Genotyping and Expression Profiling of Methylenetetrahydrofolate Reductase (C677T) in Type 2 Diabetes Mellitus and Alzheimer's Disease

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A thesis submitted as a final year project in partial fulfillment of the requirement for the degree of Masters of Science

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2017

I dedicate this work to my parents for their unconditional love and support.

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LIST OF ACRONYMS

%	Percentage
µl	Microliter
5m-THF	5-Methyl Tetrahydrofolate
ABAD	Aβ-Binding Alcohol Dehydrogenase
AD	Alzheimer's Disease
AGE	Advanced Glycated End products
APP	Amyloid Precursor Protein
ATP	Adenosine Tri-Phosphate
Aβ	Amyloid Beta
BMI	Body Mass Index
Bp	Base pair
CAA	Cerebral Amyloid Angiopathy
cDNA	Complementary DNA
CNS	Central Nervous System
CpG	C-phosphate-G
Ct	Cycle Threshold
DEPC	Diethylpyrocarbonate
DMR	Differentially Methylated Regions
DNA	Deoxyribonucleotide
dNTP	Deoxynucleotide Triphosphate
GWAS	Genome Wide Association Study

	Haemoglobin Type A1C
Hb1Ac	Glycated Haemoglobin / Glycosylated
Hcy	Homocysteine
IDE	Insulin-Degrading Enzyme
IGT	Intolerance Glucose Test
	internal control gene)
LOAD	Late Onset of Alzheimer's Disease LOAD
$MgCl_2$	Magnesium Chloride
ml	Millilitre
mM	Micro Molar
MRI	Magnetic Resonance Imaging
MRI	Mild Cognitive Impairment
MTHFR	Methyltetrahydrofolate reductase
NCBI	National Center of Biotechnology Information
NEFA	Non-Esterified Fatty Acid
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OGTT	Oral Glucose Tolerance Test
PCR	Polymerase Chain Reaction
	products
RAGE	Receptors of Advanced Glycated End
RI	RNAse Inhibitor
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

Rpm	Revolutions Per Minute
RT	Reverse Transcriptase
SEM	Standard Error of Mean
SNP	Single Nucleotide Polymorphism
T2DM	Type 2 Diabetes Mellitus
TAE	Tris base, Acetic acid and EDTA
ΔCt	Delta Ct (Ct of gene of interest – Ct of ΔCt of control sample)
ΔΔCt	Delta delta Ct (ΔCt of experimental sample – ΔCt of control sample)

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ABSTRACT

Type 2 diabetes mellitus, considered as a multifactorial metabolic disorder where failure of β cell and resistance of peripheral insulin takes place. It is one of the most prevalent diseases in the world. It is associated with other major macrovascular as well as microvascular complications affecting liver, kidney, adipose tissues, heart and even brain. On long-term basis, it causes neurodegenerative disorders including Alzheimer' Disease (AD). Many genetic as well as epigenetic and environmental risk factors are involved in T2DM leading towards the condition of AD which comes as a dementia form and considered by neural loss and amyloid beta plaques. One of the genetic risk factors is methylenetetrahydrofolate reductase (MTHFR) which has a common polymorphism C677T that is responsible for the onset of both the diseases i.e. T2DM and AD. This present study aims to evaluate the expression level of MTHFR gene in the study groups of T2DM and AD compared with control group. For this, real time PCR was carried out on all the samples and their cycle threshold (Ct) values have been analysed. This study has reinforced the hypothesis that MTHFR has elevated expression level in T2DM and AD than control group. Although expression of MTHFR in AD was not significantly high but still it shows increased expression than control. Therefore, it concludes that MTHFR polymorphism C677T, a significant genetic risk factor for incidence of both disorders i.e. T2DM and AD. This study also predicts that it further needs to be extended on greater sample size and various ethnic groups to evaluate more about such association study. Such association study will help in future to find out the shared risk factors so that better therapeutic approaches can be designed to target both the ailments.

CHAPTER 1

INTRODUCTION

1.1 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM), considered as defined by insulinopenia and resistance of insulin, is due to failure of pancreatic β cell in skeletal muscles, adipose tissues, liver and is also associated with other metabolic derangements e.g. heart and kidney failure (Eriksson *et al.*, 1989). It is complex multifactorial disorder which is causing high risk of the co-morbidities all over the world (Dixon *et al.*, 2011).

T2DM is involved in reducing the uptake of peripheral glucose, increase in producing hepatic glucose, decrease in the secretion of insulin along with sensitivity of the insulin. Generally, when glucose levels in the blood increase, it is compensated by elevated insulin secretion and β cell mass also increases, whereas the individuals who have genetic predisposition to β cell dysfunction, elevated glucose levels could not be compensated by enhancing insulin secretion which thus leads towards hyperglycemia (Guillausseau *et al.*, 2008). In T2DM, the β cell mass reduces potentially (Kahn *et al.*, 2006). Regulation of β cell mass is carried out by keeping the balance between replication and apoptosis of cells along with the formation of new islets from the exocrine pancreatic ducts. When this balance of both pathways disturbs, it leads towards rise in apoptosis of β cell together with decrease in mass of the β cell (Butler *et al.*, 2003).

Apart from hyperglycemia, T2DM has some other symptoms too including polydipsia (thirst), polyuria (frequent urination), loss in weight and polyphagia (overeating) (Control *et al.*, 2012). T2DM is linked with various severe and serious complications which include retinopathy leads to potential vision loss, cardiovascular diseases, neuropathy involving Alzheimer's disease (AD), limb amputation and nephropathy leads to kidney failure. Moreover, risk of cardiovascular morbidities and the stroke increases two to four times in diabetic individuals as compared to non-diabetics (Hotta *et al.*, 2000; Zimmet *et al.*, 2001). These complications linked with T2DM cause the major socioeconomic burden onto the developing as well as developed nations (Wei *et al.*, 1998).

1.2 Pathophysiology of Type 2 Diabetes Mellitus

It is a disorder of fat metabolism and impaired carbohydrate. It is specified by impaired glucose tolerance and impaired fasting glucose. The imbalance between beta cells insulin secretion and

insulin sensitivity causes abnormalities in metabolism of glucose (Scheen, 2003). This imbalance leads to hyperglyaemia. Insensitivity of insulin is also related with accumulation of lipid in muscle, liver and visceral adipose tissues. This accumulation in muscle and liver makes them insensitive to insulin (D'Adamo and Caprio, 2011). Other than muscle, Beta cells and liver (Fig 1.1) (DeFronzo, 1988) kidney, adipocytes, brain and alpha cells play important role in T2DM in glucose intolerance.

