Despite the mammoth efforts and researches being done on AD which is a neurodegenerative and complex disorder, there is no cure of this devastating disease. The multifactorial nature of the disease makes it highly complicated to understand and device a strategy to design therapeutics which is added on by the limitation in availability of diseased sample i.e. human brain (Atz, Walsh et al. 2007). Sporadic studies all across the globe have been done in order to get insight into this debilitating disorder in. In majority of them considers most studied genes ignoring all others (Taguchi, Yamagata et al. 2005; Olgiati, Politis et al. 2011). In order to get a better insight into this complex disorder, synergistic work needs to be carried out at an earliest in order to device treatment strategies. There are numerous evidences suggesting AD link up with varied factors like obesity and gender. Obesity has been listed as one of the most significant risk factor for AD (Anstey et al., 2011). Abnormal higher levels of Aβ and τ proteins have been found in the brains of obese individuals which was comparable to people with established AD (Mrak, 2008). High BMI and obesity has been associated with the increased risk of dementia by many studies (Fitzpatrick et al., 2009, Kivipelto et al., 2005).
Elias et al have reported reduction in cognitive ability and memory function of obese individuals (Elias et al., 2003). Synaptic plasticity and cognitive functions are affected by altered insulin levels (Watson and Craft, 2003). Sex and gender have been listed as one of the significant risk factor for developing AD (Hebert et al., 2001). There are numerous prevalence studies being carried out stating women are more affected by AD than men. These associations need to be studied more to understand AD better.

This collective analysis of ADA genes comprises of functional annotation in terms of biological process, Molecular function, Cellular Component, pathway, protein classes and sequence similarity in correlation with the expression data of whole transcriptome of healthy and diseased individuals. This study is an effort to provide a holistic approach to study the property of combination of all the genes in sync thereby to have a clear picture of AD which will eventually help in better understanding of this disease.

5.1 Dataset and genes under study

Here one of the most comprehensive dataset has been taken for the study. All alzheimer associated genes taken from published studies and database search (www.alzgene.org). Alzheimer associated Y chromosome genes were taken from literature searches as were not listed in database (Vawter et al., 2004). All genes have been verified from wet lab experimentation and polymorphism studies for their association with AD. The obesity genes

were taken from the T-HOD database which comprises of gene list from literature search considering protein-protein interactions and single nucleotide polymorphism also. Sex associated gene list comprises of all the known genes encoded by sex chromosomes along with mitochondrial genome since it is known to have maternal inheritance. To further strengthen the data recently listed sex-biased genes were also included in the study.

5.2 Sequence based clustering

ADA genes taken for the study from literature and database searches were clustered according to various similarity methods. These were first clustered on the basis of sequence similarity and then on the functional basis. Total 526 clusters were obtained on performing sequence similarity search out of which majority (462 clusters) of clusters had single sequence which indicates the diverse nature of ADA genes thereby supporting the complexity and multifactorial nature of AD.

5.3 Functional clustering

Functional annotation and division of genes into function based subgroups aids in providing deeper biological insights in molecular and cellular function of the gene. The data was classified on the basis of pathways involved, molecular and biological processes, cellular component where the protein lies and the class of protein they encode. Pathway based functional clustering of ADA genes was done further to get knowledge of pathways significantly associated with AD. Clustering according to pathways revealed that maximum number of genes (47 genes) fall in the category of Gonadotropin-releasing hormone receptor pathway (GnRH) followed by Inflammation mediated by chemokine and cytokine signaling pathway with thirty-one genes. Alzheimer disease - presenilin pathway and CCKR signalling map has twenty seven and twenty six genes respectively. Other pathways with less than 25 genes are listed in the table (Table A 2.5). It is inferred that above mentioned pathways are the major pathways associated with AD with respect to the number of genes assigned and thereby need to be studied further to get some significant highlights about the disease progression. Current study states that Gonadotropin-releasing hormone receptor pathway is the most important of all the associated pathways with AD as maximum number of AD genes assigned which is a new finding. GnRH pathway was surprisingly found to be most associated pathway with AD even more than Alzheimer disease-presenilin pathway and inflammation mediated by chemokine and cytokine signaling pathway which are highly

related to AD (Rubio-Perez and Morillas-Ruiz, 2012). Results highlight GnRH to be significantly associated with AD which is relatively not studied much.

In GnRH pathway, GnRH regulates the production and release of the gonadotropins by acting on its receptor in the anterior pituitary. GnRH receptors are known to be located throughout in the central nervous system, especially in hippocampus which is involved in cognitive and sexual behavior. According to Drummond et. al., in brains of aged hypogonadal hpg mice (carrying an inactivating genetic mutation in the GnRH gene causing reduction of gonadotropins and gonadal sex hormones) were found high levels of AD markers, like APP C-terminal fragment, presenilin 1, and Aβ (Drummond, Martins et al. 2012). It has been observed that GnRH is decreased in mice hypothalamic ageing and may attenuate brain and systemic aging processes by restoring normal GnRH levels. Studies state that GnRH is involved in homeostasis of hippocampal related neurons and ageing linked health issues (Maggi 2016). GnRH pathway is directly related to sex hormones and difference of sex hormones exists in human male and female even with the age expression of sex hormone differs (Zhao, Mao et al. 2016). According to our study, the association of GnRH pathway with AD was found and hence supports the hypothesis that AD affects male and females differently. It can also be the target for developing therapeutics as the potential future treatment for AD.

The second most associated pathway with AD according to no. of ADA genes (Table A 2.5) is Inflammation mediated by chemokine and cytokine signaling pathway. There are studies speculating role of the inflammation related genes in AD and thereby Inflammation mediated by chemokine and cytokine signaling pathway (Rubio-Perez et al., 2012). It was quite obvious to find that large number of AD genes were linked with inflammation and hence justifies current approach. Inflammatory responses are known to accelerate the pathogenesis of AD and are a hallmark of AD brain. Inflammatory components related to AD neuroinflammation include brain cells as well as cytokines and chemokines. Compelling affirmation by various studies that inflammatory processes are involved in the pathogenesis of AD, researchers are looking into the use of anti-inflammatory drugs as a treatment option for patients with AD (Rubio-Perez and Morillas-Ruiz 2012).
It should be studied more in order to have a promising solution to AD mystery and in development of an effective drug target.

