Association of Raptor to mTOR by its phosphorylation is physically stimulated by Rheb and inhibited by activated AMPK. The activation of mTORC1 complex is a;so regulated by the prersence of Cytoplasmic p53 and the Rag complex. This activated complex plays major role in regulating autophagy as well as DNA repair pathways contributing towards the pathogenesis of AD. Activated mTORC1 complex stimulates phosphorylation of activated UVRAG rendering it inactive. UVRAG is an essential protein in the intial steps of autophagosome formation during autophagy. Activated mTORC1 complex also inhibits the activation of another important autophagy complex, ULK1 complex.

The ULK1 complex comprises of four proteins: ULK1/2, ATG101, ATG13, and FIP200. The activation of this complex is regulated by various proteins such as PKA, AMBRA1, mTORC1, and TRAF. The activation of ULK1/2 by it ubiquitination and dephosphorylation is inhibited by PKA and mTORC1 complex and physically stimulated by AMBRA1 and TRAF. Similarly, the activation of ATG13 by its dephosphorylation is inhibited byPKA and mTORC1 complex. The third essential complex in this model is the PIK3C3 complex comprising of NRB2, VPS15, Beclin1, ATG14L, and VPS34. The activation of ATG14L by its dephosphorylation is inhibited by mTORC1 complex. The activation of Beclin1 by its phosphorylation is physically stimulated by activated AMPK, activated ULK1/2, activated DAPK, and activated UVRAG while inhibited by Bcl2/Bcl-XL complex. Beclin1 is an essential protein with significant role in autophagy, DNA repair and AD. Beclin1 knockout models in mice show Alzheimer like conditions establishing its importance in AD.

Aβ toxicity in the neuron hinders the functioning of mitochondria triggering a cascade of reaction and the activation of Caspases. These caspases are responsible in cellular apoptosis leading to the death of neuron. Elevated levels of PARP1 in the nucleus due to excessive DNA damage causes activation of NHEJ. However, excessive PARP1 activity leads to low energy in the cell due to NAD^+ deficiency, tiggering cell death. Similarly, endoplasmic reticulum under stress conditions degrades antiapoptotic miRNA triggering Caspase 2 to cause cell death. Hyperphosphorylation of Tau protein leads to the formation and accumulation of truncated NFT in the neuron, tiggering cell death. Activated Calpain plays an evident role in this hyperphosphorylation of Tau. Excess cytoplasmic Ca^{2+} ion triggers the activation of Calpain which further triggers the activation of Caspase 12 triggering cellular apoptosis. All of these incorporations in the E-cell helped build a near real biological neuron for AD pathogenesis. The constructed model was further analysed using Cytoscape for various network topology parameter such as degree, indegree, outdegree, neighbourhood connectivity, and partner of multi-edged nodes through individual histograms plotted with respect to the average nodes.

After this intensive computational investigation, an experimental routine was designed to validate the results for the key protein complexes involved i.e., mTOR, ULK1 and PIK3C3. Plant based inhibitors of mTORC1 complex and promoters of PIK3C3 complex (including Beclin 1) were searched to study their effect on cell lines. Neuro 2A (N2A) cell line, derived from neural crest cells of mouse, was put to use for the experimental work. The cell viability was determined by hemocytometry before proceeding with the experiment. The percentage of

live N2A cells came up to 94.56% indicating that most cells are in log phase of growth. The total number of cells came up to 3.202×10^7 cells/ml.

After confirming the viability of the N2A cells, MTT assay was performed to determine the viability of the cells with our samples i.e., CdS NPs, Quercetin and Gallic acid. CdS NPs were found to be cytotoxic with an increasing concentration (0.1-100 µg/ml) and the percentage cytotoxicity varied from 44.41-80.3 % on N2A cells as mentioned in Figure 24. On the other hand, Quercetin was found to have a proliferative role on the cells with an increasing concentration (50-200 µg/ml) as the percentage proliferation varied from 60.5-100% on N2A cells as mentioned in Figure 25. Similarly, Gallic acid was also found to have a proliferative role on the cells with an increasing concentration (50-200 µg/ml) as the percentage proliferation varied from 20.75-100% on N2A cells as mentioned in Figure 26. Through this experimentation, we can conclude that the selected plant-based inhibitors are non-cytotoxic, rather, they have proliferative effect on the cells.

Both Quercetin and Gallic acid showed anti-microbial activity against *Escherichia coli* (E. coli), *Salmonella typhi* (S. typhi), *Staphylococcus aureus* (S. aureus), and *Bacillus subtilis* (B. subtilis) in both well diffusion assay as well as MIC. Quercetin's minimum inhibitory concentration against S. aureus and B. subtilis is up to 1.56 µg/ml while against E. coli and S. typhi, is up to 6.25 µg/ml and 3.13 µg/ml. Similarly, Gallic acid's minimum inhibitory concentration against S. aureus, E. coli and B. subtilis is up to 3.13 µg/ml while its 6.25 µg/ml against S. typhi. Quercetin is a flavonoid group of poly-phenol which contributes towards its anti-microbial activity. Similarly, Gallic acid is phenolic compound and the phenolic group contributes towards the anti-microbial properties.

Comet assay is a single-cell gel electrophoresis technique which allows the in-vitro evaluation of DNA damage and repair. Results from alkaline comet assay showed that the comet tail of Aluminium chloride-treated N2A cells (control) was longer (32 mm) and had higher DNA intensity, suggesting a substantial accumulation of fragmented DNA due to the DNA damaging activity of Aluminium chloride. However, when the cells were further treated with Quercetin and Gallic acid, the comet tail was found to be shorter i.e., 23 mm and 21 mm respectively. This signifies that treatment with Quercetin and Gallic acid helped reduce DNA damage caused by the Aluminium chloride. Through this assay, we can conclude that Quercetin and Gallic acid not only has proliferative activity towards N2A cells but also has DNA repairing role towards N2A cells.

These findings support the results found through our computational investigation suggesting the DNA damage and repair activity of mTORC1 and Beclin1. As Quercetin is an efficient compound known for its inhibitory role towards mTORC1 and by inhibiting the activity of mTORC1 complex in N2A cells, the excessive DNA damage caused due to Aluminium chloride treatment was reduced. This suggests favourable role of Quercetin in treating AD. Similarly, Gallic acid is known to have promoting role towards Beclin1, protein associated to the PIK3C3 complex. This suggests that promoting Beclin1 complex using Gallic acid can help treat AD condition.

CHAPTER 6: CONCLUSION

An exclusive E-cell model was constructed to demonstrate the molecular machinery of AD with special relevance to autophagy and DNA repair. The key regulatory protein/protein complexes were determined through this model and plant-based inhibitors/promoters for the same were tested against N2A cell line to determine their activity. Further, the N2A cells were conditioned with degenerative conditions to determine the DNA damage through Comet assay and to study the effect of the selected plant-based inhibitors/promoters on N2A cells w.r.t our key regulatory protein complexes.

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