1.2.1 β-cell Function

Beta cell dysfunction takes place gradually, and hence becomes a progressive disorder (DeFronzo, 2009). Different studies have shown that when Beta cells do not respond properly to peripheral insulin resistance than T2DM occurs. Secretory potential and mass of Beta cells affect their ability to release insulin (Kahn *et al.*, 2006). It is mainly influenced by environmental and genetic factors (Lyssenko *et al.*, 2008).

1.2.2 Insulin Resistance

Although T2DM pathogenesis is not still understood, it is obvious that insulin resistance has important role in its development. Many longitudinal and cross sectional studies show that resistance of insulin developed 10-20 years before T2DM onset (Shulman, 2000). In order to balance the peripheral insulin insensitivity, Beta cells must have to increase insulin secretion, therefore influencing the Beta cells functioning efficiency. The exact mechanism by which insensitivity of insulin leads to failure of Beta cell remains unknown. However, it is believed that lipotoxicity, which has a strong role in developing the insensitivity of insulin is a cause of Beta cell failure (DeFronzo, 2009; Kahn *et al.*, 2006).

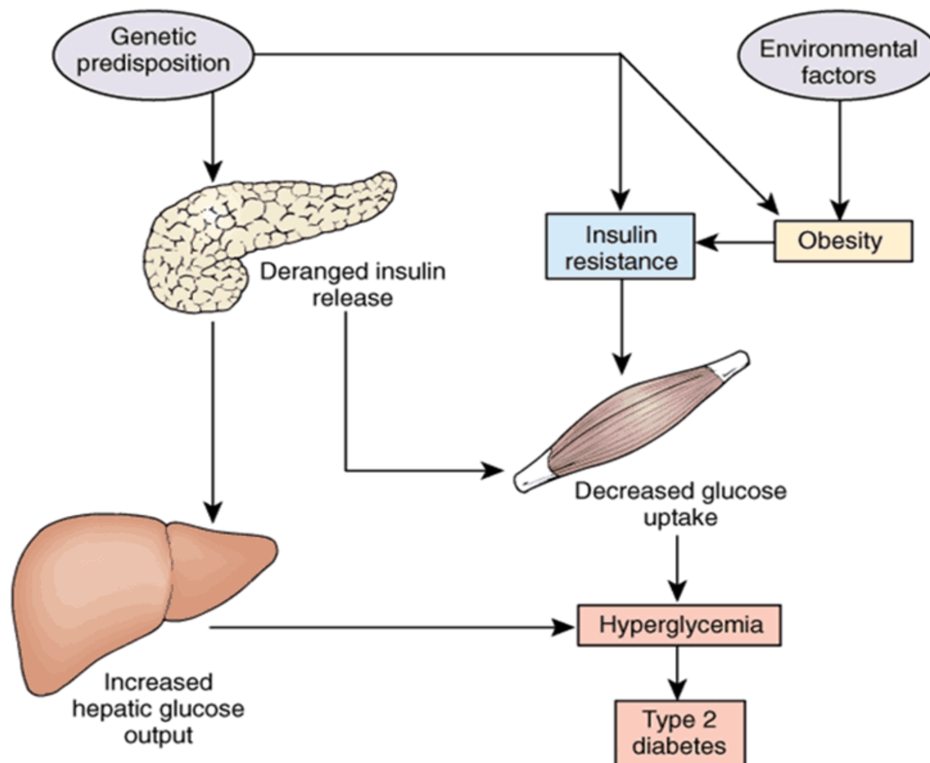


Figure. 1.1 Pathophysiology of T2DM: ach arrow shows the presence of defect that contributing towards the elevated glucose levels i.e. hyperglycemia. Insulin resistance in muscle and liver and impaired insulin secretion represent core defects in T2DM

1.3 Prevalence of Type 2 Diabetes Mellitus

In the world, every 12th individual is suffering from diabetes and their number is increasing day by day. International Diabetes Federation Atlas reported in 2014 that about 387 million in the world become infected with T2DM and this number is going to increase up to 592m in 2035. It was responsible for deaths of 4.9 million people in year 2014 and after every 7 seconds a person dies from it. It mainly affects people of low and middle income countries with age between 40 and 59 years. It is projected that the number of persons affected with DM will rise up to 439 million by the year 2030 (Figure1.2) (Chen *et al.*, 2012), which is constituting 7.7% of global adult population having age range 20-79 years (Shaw *et al.*, 2010).

As reported in "Dawn News" on Feb 10th 2017 diabetes is increasing at terrifying rate in Pakistan. In Pakistan, approximately 7.1 million people have diabetes and it is believed that this number will increase up to 14.4 million people in 2040 (https://www.dawn.com/news/1296299).