Alzheimer disease-presenilin pathway and CCKR signalling map pathways are of equally importance since have similar number of ADA genes clustered together. As the name suggests this pathway is highly implicated in AD. Presenilin (PSN) is the multifunctional transmembrane proteins which are part of core catalytic unit of γ secretase involved in cleavage of amyloid precursor protein (Wolfe, Xia et al. 1999). It has been reported that animal brains in which presenilin genes are deleted were accompanied by age-associated neuronal death leading to loss in memory, learning and synaptic functions (Saura et al., 2004). Studies have also reported that PSN mutations affect calcium homeostasis and altered calcium signalling leads to neuronal death in AD (Mattson, Chan et al. 2001).

According to this study CCKR signalling map pathway is the third most significant with respect to no. of associated AD genes. Cholecystokinin (CCK) is one of the regulatory peptide found in the cerebral cortex in high concentrations and is known to be involved in learning and memory as well as neurodegenerative processes (Lofberg, Harro et al. 1996). CCKR signaling map can be the target for drug development and therapeutics to get a promising solution for this traumatic disease. Other significantly AD associated pathways (Table A 2.5) are not focussed much in AD research and should be explored more for better understanding of the disease.

The clustering studies on the basis of category biological process revealed that 320 and 280 genes were involved in cellular process and metabolic process category respectively (Fig. 4.2). The cellular process category comprises of cell communication, cell cycle, cell growth, cell proliferation, cell recognition, cellular component movement, cytokinesis and chromosome segregation. Metabolic process comprised of the chemical reactions and pathways by which living organisms transform chemical substances. Presence of protein aggregates inside the cell which is the hallmark feature of AD is due to the alteration in cellular process only. Misfolding and aggregation of tau protein is because of alterations in cellular processes in diseased brain (Yan, Chen et al. 1994). Protein transport and cell signaling processes are also affected in AD (Godoy, Rios et al. 2014).
It implies that majority of ADA genes are involved in cellular and metabolic processes, both of these processes cover major and diverse sub-processes in the cell which adds upto the multifactorial nature of the disease.

Clustering according to protein classes was done and observed that hydrolase and signaling molecule were the protein classes with 75 genes each, stating type of proteins encoded by the majority of AD related genes (Table A 2.6). Hydrolase includes enzymes catalyzing hydrolysis of a variety of bonds, such as esters, glycosides, or peptides like, amylase, deaminase, deacetylase, esterase, glucosidase, lipase, phosphatase, phosphodiesterase, glycosidase, galactosidase, protease and pyrophosphatase. Lysosomal hydrolases and proteases are highly significant in progression of AD (Bachovchin and Cravatt 2012). Serine hydrolases that cleaves and inactivates neurotransmitter acetylcholine are currently used for treating AD and dementia (Bar-On, Millard et al. 2002). Signaling molecule is the molecule that transduces a signal between cells including cytokine, growth factor, peptide hormone and membrane bound signaling molecule.

According to molecular function based clustering most of the ADA genes were enzymatic molecules involved in catalyzing physiological reaction. Classification on the basis of molecular function revealed that catalytic activity (276 genes) was the most enriched class (Fig. 4.1). The catalytic activity is further classified into subtypes: deamainase, enzyme regulator, helicase, hydrolase, isomerase, ligase, lyae, oxidoreductase and transferase activity. Majority of changes in AD brain is due to altered enzymatic activity. Aggregation of amyloid beta, an outcome of hydrolases enzymatic reaction is an important example of it (De Strooper, Vassar et al. 2010). Second most enriched class (218 genes) was binding (molecular function), is defined as the selective, non-covalent, interaction of a molecule with one or more specific sites on another molecule (Fig. 4.1). It includes antigen binding, calcium ion binding, calcium dependent phospholipid binding, carbohydrate binding, chromatin binding, lipid binding, nucleic acid binding, etc. Calmodulin-binding proteins are very well associated with AD as responsible for the formation of amyloid-β plaques (O'Day, Eshak et al. 2015). Binding of zinc ion to tau protein leads to it's hyperphosphorylation resulting in tau tangles in AD brain (Cristovao, Santos et al. 2016). Association of high number of ADA genes with different binding functions and catalytic activities again suggests multifactorial and complex nature of AD.

Cell part (138 genes), membrane (84 genes) were the most enriched classes in clustering on the basis of cellular component (Fig. 4.3). These results are in concordance with the clustering on the basis of other clustering methods. In biological process clustering most of the genes were in the catalytic and metabolic activity which primarily occurs in the cell part

and its membrane. Thereby these results are in concordance with molecular function based annotation and states that genes of these classes are highly associated with AD. Cell part is defined as any constituent part of a cell, the basic structural and functional unit of all organisms like cell projection, coated pit, apical part of cell, neuronal cell body and plasma membrane. Membrane is a double layer of lipid molecules that encloses all cells and also includes associated proteins. Numerous studies state brain cell membrane degeneration is accelerated in AD (Nitsch, Blusztajn et al. 1992).

5.4 ADA genes

Further expression of ADA genes in diseased and control brains was evaluated in order to understand how their expression is altered in AD. Generally there are expression studies focusing on genes differentially expressed on transcriptomic data but none of them have studied the expression of all the genes associated with AD (Liang, Dunckley et al. 2007; Winkler and Fox 2013; Sekar, McDonald et al. 2015). Even expression studies of majority ADA genes have not been carried out in AD and control samples. Although neuroimaging techniques have helped a lot in gathering large amount of knowledge about AD by allowing the study of the brain in living subjects but molecular changes can only be studied by transcriptome studies (Ferreira and Busatto 2011). The data fulfilled the quality criteria and passed the quality check performed. It is good collection of sample as comprises of three dataset from different brain lobes (Table 4.1).