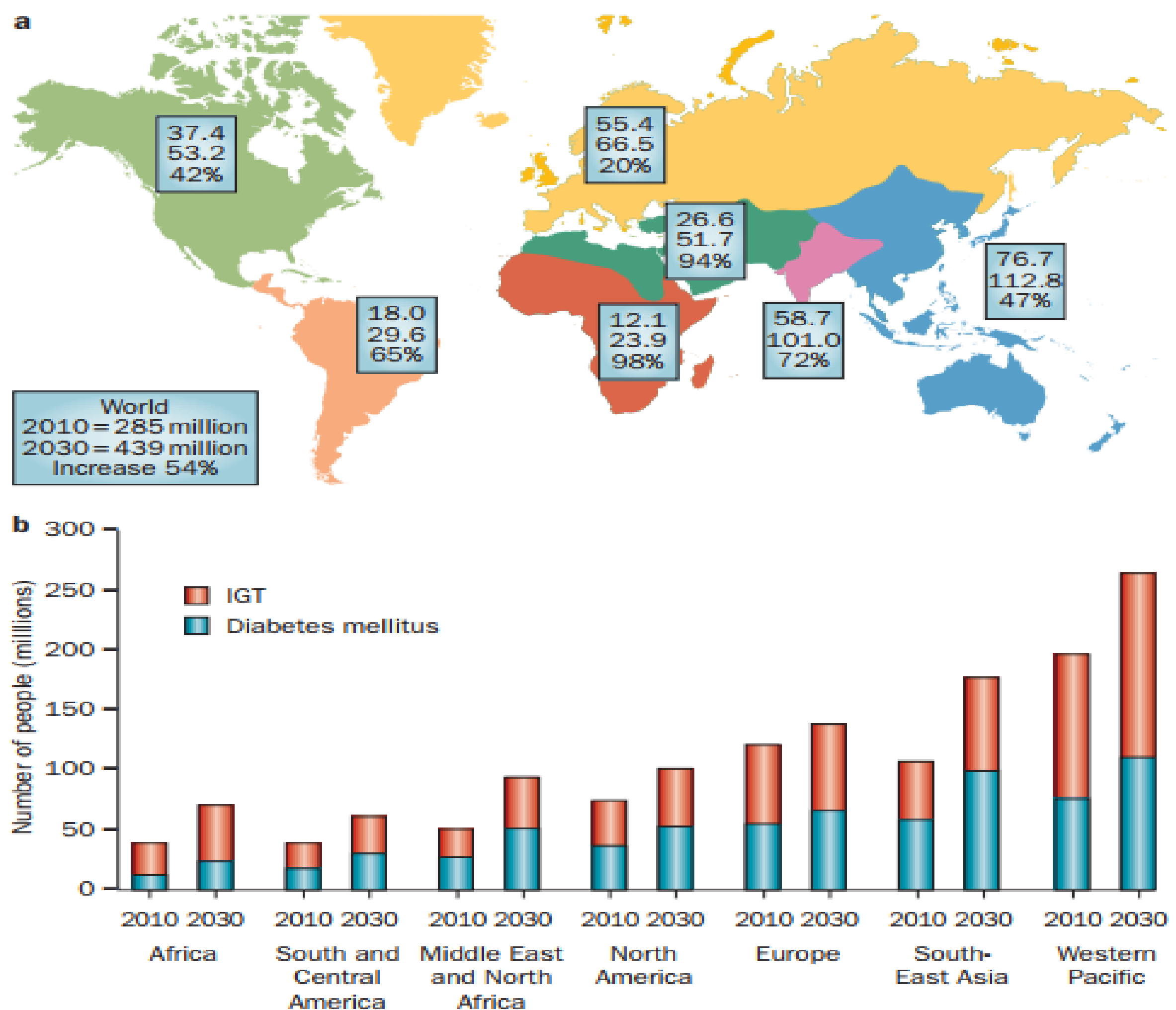


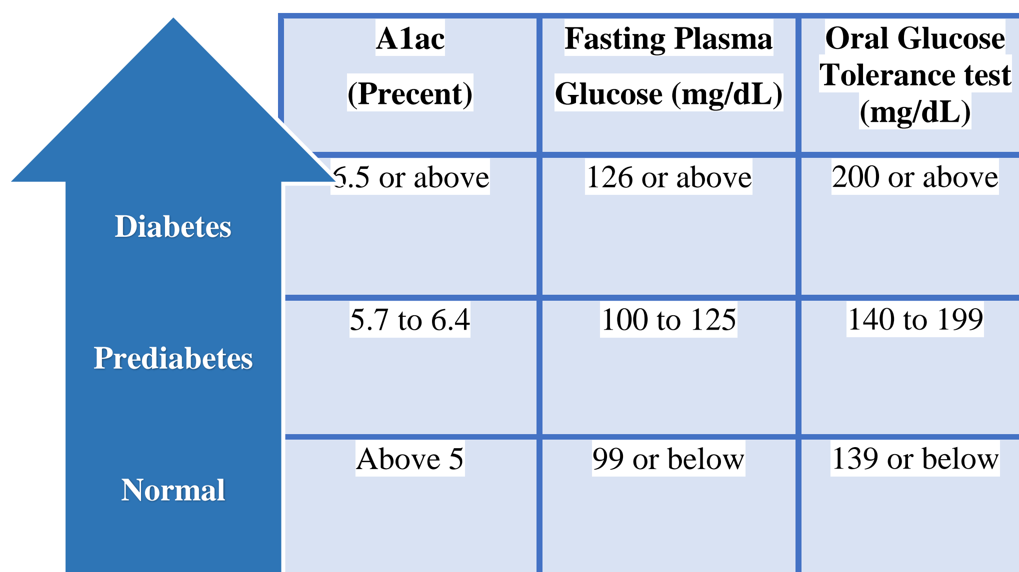
Figure 1.2 Global projections of Diabetes Mellitus epidemic: 2010-2030 (a) Top and middle values depict no. of diabetic individuals (in millions) in year 2010 and 2030 respectively; while bottom values are showing percentage increase from year 2010 to 2030. (b) No. of individuals having diabetes mellitus and Impaired glucose tolerance (IGT) among the adults aged between 20-79 years for year 2010 and 2030.

1.4 Clinical Diagnosis

The symptoms of diabetes include frequent urination, loss of weight, increased thirst, fatigue, slow healing of wounds and blurred vision (Organization, 1999).

Tests for diagnosis involve concentration of blood glucose either 2-hour post intake of glucose during an oral glucose tolerance test (OGTT) or fasting or both (Table 1.1). Blood drawn after fasting overnight is used to check plasma glucose concentration (Consultation, 1999; Group, 1979). Testing can help health care providers finding T2DM and prediabetics and thus help them to prevent complications and control to occur.

In the blood, Glycated haemoglobin (haemoglobin bind to glucose molecule) is checked by a test called HbAIC. We can get overall picture of glucose levels in blood in last 8 to 12 weeks by this test and it can be done at any time in a day without any kind of preparation like fasting (Committee, 2009; Group, 1979; Nathan *et al.*, 2007; Organization, 2013). Less than 6.5 % value of HbAIC does not abolish presence of diabetes in a person having glucose tests indicating diabetes (Organization, 2013). In a person, higher glucose levels in blood, on red blood cells sugar coat will be thicker. Oral glucose tolerance test (OGTT) measures the main energy source of body that is glucose.



	A1ac (Precent)	**Fasting Plasma Glucose (mg/dL)**	**Oral Glucose Tolerance test (mg/dL)**
Diabetes	6.5 or above	126 or above	200 or above
Prediabetes	5.7 to 6.4	100 to 125	140 to 199
Normal	Above 5	99 or below	139 or below

Table 1.1 Diagnostic Criteria for Type 2 Diabetes Mellitus

1.5 Causative Factors

The development of T2DM and exact causative aspect is not known but some major factors are genetic, environmental, behavioural, epigenetic and metabolic factors (Barrès *et al.*, 2009; Eriksson *et al.*, 1989; Initiative, 1998).

1.5.1 Genetic factors

T2DM is complex polygenic disorder involving multiple factors. It has been observed that genetic factors themselves are not capable of causing disease because of less penetration but with the contribution of environmental factors, they can. It is inherited disorder, so positive family history could enhance the chances of causing disease in an individual, if one parent is affected, chances increases up to 40% while it reaches up to 70% if both the parents are affected (Meigs *et al.*, 2000). Moreover, the concordance rate of the T2DM observed in monozygotic twins is around 70% while 30% in the dizygotic twins (Kaprio *et al.*, 1992). The past decade studies based on familial linkage analysis have evidenced that there are number of genetic loci associated with the T2DM. The most important is TCF7L2 in terms of playing its part in regulating the synthesis of insulin along with its secretion from the β-cells (Saxena *et al.*, 2007). It is stated that T2DM susceptibility genes containing SNP which is showing strong association with disorder have an odd ratio of 1.50. Furthermore, HNF4, ADIPOQ, CAPN10 and ENPP1 also show association with the T2DM by GWAS analysis (Grant *et al.*, 2006; Hanis *et al.*, 1996; Silander *et al.*, 2004; Vionnet *et al.*, 2000).