On comparison of DGE ADA found in current study with the available similar reported datasets in literature, evidence of 24 genes out of total 54 genes altered in AD samples was obtained. It accounts for 45% of altered gene expression of frontal and temporal lobe in diseased brain. These 24 genes followed the same pattern of altered expression as reported by earlier AD studies (Berchtold et al., 2013, Kong et al., 2009, Magistri et al., 2015, Satoh et al., 2014). Among these 24 genes which emerged as differentially expressed in this analysis and also in previously identified AD genes somatostatin (*SST*) gene was found to be down-regulated in previous studies. This gene is also known as growth hormone inhibiting hormone also plays significant role in neurotransmission. Few studies have found that upon aging, expression and activity of *SST* is down-regulated (Davies et al., 1980, Hayashi et al., 1997, Lu et al., 2004). Also according to a study published in Nature (Saito et al., 2005) *SST* gene depleted mice were found to have increased $A\beta_{42}$ brain level which is also a common phenotype of AD affected individuals. *SST* is found to work by modulating activity of

neprilysin, a major Aβ degrading enzyme (Hama and Saido, 2005). Based on these findings, it is proposed that down-regulation of SST expression in the AD brain might be responsible for gradual AD progression. In addition to confirming the previous AD literature this analysis reveals other AD associated genes which undergo altered expression in AD thus suggesting new target genes that might be important for maintaining healthy brain and preventing AD.

In diseased frontal lobe, temporal lobe and whole brain samples 22, 33 and 49 genes were observed to be down-regulated respectively (Table 4.5, 4.6 and 4.7). Important genes which were found to be down-regulated in AD temporal lobe namely, CXCL12, IL-1B, HSPA5 and BDNF are discussed below.

CXCL12 is a serum chemokine was found to have decreased expression in diseased temporal lobe. There are studies stating its decreased expression in AD (Laske, Stellos et al. 2008). AD is characterized by massive neuronal loss in the brain. Further studies are needed to examine whether a manipulation of chemokines could be a promising new therapeutic strategy for AD.

In diseased temporal lobe expression of IL-1B was found to be down-regulated. Studies have generated an elegant mouse model in which local hippocampal overexpression of IL-1B in an Alzheimer disease (AD) transgenic mouse model resulted in plaque amelioration instead of expected exacerbation of the amyloid β plaque deposition common to AD (Liu, Cui et al. 2014). Specific activation of immune cells may have potential protective benefits for AD. IL-1B overexpression is beneficial in AD brain (Lemere 2007). The IL-1B manipulation results provide much food for thought and undoubtedly support much-needed future investigation into a positive role of inflammation in neurodegenerative diseases, including AD. Thus, to protect against AD, manipulation of the immune system may be a successful therapeutic approach.

Endoplasmic reticulum (ER) chaperone heat shock 70 kDa protein 5 (HSPA5) encodes glucose regulated 78KDa (GRP78) was found to be down-regulated in diseased temporal lobe. It is known to be involved in the metabolism of amyloid precursor protein and neuronal death in AD could arise from dysfunction of the ER (Hsu, Wang et al. 2008). In AD HSPA5 has been found to play a supportive role for the progression of tau phosphorylation and

neurodegeneration. Thus HSPA5 represents a viable target for anticancer, antiviral, and anti-Alzheimer's therapeutics (Booth, Roberts et al. 2015). Certainly, heat shock family of proteins, which are supposed to play significant role in protein folding are ought to find seat in the protein misfolding and aggregation disorders like AD.

Brain-derived neurotrophic factor (*BDNF*) gene was found to be down-regulated in diseased temporal lobe. Levels of *BDNF* were found to be reduced in specific brain regions in AD and also it's polymorphisms have been suggested to influence AD risk, hippocampal function, and memory. *BDNF* neuroprotective role can be implicated in development of drug targets for AD (Nagahara, Merrill et al. 2009).

In diseased temporal lobe *AHSG* and *ABCA12* genes were found to be up-regulated (Table 4.2). *ABCA12* is a member of the A-subfamily ATP-binding cassette transporter family (ABCA), and members of the ABCA subfamily are known to have closely related functions as lipid transporters and translocate variety of substrates across extra- and intracellular membranes. There are researches pointing significant contribution of several A-subfamily ABC transporters to neurodegenerative diseases, in particular AD (Piehler, Ozcurumez et al. 2012).

AHSG (Alpha2-Heremans-Schmid glycoprotein) is a glycoprotein present in the serum which is known to be involved in brain development and the formation of bone tissue. The AHSG protein is commonly present in the cortical plate of the immature cerebral cortex and bone marrow haematopoietic matrix, and it has therefore been postulated that it participates in the development of the tissues. *AHSG* gene polymorphism was found to be associated with AD in Italian patients and decrease in expression in the cerebrospinal fluid of patients was observed (Geroldi, Minoretti et al. 2005). In AD whole brain 49 genes were down-regulated (Table 4.7) out of which *AKT2*, *LRP6* and *TP73* genes were significant in AD etiology.

AKT (Protein kinase B) is a serine/threonine kinase which plays a significant role in regulating cell survival, insulin signaling, angiogenesis and tumor formation. It also regulates cell survival via the phosphorylation of MAP3K5 which is an apoptosis signal-related kinase (Rickle, Bogdanovic et al. 2004).

Low-density lipoprotein receptor-related protein 6 (*LRP6*) gene was down- regulated in diseased whole brain which is also known to be associated with Alzheimer disease presenilin pathway. The *LRP6* is an essential co-receptor for Wnt signaling and (Liu, Tsai et al. 2014) is found to affect synaptic function and cognition properties by altering neuronal *LRP6* mediated Wnt signalling. Significant increase in loss of cognition, high amyloid β load and poor synaptic function has been reported with decrease in *LRP6* expression. However, the functional *in vitro* studies for understanding the role of *LRP5/6* in AD pathology are not carried out (Zhang, Bahety et al. 2015) and provide scope for developing promising therapeutics for AD.

Tumor protein 73 (*TP 73*) genes also known as p73 encodes a member of the p53 family of transcription factors involved in key physiological events like DNA repair, cellular responses to stress and development and apoptosis. Studies have shown that the loss of one allele of the p53 family member, p73, makes mice susceptible to neuro degeneration as a consequence of aging or AD as brains of these mice showed neuronal degeneration and early and robust formation of tangle-like structures containing P-tau. Also p73 has been clearly stated essential for preventing neurodegeneration and its haplo sufficiency might be a susceptibility factor for AD and other neurodegenerative disorders (Wetzel, Naska et al. 2008).

In whole brain three genes were observed to be up-regulated (Table 4.4) out of those *MT-ATP8* and *APOA2* are discussed below. *MT-ATP8* gene encodes mitochondrial membrane ATP synthase F0 subunit 8. It is a component of ATP synthase which is responsible for producing ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. Role of mitochondrial genome in AD has been highly studied and it's dysfunction is related with oxidative stress. Altered mitochondrial function causes irregularities in ATP synthesis, dysregulation of cytoplasmic calcium concentration, and induction of cytotoxic reactions (Kubota, Kasahara et al. 2006).
A growing body of evidence suggests that mitochondrial dysfunction is involved in various neurodegenerative disorders (Ben-Shachar and Laifenfeld 2004).

Apolipoprotein A2 gene (*APOA2*) is a very common protein in high-density lipoproteins. It appears to hamper the reverse cholesterol transport and antioxidant function of high-density

lipoprotein (Zaki, Amr et al. 2013). A study states that individuals with increased level of *APOA2* had a higher risk of undergoing cognitive impairment (Ma, Li et al. 2015).

In diseased frontal lobe 22 genes were down-regulated (Table 4.5) out of those EGR2, the most significant one. According to the studies regulation by muscarinic acetylcholine receptors (AChR) activity of EGR-dependent genes in postsynaptic cholinergic target cells was observed, expression of such genes may be decreased in Alzheimer's disease brains (Von Der Kammer, Mayhaus et al. 1998). There is no expression studies of *EGR2* gene performed yet with respect to AD.

In AD frontal lobe nine genes were up-regulated (Table 4.2) out of which G protein beta 3 subunit genes (*GNB3)* is important in disease etiology and is mentioned below. *GNB3* was found to be up-regulated in frontal lobe of disease sample. There are genetic and functional studies indicating strongly that together GNB3 and ADRB1 polymorphisms is prone to AD susceptibility pointing the modulation of brain adrenergic receptors as a potential target for novel AD therapeutic strategies. Exclusive expression studies of *GNB3* gene with respect to AD need to be done in order to have better understanding of the disease.

Other significant genes which were found to be down-regulated in two AD samples are mentioned below. Genes very low density lipoprotein receptor (*VLDLR)* and *CHRNB2*, were observed to be down-regulated in frontal and temporal lobe (Fig. 4.8). *VLDLR* gene has a potentially important role in lipoprotein metabolism and Alzheimer's disease (Near, Wang et al. 2001). It also plays important role in reelin signaling pathway. Reelin is an extracellular matrix protein essential for maintaining neuronal position and synapse plasticity and function (Doehner and Knuesel 2014). Its association with AD was explored in twelve studies with late onset of AD (Olgiati, Politis et al. 2011). *CHRNB2* belong to a superfamily of ligand-gated ion channels which in response to ligands such as acetylcholine controls the flow of sodium and potassium across the plasma membrane. This gene encodes one of the beta subunit. Cholinergic deficit associated with loss of nicotinic acetylcholine receptors (nAChRs) has been associated with AD (Engidawork, Gulesserian et al. 2001). The association between *CHRNB2* and AD was originally investigated in an Asian sample of 58 patients and 51 controls with negative results (Kawamata and Shimohama 2002). Significant association of this gene with AD has been reported by Cook et., al. (Cook, Ho et al. 2004).

AGT is down-regulated in both frontal and whole brain diseased samples. It encodes angiotensinogen precursor which is expressed in the liver and is cleaved by the enzyme renin in response to lowered blood pressure. Its role with AD is yet to be explored as information about its gene expression in AD samples is not available.

Genes *TTR* (Transthyretin), *EFEMP1* and *ADAM9* were found to be down-regulated in all the three brain samples (lobe, temporal lobe and whole brain) (Fig. 4.7). These results are in concordance with previous studies. *TTR* gene is reported to reduce Aβ concentration in the brain and thereby alleviate AD symptom (Wang, Cattaneo et al. 2014). Research outcomes suggest that *TTR* has protective role in neurons attribute to its capacity to bind toxic or pretoxic Aβ aggregates in both the intracellular and extracellular environment (Buxbaum, Ye et al. 2008). This gene could be therapeutically useful and may be helpful to design therapeutic strategy against AD (Li, Masliah et al. 2011).

EFEMP1 gene is a member of family of genes encoding extracellular matrix glycoprotein. It is supposed to play role in cell adhesion and migration and function as a negative regulator of chondrocyte differentiation. Studies conclude that *EFEMP1* plays an important role in the etiology of Age related Macular Degeneration (AMD) which share similarities with Alzheimer's disease (Marmorstein, Munier et al. 2002).

ADAM9 is a member of disintegrin and metalloproteinase (ADAM) family which can cleave the amyloid precursor protein (APP) and preclude generation of Aβ peptides (Hotoda, Koike et al. 2002). Studies suggest that human ADAM9s has an alpha-secretase-like activity for APP (Asai, Hattori et al. 2003). Alpha-secretase conducts an alternative proteolytic cleavage that prevents A-beta production and accumulation (Zhang and Saunders 2009). Gene specific studies targeting *ADAM9* expression alteration in AD samples have never been reported so far. Also this gene is involved in Alzheimer disease-amyloid secretase pathway which is very well associated with AD progression. As this gene was found to be down-regulated in all the diseased samples hence elevating levels of alpha-secretase cleavage might be a potential therapeutic strategy to treat AD (Esler and Wolfe 2001).