1.5.2 Epigenetic factors

It involves meiotic and mitotic heritable changes in gene function and expression, without any changes in sequence of nucleotide (Bird, 2007; Eccleston *et al.*, 2007). It causes changes in gene expression and hence determines functions of different cells in an individual with same genetic sequence.

Its mechanism consists of histone tail covalent modifications (like methylation, acetylation, phosphorylation), DNA packaging around nucleosomes and folding of chromatin, impact of micro RNAs on gene transcription (Dolinoy and Jirtle, 2008). The potential areas where epigenetic changes take place include CpG islands and include housekeeping genes promoter regions, imprinted genes cis-acting regulatory elements and transposable elements (Dolinoy and Jirtle, 2008).

These changes may continue to exist for whole cell's life, transmitted to daughter cells for many generations. Epigenetics regulate expression of gene and provides a control adaptive layer allowing the organism to adjust the changing environment. This additional layer is influenced by tissue type, disease status of person developmental stage and environmental conditions (De Bustos et al., 2009; (Keshet *et al.*, 2006; Mikkelsen *et al.*, 2007).

By using methylated DNA immune precipitation sequencing (MeDIP-seq) in monozygotic twins with type 2 diabetes a genome wide analysis computed differentially methylated regions (DMR). In T2D patients they suggested that there are 3,597 genes in gene body or in promoter region with at least one differentially methylated area. More precisely, 0.8% of T2D DMRs were present within 100bp of transcription start region (Yuan *et al.*, 2014). Therefore, on T2D DNA methylation has large impact.

1.5.3 Environmental factors

1.5.3.1. Obesity

It is increasing into a global epidemic disturbing drifts are being seen across world. Over agglomeration of adipose tissue is obesity. It affects psychological, social, metabolic and physical health (Naser *et al.*, 2006). In obese people, adipose tissues produce more inflammatory cytokines which causes resistance of insulin in target tissues. As the target tissues, do not answer to insulin and therefore glucose uptake from blood does not take place thus, causes increase in glucose levels of blood. Pancreatic beta cells elevate secretion of insulin into blood stream to maintain normal glucose level (Perley and Kipnis, 1966). For an insulin-resistant and obese person to develop T2DM, insulin released from beta cells will not be sufficient to compensate its lack of action and sensitivity. In obesity, adipose tissues release NEFAs into blood stream and induce insensitivity of insulin in target tissues (Kahn, 2001).

1.5.3.2 Hypertension

It is considered that hypertension is prevalent with obesity and T2D. It is an insulin resistant state (Ferrannini, 1995). In hypertensive state insulin resistance is restricted to glucose metabolism and it only affects tissues of periphery but not the liver (Ferrannini *et al.*, 1987).

Research studies shows that an inflammatory marker C-reactive protein has high levels in both hypertension (Blake *et al.*, 2003) along with T2DM (Hu *et al.*, 2004). Another link between these two is endothelial dysfunction (Gokce *et al.*, 2001).

1.5.3.3 Dietary Patterns

Type of food has a large impact on health of a person. Research studies have shown that development of T2DM is largely associated with dietary patterns. Individuals eating more of dairy products with high fat, red meat, sweets, processed food have high chances of T2DM (Liese *et al.*, 2009; Montonen *et al.*, 2005; van Dam *et al.*, 2002). It has been suggested that in order to avoid T2DM risk, food with high fiber content, low glycemic index and processed whole grain should be consumed as diet (Hu *et al.*, 2001).

1.5.3.4. Physical inactivity

Various studies have shown that physical activity is strongly related to T2DM (Fretts *et al.*, 2009; Jeon *et al.*, 2007). It causes reduction in weight and tends to change BMI Weight reduction improves sensitivity of insulin and decreases resistance in target organs resulting in normal glucose tolerance.

1.6 Treatment strategies

1.6.1 Life style Alteration

First approach for controlling T2DM is to balance the lifestyle and dietary intake. In a research study done in 2006, non-diabetic individuals with high level of blood glucose were given lifestyle modification program and placebo. In comparison to placebo the group with modified lifestyle showed decrease T2DM onset by 58% (Group, 2003).

1.6.2 Anti –diabetic Medications

The other approach is oral anti diabetic medications. The main drugs which are used are heterogeneous in tolerability, action and safety profile. These drugs consist of those which improve tissue sensitivity against insulin (thiazolidinediones), delay carbohydrates absorption and digestion in the gut (alpha glucosidase inhibitors), insulin secretion agents (rapid acting secretagogues) and reduce production of glucose from liver (biguanides). Currently no licensed

antidiabetic drugs against prediabetics are available (Krentz and Bailey, 2005). When drug intake does not make HbAIC normal, then insulin is the only choice.

1.6.3 Insulin therapy

Normally pancreatic beta cells produce insulin when there is high concentration of glucose in blood. In T2D, when deficiency of insulin occurs, external insulin is given to the patients. This insulin is injected subcutaneously. Previously, the activity of insulin was decreased down by adding chemicals that made crystals in buffer, results in decreased body absorption (cloudy insulin). Currently, insulin analogues with changes in sequence of amino acids are used. The types of insulin include slow absorbing analogues (glargine) and fast absorbing analogues (aspart and lispro). Galgarine can be eaten at any time but regular insulin is eaten 30 minutes before any meal (Sasali and Leahy, 2003).

1.7 Aims and Objectives

Diabetes is an endemic and over the last three decades, the number of people with DM has more than doubled globally. Most commonly occurring form of dementia is Alzheimer's disease (AD), which increasingly causes threat to global health. We need to check their genetic associations and risk factors to reduce the risks and to go for their therapeutic strategies. Thus, the objectives of my study are as follows:

- To find out the genetic predisposition of methylenetetrahydrofolate reductase gene in T2DM and AD
- To identify the expression levels of methylenetetrahydrofolate reductase gene in both T2DM and AD.

1.8 Impact of the Study

This present study is helpful in providing much required information for the design of specific future novel therapeutic approaches, significantly focusing or targeting a population of T2D and AD individuals. It will also add to the overall understanding and research studies of shared etiology between T2D and AD by considering its RNA Expression and SNP Genotyping. There is a wide spectrum of genetic heterogeneity for this disease due to which it is difficult to identify the main causative agents. Thus, such association analysis of these SNPs will be an addition to already reported studies and provide us a comparison of protein products of such genes in

normal and affected individuals. This also helps us to determine whether this SNP C677T is a risk factor towards the susceptibility of the disease or not. Such studies are basis of mutation screening leading to designing of genetic markers for disease diagnosis that further could be used for better clinical detection of T2D and AD.