MT-TI (Mitochondrially Encoded TRNA Isoleucine) gene, a non coding RNA gene was observed to be up-regulated in all the three brain samples, frontal lobe, temporal lobe and whole brain respectively (Fig. 4.6). None of the studies have ever reported differential

expression of *MT-TI* gene in AD samples. It is involved in biosynthesis of isoleucine amino acid. Isoleucine is an amino acid of predominant importance in human mitochondria. Furthermore, an isoleucine codon initiates ND2 synthesis (subunit 2 of NADH coenzyme Q reductase) which is the first complex in electron transport chain. Complex I is one of the main sites of production of superoxide. Superoxide is the reactive oxygen species which leads to oxidative stress and it has been well established fact that in AD cells is under oxidative stress (Bonilla, Tanji et al. 1999) and thereby has effect on energy production. Hence this gene is proposed as a drug target for AD.

Significantly (statistically) differentially expressed ADA genes were also correlated with pathway based clustering results (Fig. 4.4). According to p-value (<0.05) cut-off two genes *FOS* and *PRKAB2* come under GnRH pathway and are down-regulated in temporal and whole brain diseased sample respectively. With the increasing age change in the production of gonadal steroids may be associated with cognitive senescence and it has been proposed to be implicated in the neuropathology of AD (Nuruddin et al., 2014). Genes AKT2 (down-regulated in AD whole brain), IL-1B (down-regulated in AD temporal lobe) and CXCL12 (down-regulated in AD temporal lobe) were grouped in Inflammation mediated by chemokine and cytokine signaling pathway. The dysregulation of these genes is in concurrence with their function in the pathway. The generation and secretion of proinflammatory mediators may interact at multiple levels with neurodegeneration such as APP processing and τ phosphorylation (Rubio-Perez and Morillas-Ruiz 2012).

EGR2 (down-regulated in AD frontal lobe), *GNB3* (up-regulated in AD frontal lobe) and *AGT* (down-regulated in AD whole brain) were found to be implicated in Angiotensin II-stimulated signaling through G proteins and beta-arrestin pathway. G protein coupled receptors (GPCRs) represent the most important targets in modern pharma studies because of the different functions they mediate, especially within brain and peripheral nervous system and make such receptors ideal therapeutic targets for different diseases.
Most importantly, among all hormones and neurotransmitters it has been estimated that 80% of them exert their effects via interacting with GPCRs.

Differential expression of ADA genes was studied in different AD and control samples. Several genes were found to be differentially expressed with significant p-value. Majority of DGE (116 genes) were found to be down- or up-regulated which was in sync with their

functional role in the AD. During present investigations it was found that more genes were down-regulated as compared to the up- regulated ones in diseased brain than healthy brain, may be potentially due to lowering of transcriptional activity in the former. Most of them were not studied earlier with respect to their expression in diseased condition. These DGE genes will be helpful in understanding disease etiology. Few genes were up- (1 gene) or down- (3 genes) regulated in all the AD samples taken for the study which strongly states them as a marker and therapeutic target for e.g. *MT-TI* gene as drug target.

5.5 Obesity genes

Even after innumerable efforts going on all over the world, millions of dollar being pumped for AD research its etiology stands as a grave challenge to scientists in 21st century. Obesity is listed as one of the modifiable risk factor for AD. If explored more about the molecular mechanisms associating the two diseases it would be helpful in understanding, preventing and designing therapeutics for AD. AD is affecting lives of millions globally. Does lifestyle factors enough to justify the record number of people suffering from AD and obesity world over? Since no study examines obesity and AD associated genes together this is an effort to find the link speculated between AD and obesity. Hence it will be an effort to quench the thirst of AD cure and promising therapeutics.

More than hundred (117 genes) AD genes were found to be common with obesity (Fig. 4.9). It indicates significant association of two complex diseases and justifies current study. It clearly pinpoints that there exists an unexplored path linking AD and obesity. These genes could be the best target to elucidate this association through further experimentation. Pathway analysis of these genes revealed that about 66% genes were associated with signalling related pathways (Fig. 4.10) emphasizing on the fact that altered signalling is responsible for the two incurable diseases. Also disturbances in such signalling pathways lead to inflammatory disorders which were found to be pronounced in both obesity and AD (Cooper-Knock et al., 2012, Turner et al., 2014).

Metabolism related pathways were the second most enriched pathways which include 14% of common AD and obesity genes (Fig. 4.10). Obesity is an established metabolic disorder however various studies also reports altered metabolic pathways and their pathogenic relevance in progression of AD (Oresic et al., 2011, Trushina et al., 2013).

Total 153 obesity related genes were found to be differentially expressed according to p-value ($p < 0.05$) in AD with comparison to normal samples. Most of the genes were down-regulated (130 genes out of 153 genes) suggesting repressed transcriptional activity of AD brain which was also supported by earlier studies (Twine et al., 2011). Further in diseased temporal lobe relatively more genes were down-regulated (43 genes, Table 4.9) as compared to frontal lobe (27 genes, Table 4.8) and only three genes were up-regulated in temporal lobe. AD is known to start from hippocampus which is the part of temporal lobe of brain (Mu and Gage, 2011). In AD, this part of the brain is most affected region which is known for reduced brain activity and neuronal death as indicated by the results i.e. most genes were down-regulated, which supports reduced activity in temporal region of brain.