CHAPTER 2

REVIEW OF LITERATURE

2.1 TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus (T2DM), prevailing complex metabolic disorder, is increasing dramatically. Around 312 million individuals are suffering from T2DM worldwide, so it has become an epidemic because of this global increase in occurrence. It is distinguished by early insulin insensitivity that is also linked to obesity as well as gradual β-cell failure occurring before onset of the clinical hyperglycemia. There are various mechanisms underlying T2DM i.e. increase in adipokines, NEFAs, inflammatory cytokines, glucotoxicity, lipotoxicity and amyloid formation for the β-cell dysfunction. It has polygenic inheritance i.e. multiple gene polymorphisms are involved in the development of disease and also predispose the individuals towards the disease (Gerich, 1998). On average, there is one single nucleotide polymorphism (SNP) after every 100-300 bases. In the genetic studies of complicated diseases e.g. T2DM, there is immense importance of SNPs (Ban *et al.*, 2010). The genes taking part in causing T2DM are; peroxisome proliferator-activated receptor γ, insulin-receptor substrate-1 and Calpain 10. Epigenetic factors are also recognised for playing a significant role in the development of T2DM. Epigenetics cause reversible changes together with heritable changes in expression of gene without altering primary DNA sequence. The state of chromatin lying in particular locus changes with gene expression changes. Thus, status of the histone modifications and DNA methylations are able to control the state of the chromatin unfolding or folding, and however; regulate the expression of gene.

2.1.1 Insulin Signalling Pathways:

Glucose levels in the blood ranges from 4 to 7mM normally irrespective of the feeding and fasting periods' length. This blood glucose control mechanism is carried out by balancing the glucose absorption from intestine, the production from liver, uptake and the metabolism by fat and muscle. Such balance is kept by insulin ability, insulin which is considered as the most potent and effective physiological anabolic hormone, for regulating the uptake of glucose in fat and muscle, in addition to its ability of decreasing its production from liver. Glucose uptake is enhanced by insulin through glucose transporters i.e. GLUT4 which are recruited into plasma membrane as result of the insulin signalling. Apart from blood glucose regulation, insulin is

working in stimulating the cell growth, cell differentiation, storage of proteins, lipids, and carbohydrates in the muscle, fat tissue and liver (Saltiel and Kahn, 2001). Any sort of insulin function abnormality results in various dysregulation of processes resulting in the metabolic dysregulation that leads towards the many metabolic disorders. Thus, insulin is the most potent and significant player in the maintenance of metabolic homeostasis.

2.1.1.1 Insulin receptor signalling

Insulin receptors belong to a subfamily of the receptor tyrosine kinases that also constitutes insulin receptor-related receptor as well as insulin like growth factor denoted as IGF-1 (Patti and Kahn, 1998). Insulin induces transphosphorylation of a β-subunit by another locating at the specific tyrosine residues, this happens when the insulin binds to α-subunits. Kinase activity of protein is enhanced due to transphosphorylation (Lietzke *et al.*, 2000; Myers *et al.*, 1992). The process of auto phosphorylation may also occur at the other tyrosine residues residing within intracellular tail of receptor and the juxta membrane region (Chang *et al.*, 2004). Tyrosine residues on the downstream signalling molecules as well as substrates including insulin receptor family i.e. IRS5/DOK5, Gab-1, IRS/DOK5, APS, Cb1 and Shc isoforms, are phosphorylated by the activated insulin receptors (Saltiel and Kahn, 2001). Tyrosines of these mentioned substrate molecules when phosphorylated, they act as the docking sites for the proteins having Src-Homology-2 (SH2) domain, e.g. p85 is regulatory subunit of the P13K (Saltiel and Kahn, 2001).

2.1.1.2 Phosphatidylinositol-3-Kinase Pathway

Phosphatidylinositol-3-kinase denoted as PI (3) K has a fundamental and significant role in the mitogenic and metabolic functions of insulin. If there is any inhibition or it is knocked out, studies reveal that it could lead towards the disruption of many metabolic activities of the insulin involving uptake of glucose, synthesis of lipid and glycogen (Shepherd *et al.*, 1995). It consists of two subunits i.e. p85 regulatory and p110 catalytic subunit. Regulatory subunit p85 has two domains of SH2 which identifies and binds to phosphorylated tyrosine residues of the IRS proteins leading to activating the PI (3) K enzyme (Shepherd *et al.*, 1995). Once it is activated, it begins to catalyse phosphorylation of the phosphoinositides on 3 positions for producing phosphatidylinositol 3,4,5-triphosphate i.e. PIP_3 that subsequently alters activity or the subcellular localization of many proteins (Lietzke *et al.*, 2000; Shepherd, 2005).

PIP3 binds with pleckstrin homology (PH) domain of many proteins that includes signalling molecules, adapter proteins, enzymes, and recruits them to plasma membrane (Lietzke *et al.*, 2000). These proteins are including Ser/Thr PDK1 that in turns phosphorylating and activating the different downstream signalling molecules constituting Akt1 through 3, glucocorticoid-inducible kinase (SGK), plus protein kinase C (PKC) (Mora *et al.*, 2004). PIP3 has also been appeared for mediating translocation of Akt towards plasma membrane through its PH domain (Corvera and Czech, 1998). This eventually results in the translocation of the GLUT-4 towards plasma membrane from the intracellular sties allowing for glucose uptake into the cell.

2.1.1.3 Insulin Signalling from the Lipid Rafts

Studies reveal that another insulin signalling pathway has been found within lipid rafts present in cell membrane. These lipid rafts are the regions which are rich in the cholesterol, glycolipids, sphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins (Smart *et al.*, 1999), and interacts with lipid raft protein i.e. caveolin. Insulin receptor activation activates adapter protein APS that exists as homodimer and contain both the domains of SH2 and PH (Baumann *et al.*, 2000; Kimura *et al.*, 2002).

On activation, APS shows interaction with three phophotyrosines on activation loop of insulin receptor through its SH2 domain. Every β-subunit of insulin receptor shows interaction with one APS homodimer, thus allows the receptor for recruiting two APS homodimers simultaneously (Hu *et al.*, 2003). Upon binding to receptor, C-terminal tyrosine of the APS phosphorylates and acts as docking site for the Cb1 protein that binds through its SH2 domain, and subsequently phosphorylates at the three tyrosine residues (Liu *et al.*, 2002). With the activation of the insulin receptors that are lying in lipid rafts, c-Cb1 and Cb1-b i.e. proto-oncogenes; also, get activated. Cb1 is associated with CAP i.e. Cb1 associated protein and both are recruited to APS. CAP shows binding with proline-rich sequences of the Cb1 through C-terminal of SH3 domain. CAP has been observed as predominately expressing in the insulin-sensitive tissues (RIBON and SALTIEL, 1997). Cb1-CAP complex is translocated towards lipid rafts located in cell membrane (Baumann *et al.*, 2000) which persuades the adaptor protein (CrkII) towards the lipid rafts. CrkII conjoins with guanyl nucleotide-exchange protein (C3G) forming a combination or complex, when it is translocated, it becomes proximate with the G protein (TC10) (Chiang *et al.*, 2001). TC10 delivers subsequent signal towards GLUT4 protein

which is translocated to plasma membrane as well as allowing glucose uptake by cell (Chiang *et al.*, 2001).