Fourteen genes were down-regulated in more than one brain samples (Table 4.14) out of which eleven were directly or indirectly related to AD. Remaining genes were not studied for AD in literature. Three out of fourteen DGE genes were known to be associated with AD and also listed in Alzgene database namely, Very-low-density lipoprotein receptor (*VLDLR*), Angiotensinogen (*AGT*) and Stearoyl-co A desaturase (*SCD*) (Bertram et al., 2007). Other eight genes (*VGF, GAD2, CNR1, CDH13, TIMP3, NOS2, MCHR2* and *NR3C2*) were found to be associated with AD, cognition, neuron growth or synaptogenesis. Nerve growth factor (*VGF*) is expressed in neurons where it promotes growth and survival of neurons, also is involved in energy homeostasis, neurogenesis and synaptogenesis (Ferri et al., 2011). VGF peptides may also be biomarkers for AD since previous studies have shown that altered secretion of these factors occurs in neurological and psychiatric disorders, (Bartolomucci et al., 2010). VGF is also found to play a significant role in the control of body weight and basal metabolism, hence, could represent a novel target for pharmacologic intervention to prevent human obesity (Hahm et al., 1999). Glutamic acid decarboxylase 2 *(GAD2)* encodes GAD65 (glutamate decarboxylase 2 brain 65 kDa) which catalyzes the production of a neurotransmitter gamma-aminobutyric acid (GABA)

which interacts with a neuropeptide in the hypothalamus to help stimulate appetite (Boutin et al., 2003). Few studies suggest that reduced inhibitory neurotransmission during aging and in AD may be the result of compensatory responses that render the neurons vulnerable to Ca^{2+}-mediated degeneration.

Cannabinoid receptor-1 *(CNR1*) gene encodes cannabinoid receptor type 1 (CB1 receptor) which has the greatest expression in the central nervous system but also in several peripheral organs including liver, muscle and adipose tissue (Di Marzo and Matias, 2005). The polymorphic variants of this gene may contribute to individual susceptibility to obesity and related metabolic disorders (Russo et al., 2007). The CB1 protein levels (Kalifa *et al*., 2011) and receptor binding (Westlake *et al*., 1994) was found to be decreased, in the hippocampus, neocortex and basal ganglia during Alzheimer's disease progression (Lee et al., 2010, Westlake et al., 1994).

CDH13 was found to have significant association with MRI derived measure of temporal lobe volume (Kohannim et al., 2012). Tissue inhibitor of metalloproteinase 3 (*TIMP3*) has been implicated in cell death of ischemic neurons (Wallace et al., 2002). Nitricoxide Synthase 2 *(NOS2*) generates nitric oxide (NO) which is found to play significant role in neuroinflammation and redox activites in brain. In the mice decrease in levels of NO has been found leading to increased Aβ mediated damage (Colton et al., 2008). Melanin concentrating hormone receptor 2 (*MCHR2*) was found to be associated with pathways implicated in AD (Magistri et al., 2015). On the basis of meta-analysis from GWAS Nuclear Receptor subfamily 3 group C member 2 *(NR3C2)* gene has been identified as potential risk gene involved in AD pathogenesis (Sun et al., 2014). Hence all fourteen genes should be taken for further wet-lab experimentation to discover their precise role in AD.

Four genes were found to be down-regulated in all three AD samples. Two (*HDAC9* and *THBS1*) out of four genes down-regulated in all AD samples (Table 4.15) have been previously associated with AD which justifies the current approach and suggests further investigation of these genes. Histone deacetylase 9 (*HDAC9*) is a member of the class II family of HDACs (Chatterjee et al., 2014) and recently several studies have suggested that histone acetylation is significantly involved in the etiology of AD (Johnstone and Baylin, 2010, Stilling and Fischer, 2011).

By the use of histone deacetylase (HDAC) inhibitors it is a target for development of drugs specifically for cognitive enhancement (Graff and Tsai, 2013) in AD. *HDAC9* also acts as molecular mediator of impaired adipogenic differentiation in obesity and hence emerging as a critical regulator of adipose tissue health and a novel therapeutic target for obesity-related disease (Wang et al., 2005). These findings suggest the possibility of therapeutically targeting *HDAC9* in obesity and AD.

Thrombospondin-1 *(THBS1)* is a circulating glycoprotein highly expressed in hypertrophic visceral adipose tissues of humans and mice and its expression is elevated in insulin-resistant, obese humans (Varma et al., 2008). *THBS-1* expression is markedly decreased in neuronal populations in the brains of AD patients (Buee et al., 1992). Since this gene shows dysregulation in AD and obesity it can lead to a common cure for both disorders. The other DGE genes in more than one brain samples (*F3, CCDC80, PDP1, HBB* and *ABCC11*) have not been studied earlier in context to AD and should be taken for further experimentation to elucidate their role in AD.

Two genes, *PSME2* and *CEBPB*, up-regulated in frontal lobe and whole brain region have not been studied directly in context with AD. Proteasome activator complex subunit 2 encoded by gene (*PSME2*) was up-regulated in AD induced pluripotent stem cell (iPSCs) derived neuronal cell line (Hossini et al., 2015). *CEBPB* gene product is involved in regulation of interferons and cytokines which play a significant role in inflammatory response in AD (Li et al., 2015). Gene *AZGP1* was up-regulated in all the AD brain regions. It encodes a 42 kDa protein AZGP1 which in human adipocytes functions as a key player in lipid mobilization (Bao et al., 2005). Data from genetic studies suggest that *AZGP1* expression appears to be inversely correlated with adiposity. Moreover, it has been identified as a prognostic marker for prostate cancer (Yip et al., 2011) and over-expression induces apoptosis and cell cycle arrest (Chang et al., 2014). Earlier no study has reported this gene with AD perspective. Since this study first reports it to be DGE in AD, a detailed study is suggested to be carried out for the sake of understanding AD better and exploring a promising drug target.

High number of obesity genes has been found to be DGE in AD brain samples. 21obesity genes were down-regulated in two or more AD samples. Out of those 15 genes has been found to be associated with AD. These genes should be the first priority of research in order to explore the proposed link between AD and obesity.

High number of DGE of obesity genes and majority of them getting repressed in AD brain samples which is in concurrence with published literature to supports this study (Gonzalez-Zuniga et al., 2014). Results obtained are in sync with the earlier study reporting presence of Aβ and τ protein in obese individuals which was comparable to people with established AD (Mrak, 2008). Hence these results supports the idea behind the study that there exists a strong

link between AD and obesity which if inferred would aid in understanding the disease and devising a curative strategy for AD. These genes should be further explored in different experimentation for their exact role in AD. This outcome warrants the further study of these identified genes for their role in AD through experimentation to unveil AD pathology and to discover new therapeutic targets.

5.6 Sex-associated genes

110 years after the discovery of AD, scientists and researchers stand bare-handed as far as therapeutics and treatment of disease is concerned. No cure on horizon, millions of people suffering from the disease and numerous more affected every day poses a grave threat to researchers in 21st century. Gender and sex have been linked with AD innumerable times in past. The exact molecular mechanisms of how gender influences AD progression are yet to be explored. AD is significantly prevalent in women as compared to men. A recent study has stated that out of total individuals suffering from AD two-third are women (Association, 2016). Also an ageing related study have stated that rate of progression from mild cognitive impairment (MCI) to AD was higher in women than men after 80 years of age (Roberts et al., 2014). Exactly how sex hormones and sex specific genes influences and affects AD etiology is poorly understood (Li and Singh, 2014). Since no study examines exclusively sex related genes with respect to AD this is an effort to explore this association which would be helpful in understanding, preventing and designing therapeutics for AD.

Rate of mapping of reads of 95-97% to reference genome (Table 4.1) met quality standards of RNA-Seq technique (Mortazavi et al., 2008). It indicates the reliability and quality of assembly. In current study all possible sex-associated genes were included. These comprises of X, Y chromosome encoded genes along with mitochondria genes, which has maternal inheritance hence can be taken as associated with sex and included in the study. Also, genes listed as a sex-biased genes by a recent study (Kang et al., 2011) were also placed in the current work. Total 164 sex-associated genes were found to be differentially expressed according to p-value ($p < 0.05$) in AD with comparison to normal samples (Table 4.17 – 4.34). Most of the genes were down-regulated (149 genes out of 164 genes) suggesting repressed transcriptional activity of AD brain which was also supported by earlier studies (Twine et al., 2011). Among the DGE only 65 genes were protein coding genes and rest (99 genes) belong to the category of psuedogene, anti sense RNA, intronic transcript, uncharacterized locus, Long intergenic non protein coding RNA and micro RNA (Fig. 4.16).

Noticeable number of these non- protein coding genes (non-pcg) is DGE stating their significance in disease progression. Majority of studies ignore these genes over the protein coding counter parts thereby little is known about these genes with respect to AD. These non protein coding genes need to be explore more to know how these operate at cellular and molecular level in order to have promising therapeutics and treatment for AD (Luo and Chen, 2016). Also antisense transcripts and micro RNA are studied for their role in gene expression regulation in AD samples and their potential for future treatment of AD (Esteller, 2011, Guo et al., 2006).

In X chromosome total 119 genes were down–regulated and only 8 genes were up-regulated (Table 4.17- 4.22). Out of total DGE, 127 (77.4%) genes were encoded by X chromosome suggesting that X chromosome gens are more associated to AD. Maximum numbers of DGE encoded by X chromosome were repressed indicating that expression of these genes was skewed towards down–regulation. Literature also supports that female brain with aging shows more repressed transcriptional activity than males (Zhao et al., 2016). Few of these DGE genes namely, *STS*, *CHRDL1*, *ARHGEF9* were also found to be repressed in another AD study suggests these genes should be explored on priority for finding novel drug targets for AD cure (Satoh et al., 2014). In X chromosome fifteen genes were down-regulated in two AD samples among them only five genes were protein coding namely, *YY2, KLHL34, ZNF449, HEPH* and *AMOT.*

YY2 encodes for a zinc finger transcription factor and has not been studied in AD context. It is found to be highly similar to *YY1* gene which has been known to activate beta-site amyloid precursor protein-cleaving enzyme 1 (*BACE1*) expression (Yao et al., 1998). Kelch like family member 34 (*KLHL34*) gene is found to be down-regulated in Ullrich congenital muscular dystrophy samples (Paco et al., 2013). No study has been carried out to explore association of this gene with AD.

Angiomotin encoded by gene *AMOT* is a transmembrane protein associated with actin. This gene is found to play role in cell motility, angiogenesis and tight junction formation. It was found to be expressed in brain vasculome and suggested that should be explored more in context with AD to as its reduced expression is found to be responsible for dysfunctional remodelling of brain vessels (Guo et al., 2012). Gene *ZNF449* encodes a nuclear protein that contains N-terminal SCAN domain. It is likely to function as a transcription factor (Luo et al., 2006). According to linkage analysis this gene has been observed to be located in the disease

locus of X chromosome. No expression study has been done for this gene in AD context. It should be explored more as a therapeutic target for AD.

Hephaestin (*HEPH*) gene is named as sex-linked anaemia candidate gene according to studies by Vulpe et al (Vulpe et al., 1999). It encodes a ferroxidase necessary for iron release from intestinal epithelial cells also it has been stated that to maintain iron homeostasis *HEPH* plays critical role (Oshiro et al., 2011). AD related study have observed this gene to be repressed in diseased condition (Magistri et al., 2015). This gene could be studied more in order to understand iron homeostasis in AD better and have promising therapeutics for the disease.

Reduced gene content of Y chromosome has been established with time that it has undergone massive gene decay (Bachtrog, 2013). In Y chromosome none of the genes were up-regulated and only eight genes were down-regulated (Table 4.24 - 4.25). Out of eight down-regulated genes in AD samples two were protein coding, *NLGN4Y* and *PCDH11Y*, were also listed in sex biased study. Gene NLGN4Y belongs to the family of neuroligins, which are cell adhesion molecules essential for the formation of functional synapses. Gene *PCDH11Y* belongs to protocadherin family and is very closely related to its paralog on the X chromosome. The protein is thought to play a role in cell-cell recognition during development of the central nervous system. According to Magistri et al., both *NLGN4Y* and *PCDH11Y* were down-regulated in AD brain and are also listed in alzgene database (Bertram et al., 2007) suggesting *NLGN4Y* and *PCDH11Y* genes as target for AD therapeutics (Magistri et al., 2015).