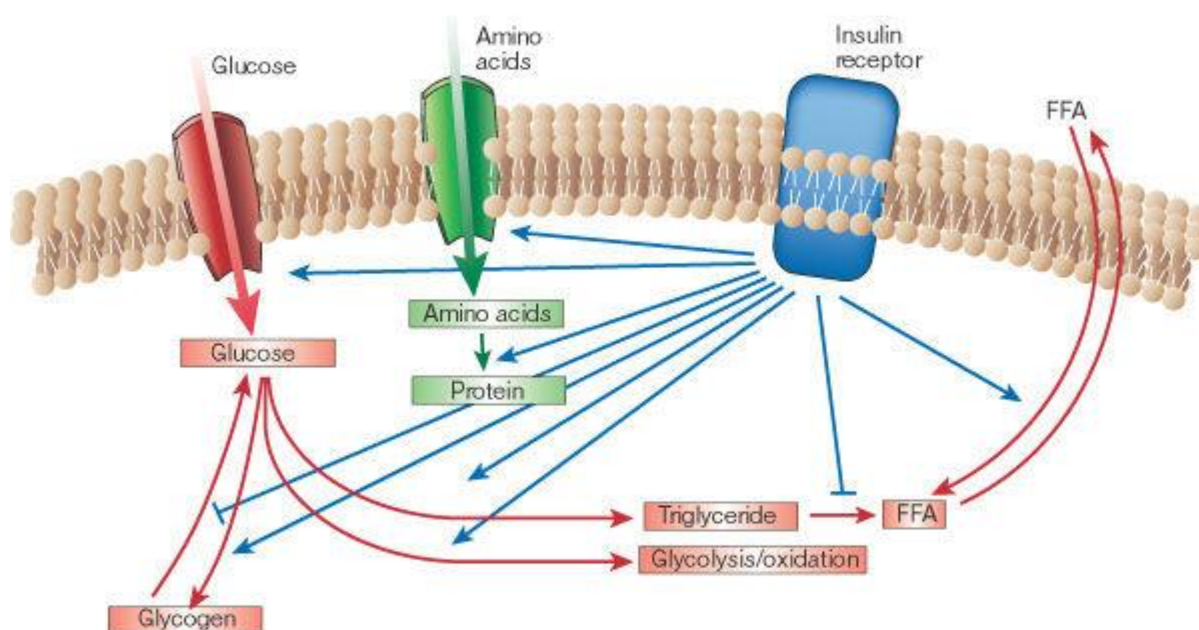


Figure 2.1 Regulation of the metabolism by insulin. Insulin being most significant anabolic hormone that promotes synthesis as well as storage of proteins, lipids and carbohydrates; whereas inhibits their degradation and then releases into circulation. Insulin stimulates glucose uptake as well as uptake of fatty acids and amino acids into the cells, increasing activity and expression of the enzymes which catalyses lipid, glycogen and protein synthesis, while inhibits the expression and activity of those which catalyse degradation.

2.2 Complications of Type 2 Diabetes Mellitus:

There are various short-term as well as long-term complications linked with the T2DM which develop with the progression of disease. Short-term complications may include hyperglycemic non-ketonic syndrome as well as hypoglycaemia in which blood glucose level gets high and low respectively. Over the long-term basis, T2DM is considered a great risk factor for many other disabling and life-threatening macrovascular as well as microvascular complications too. Long-term only appears when the disease of T2DM persists for longer period. It includes nephropathy, retinopathy, cardiovascular diseases, stroke, peripheral vascular disease, heart attack and neuropathy. One of them is enhanced hazard of the progression of Alzheimer's disease (AD) (Luchsinger and Gustafson, 2009; Vagelatos and Eslick, 2013).

2.2.1 Alzheimer's Disease

AD, accounts for commonly occurring dementia (related to age) form which accounts for above than 80% of all dementia cases. It is an estimation that globally 44.4m people suffer from dementia and AD (Larson *et al.*, 2013). AD is characterized with numerous pathophysiological hallmarks i.e. loss of neurons, senile plaques formation due to extracellular deposition of amyloid beta, formation of intracellular neurofibrillary tangles due to aggregation of hyper phosphorylated tau proteins inside the brain, activation of microglia and proliferation of astrocytes. All these features are complemented with the alterations in the neuronal synapses and mitochondrial dysfunction (Mattson, 2004).

According to the recent studies, there are shared links and association between both the diseases i.e. T2DM and AD. However, crosstalk between the mechanisms and factors contributing to diabetes-related central nervous system (CNS) complications is yet elusive and under discussion (Gage *et al.*, 2012; Hao *et al.*, 2015) Both the diseases (T2DM and AD) involve various etiological contributing factors and thus making it complex diseases. There are some genes which have been found as showing association with both the diseases (T2DM and AD) including APOE, TOMM40, HMHA 1, PICALM, CLU and MTHFR (Peila *et al.*, 2002) and have a substantial role in brain physiology as well as lipid metabolism. There are many commonalities between both the diseases (T2DM and AD) e.g. disturbance in insulin signalling pathways and amyloid beta (Aβ) abnormality.

Both T2DM and AD are common degenerative diseases; T2DM involves destruction of the pancreatic β cells and AD being a neurodegenerative disorder involves the extensive loss of neurons. Prevalence levels of these diseases increase with an increase in age (Kukull *et al.*, 2002). Therefore, commonalities between these complications (T2DM and AD) regarding their epidemiology are aging diseases, certain cardiac risk factors, high cholesterol and degenerative changes encourages us to explore and investigate the common molecular mechanisms which would lead to shared and common therapies for both the diseases. They also share several physiological mechanisms. Thus, AD is now referred as "Type-3 diabetes mellitus" that signifies the efficiency of common therapeutic approaches.

2.3 Correlation of Type 2 Diabetes Mellitus and Alzheimer's Disease

2.3.1 Epidemiological Evidences

The biological and epidemiological indications hold up the connection present in both the above indicated diseases associated with aging. T2DM relates to the variations in the cognition as well as to the cognitive dysfunction. Individuals suffering from T2DM have been reported for holding greater chances of the cognitive decline, and subsequently AD. T2DM holds strong evidences of showing association with higher hazard of progression of almost entire forms of the dementia, together with AD (Akomolafe *et al.*, 2006; Kukull *et al.*, 2002; Ott *et al.*, 1999; Wrighten *et al.*, 2009). There is a systematic review which included 14 appropriate studies (longitudinal population-based) containing diverse methodological quality ascertained that utmost studies showed higher occurrence levels for evolving "any dementia" into diabetic individuals as compared to non-diabetic individuals (Biessels *et al.*, 2006). Some of the studies depended on self-reported assessment for the T2DM, and many patients having diabetes remained undiagnosed in the elderly population, this is the reason because of which the perpetuation period of T2DM is hard to determine in aged patients (Allen *et al.*, 2004). According to one longitudinal cohort study which lasted for 9 years, the AD development risk was 65% more in diabetic persons as compared to non-diabetic controls (Arvanitakis *et al.*, 2004). A community based controlled study was made and examination of prevalence of glucose intolerance and diabetes was performed in persons having AD and control individuals not having AD and it proposed that glucose intolerance (46%) and frank diabetes (35%) was present in more than 80% patients with AD (Janson *et al.*, 2004). Even limitations exist, still there are various studies which suggest that risk of developing dementia becomes higher when

diabetic condition stays for longer time (Bruce *et al.*, 2008a; Bruce *et al.*, 2008b; Ott *et al.*, 1999).

In random effects models, at the baseline, T2DM was associated with the decreased levels of global cognition, working, semantic, episodic memory and visuospatial ability (Arvanitakis *et al.*, 2004). In T2DM, cognitive deficits mainly influence the psychomotor efficiency, learning, memory, attention, executive function, speed, and mental flexibility (Convit, 2005; Kodl and Seaquist, 2008). Recently, studies have shown that there is positive correlation in mild cognitive impairment (MCI) and T2DM, which could subsequently lead towards the dementia at an accelerated rate (Alagiakrishnan and Sclater, 2012). A survey was performed on people having diabetes living in England's nursing homes. It exhibited substantial levels of comorbidity and disability. It observed that the most commonly existing comorbidity was dementia (Gadsby *et al.*, 2011).

2.3.2 Pathophysiological Link

Strong epidemiological correlation suggests the presence of pathophysiology links too. Yet, determinants for enhanced cognitive decline are less evident in the T2DM individuals. Most of the hypotheses claim that primary root of correlation might be connected with the diabetic vascular disease as well as with inadequate cerebral circulation, which subsequently lead to silent ischemic damage caused by diabetes. But several studies which were done on cerebral structures of diabetic persons, have shown evidences that besides increased leukoaraiosis; there is an increase in cortical and subcortical atrophy even cardiovascular risk factors were controlled, this suggest the association of diabetes with the impaired cognitive function (Cherbuin *et al.*, 2012; den Heijer *et al.*, 2003).

Recent studies focus on insulin role. A strong probable link in both AD and T2DM is insulin resistance. Regardless of presence of T2DM, Hyperinsulinemia has shown association with worse cognitive performance. Literature is rapidly pointing towards insulin resistance and insulin deficiency as AD mediators. De la Monte suggests AD as "Type-3 Diabetes" representing AD as diabetes form which is predominantly involving brain with biochemical and molecular features overlapping with T2DM (de la Monte and Wands, 2008). In brain ageing, the insulin role has been known since long time and it has very substantial neurotropic properties in brain. Through blood-brain barrier, insulin is transported quickly towards the CNS by transport mechanism through insulin receptors. The fascinating point to bear in mind is the

localization of these receptors i.e. hippocampus, frontal area and entorhinal cortex which are taking part in learning and memory. Insulin has another role in producing some significant neurotransmitters e.g. acetylcholine and norepinephrine. In post-prandial period, circulating insulin increases which help in determining physiological parallel increase in hormonal concentration in brain. In condition of chronic hyperinsulinemia, insulin receptors located at the blood-brain barrier show down-regulation as it also happens in insulin-resistance state and in T2DM, thus reduces insulin transport in brain.

Evidence grows a link with the alteration levels of deposition and metabolism of amyloid precursors inside the brain which happens in individuals having diabetes, that could be proposed as involved in pathogenesis of the AD in T2DM. APP (Amyloid precursor protein) consisting of 770 amino acids is transmembrane protein and is known as precursor for amyloid beta (Aβ). It is involved in subsequent development of the AD. Though it hasn't been elucidated yet what kind of role, Aβ and isoforms are playing, it might be involved in many physiological processes. Insulin is the neurotropic factor at modest concentrations while hyperinsulinemia in brain might be linked with reduced Aβ clearance because they are competing for common mechanism of degradation exhibited by Insulin-Degrading Enzyme (Qiu and Folstein, 2006). IDE might be a link between hyperinsulinemia and the AD. Even though IDE selects insulin more instead of amyloid beta, so the condition of brain hyperinsulinemia may cause hindrance in clearance mechanism of amyloid beta which lead towards its assembly in brain, thus cause neurotoxic effects (Craft and Watson, 2004).

In the brain, mechanism of insulin signalling when get disturbed it represents the early and progressive abnormalities which could constitute many histopathological, biochemical, and molecular lesions in condition of AD. Increased hyperinsulinemia and resistance of insulin indicate links with additional amygdala and hippocampal atrophy in diabetic individuals as compared to non-diabetic individuals when magnetic resonance imaging (MRI) was performed (Convit, 2005; den Heijer *et al.*, 2003). So, these links suggest a commonly occurring procedure that influences the Aβ disposition inside the brain as well as pancreatic islet (Janson *et al.*, 2004). A risk factor for the dementia and cognitive dysfunction is change in glucose levels too. In cohort study based on communities showed the association of higher plasma glucose concentration with higher risk of dementia and AD in population having diabetes or not, it indicates the deleterious effects of higher glucose levels on aging brain (Crane *et al.*, 2013). Studies claim that the risk of AD increases in elderly patients which are having prediabetic

conditions. So, it is claimed that hyperinsulinemia and hyperglycemia can increase brain aging by inducing amyloid oligomerization and tau hyper phosphorylation and also lead towards extensive brain microangiopathy., thus diabetic individuals are more prone for developing accelerated leukoaraisos (Roriz-Filho *et al.*, 2009).

2.3.3 Cerebrovascular Mechanisms

2.3.3.1 Brain infarcts

By clinical history (Honig *et al.*, 2003) strokes are evidenced as brain infarcts when performed MRI test (Vermeer *et al.*, 2003), are correlated with elevated likelihood of the onset of dementia including LOAD (Late onset of Alzheimer's Disease). Still association processes and means aren't evident. Still, pathological investigations have revealed the lower occurrence of amyloid plaques in the brains of people suffering from dementia and also inflicted with infarcts (Schneider *et al.*, 2007; Snowdon *et al.*, 1997), It suggests that occurrence of infarcts has been considered as disgrace for dementia because it lessens amyloid threshold in the brain.