Four mitochondrial genes were found to be up-regulated in all the AD samples (Table 4.26-4.28) and none were down-regulated with respect to $p<0.05$ value cut-off. According to literature increased level of mitochondrial gene expression and oxidative damage in a transgenic mutant mouse model of AD have been demonstrated (Reddy et al., 2004) which justifies curent results. *MT-TI* (Mitochondrially Encoded TRNA Isoleucine) is the non coding RNA gene which is observed to be up- regulated in all the three brain samples, frontal lobe, temporal lobe and whole brain (Fig. 4.17).

It is involved in biosynthesis of isoleucine amino acid. Isoleucine is an amino acid of predominant importance in human mitochondria. Furthermore, an isoleucine codon initiates

ND2 synthesis (subunit 2 of NADH coenzyme Q reductase) which is the first complex in electron transport chain. Complex I is one of the main sites of production of superoxide. Superoxide is the reactive oxygen species which leads to oxidative stress and it has been well established fact that in AD cells are under oxidative stress (Bonilla et al., 1999) and thereby have effect on energy production. Hence this gene is proposed as a drug target for AD. Alteration in expression of *MT-TI* is found to be associated with various disorders like hypertension (Liu, Li et al. 2014), juvenile parkinsonism, leigh syndrome (Martikainen et al., 2013) and cardiomyopathy (Taylor et al., 2003). *MT-T1* gene was found to be up-regulated in all AD samples and have role in oxidative stress which is major factor in AD. None of the studies have ever reported differential expression of *MT-TI* gene in AD samples. This gene can be used as a drug target since reduction in oxidative stress may have therapeutic effect in AD.

In sex-biased genes 22 were down-regulated and three were up-regulated (Table 4.29-4.33) Out of these twenty genes were encoded by autosomes and two genes (*NLGN4Y* and *PCDH11Y*) were encoded by Y chromosome. Sex biased *P2RY12, NR3C2, MEGF10* and *SDC10* genes were also found to down-regulated in more than one AD sample (Table 4.34). Functional annotation of DGE on the basis of molecular function (Fig. 4.18) classifies them into seven different classes. These classes are diverse in nature hence this outcome states the multifactorial nature of the disease as the DGE genes possess varied functional properties. Biological process based functional annotation (Fig. 4.19) classifies maximum sex-associated genes into cellular process and metabolic process which is in sync with obesity and ADA gene analysis. The functional annotation on the basis of cellular component and protein class is also similar to obesity and ADA gene analysis (Fig. 4.20 and fig. 4.21).

Six DGE (*FOXO4, POU3F4, MBTPS2, YY2, LOC101928917* and *ZNF449*) were found to be encoding a transcription factor (Table 4.35). Transcription factors include proteins that initiate and regulate transcription of genes. *FOXO4* Tc factor plays role in regulation of insulin signalling pathway and oxidative stress (Manolopoulos et al., 2010). It down-regulates expression of HIF1A (hypoxia induced factor 1alpha subunit) which is involved in hypoxia mediated transcription activation of genes involved in energy metabolism, apoptosis (Maiese, 2015). These all processes are observed to be affected AD hence could be therapeutic target for the disease. *POU3F4* gene encodes POU-III class of neural transcription factors with glucagon as it's target gene. It has been observed in clinical studies that treatment with

glucagon like peptide 1(GLP1) there is increase in cognitive ability, blood glucose metabolism and synaptic function (Talbot and Wang, 2014).

It could be the promising target for AD therapeutics (Gejl et al., 2016). *MBTPS2* gene encodes intramembrane zinc metalloprotease, which is essential in development. This protein is involved in intramembrane proteolysis of sterol-regulatory element-binding proteins (SREBPs) and the ER stress response. ER stress is found to be generated by amyloid β in AD (Roussel et al., 2013). Further research to investigate the role of *MBTPS2* in AD is required which may lead to better understanding and therapeutic design.

YY2 encodes a multifunctional transcription factor that has Kruppel-like zinc fingers in its C-terminal region, may exhibit positive and negative control on a large number of target genes. May antagonize YY1 and function in development and differentiation. It is found to be expressed in AD samples but it's role in disease progression is not clear (Ray and Zhang, 2010). *ZNF449* encodes a nuclear protein that likely functions as a transcription factor (Luo et al., 2006). The protein includes an N-terminal SCAN domain, and seven C2H2-type zinc finger motifs. LOC101928917 (Heat Shock Transcription Factor, X-Linked-Like) is a Protein Coding gene and very less is known about it. As it is known that heat shock factors are involved in protein folding and stress, which are the two prime changes happening in AD brain, it could be associated with AD.

According to results, three transcription factors (*FOXO4*, *POU3F4* and *MBTPS2*) have been found to regulate target genes which are associated with AD previously. Further studies need to be done in order to explore the role of these Tc factors for better understanding and therapeutics for AD. Results obtained in current study are in sync with the earlier studies reporting significant role of sex and gender in AD pathogenesis. Hence these results supports the idea behind the study that there exists a strong link between AD and gender which if inferred would aid in understanding the disease and devising a curative strategy for AD.

High number of DGE of ADA, obesity and sex-associated genes and majority of them getting repressed in AD brain samples which is in concurrence with published literature to support this study (Gonzalez-Zuniga et al., 2014). These genes should be further explored in different

experimentation for their exact role in AD. Many of these could be studied further to unravel therapeutic or drug target for the disease. Some of these DGE are first time examined for their expression in AD. DGE obesity genes should be explored more to elucidate the link between two AD and obesity. Sex-associated DGE can be helpful in finding gender biasness of AD. Genes significantly up-regulated can be studied to develop better drug target and down-regulated might be helpful in designing therapeutic target for the disease. This outcome warrants the further study of these identified genes for their role in AD through experimentation to unveil AD pathology and to discover new therapeutic targets.