2.3.3.2 White matter disease (WHI)

It is ascertained as leukoaraiosis in brain scan which exhibits the "microvascular disease" in the brain or it could come under demyelination (a disorder of myelin sheath). Though description of WHI is up to now, a controversial subject. WHI has been considered as being ischemic in origin; same as infarcts are (Pantoni, 2006) Thus, it is suggested as being surrogate markers for cerebrovascular disease (Pantoni, 2006). Thus, contemporary studies evidence that WHI is prevalent in the LOAD, and might relate to cerebral amyloid angiopathy (CAA) (Alonzo *et al.*, 1998; Gurol *et al.*, 2006; Haglund and Englund, 2002; Nakata-Kudo *et al.*, 2006). So, some of the WHI is linked with amyloid burden and subsequently LOAD. In T2DM, WHI is very frequently occuring correlating with cognitive impairment (Manschot *et al.*, 2006), still this is not clear whether WHI is marker for the microvascular injury or else it could represent the mechanism correlated with the Aβ deposition.

2.3.4 Non-Cerebrovascular Mechanisms

2.3.4.1 Hyperinsulinemia

It is the most likely risk factor of the LOAD irrespective of the cerebrovascular disease due to following reasons. The first reason is that insulin is capable of crossing BBB (blood brain

barrier) (Park, 2001), and infusion of peripheral insulin in aged might influence the intensities of amyloid-beta $(A\beta)_{42}$ in the cerebrospinal fluid (Watson *et al.*, 2003), surrogate marker for the Aβ clearance in the brain, also indirect marker for the possibility of incidence of LOAD. The second reason is that brain has insulin receptors including hippocampus and the entorhinal cortex (Frölich *et al.*, 1998), structures have an effect early in LOAD (Small *et al.*, 1999). The third reason is the link of insulin degrading enzyme (IDE) with the Aβ clearance in the brain, also both insulin and Aβ works as competitive substrates for the IDE (Farris *et al.*, 2003). Fourth reason is insulin in brain could possibly cause rise in Aβ accumulation as well as tau protein phosphorylation, that is fundamental for pathogenesis of the LOAD (Park, 2001). Pathways related to the insulin present in the periphery with the clearance of Aβ in the brain area are complex as well as multiple. Craft and his colleagues assessed the phenomenon of peripheral hyperinsulinemia affecting clearance of Aβ in the brain (Craft, 2007). There is a potential pathway comprises on how peripheral hyperinsulinemia is involved in downregulation of uptake of insulin across BBB because of its saturation over the physiologic stages (Banks *et al.*, 1997). This could lead towards reducing the insulin levels in the brain; also, downregulating the expression of the IDE (Zhao *et al.*, 2004), thus reducing the amyloid beta clearance mediated by IDE (Farris *et al.*, 2003). Such complex observation is being used for the support of both paradoxical usage of the rosiglitazone (an insulin-sensitizer) (Risner *et al.*, 2006; Watson *et al.*, 2005), along with the intranasal insulin (Reger *et al.*, 2006) as LOAD medicine.

2.3.4.2 Advanced products of Glycosylation (AGE)

It has close association with glycaemia as well as the DM, its level elevates with the increase in glucose concentration. During hyperglycaemic conditions, diabetic animal models and the human tissues comprise elevated AGE; also, its receptors (RAGE) show upregulation (Basta *et al.*, 2004; Creager *et al.*, 2006; Negrean *et al.*, 2007). AGEs are known for its association with traditional microvascular complications of the T2DM (Peppa *et al.*, 2006; Schalkwijk *et al.*, 2002; Stitt, 2001; Thallas-Bonke *et al.*, 2004; Vlassara *et al.*, 1994). In LOAD, high expression of RAGE could be detected (Du Yan *et al.*, 1996; Lue *et al.*, 2001; Schmidt *et al.*, 2000) In LOAD, high expression of RAGE could be detected (Du Yan *et al.*, 1996; Rashid *et al.*, 2003).

2.3.4.3 Lipoprotein Related Proteins (LRP)

It is a lipoprotein receptors' group affecting the metabolism of lipids. LRP-1 which is present in liver, and the other tissues. They have a role in clearing amyloid from the plasma and mediating the movement of amyloid out of brain (Donahue *et al.*, 2006; Tamaki *et al.*, 2007).

It has been observed that LRP-1 is reduced in T2DM without disturbing the lipid levels (Tamaki *et al.*, 2007). It has been observed that LRP-1 is reduced in T2DM without disturbing the lipid levels. Clearance of Amyloid by LRP-1 is eased by soluble LRP, also, it could be therapeutic candidate to treat the LOAD (Deane *et al.*, 2004; Zlokovic *et al.*, 2010). Hence, LRP-1 is the one of potential mechanism linking T2D to amyloid beta and the LOAD.

2.3.5 Metabolic Dysfunction Enhances Risk of Dementia

Obesity and metabolic disorder (T2DM) are contributing to impairment of cognitive function and increasing risk of AD. Previous studies have shown that individuals having diabetes and features of this metabolic disorder have high risk factor for developing dementias including the AD (Arvanitakis *et al.*, 2004; Ott *et al.*, 1999). But magnitude of this risk factor is still under debate; e.g. a longitudinal study evidenced 2-fold increased risk magnitude of T2DM in development of AD as compared to non-diabetic persons (Luchsinger *et al.*, 2004). Thus, other studies show different results about the association of both the diseases (Hassing *et al.*, 2002). A recent longitudinal study has suggested 1.5-fold higher magnitude of risk factor of AD among the diabetic individuals (Cheng *et al.*, 2012). Increased risk of dementia is also affected with age factor. A study has shown that in middle-aged people, central obesity rate increases which associate with increased risk of dementia irrespective of diabetes (Whitmer *et al.*, 2008). In aged population, T2DM and obesity appears as weak association risk factor to AD (Anstey *et al.*, 2011).

Association of T2DM and its precursor circumstances are not restricted to the AD. It is also associated with vascular dementia (MacKnight *et al.*, 2002), lacunar infarcts (MacKnight *et al.*, 2002) and white matter hyper intensities (Hsu *et al.*, 2012). Together, epidemiological and clinical findings have demonstrated the T2DM as high risk factor for cognitive function deterioration and subsequently dementia. These insights have been found out in researches performed on the animal models.

2.4 Linked Pathways of Type 2 Diabetes Mellitus and Alzheimer's Disease

2.4.1 Dysregulation of Glucose and Insulin

Diabetes has key features i.e. dysregulation of insulin along with glucose and it leads to impaired insulin signalling as well as insulin resistance which have been associated to elevated AD pathology (Schulingkamp *et al.*, 2000). With normal aging, levels of insulin and its receptors in brain decreases (Frölich *et al.*, 1998). Moreover, expression level of insulin receptors in the brain declines further with the AD (Steen *et al.*, 2005).

There is a zinc-binding metalloprotease named as IDE, both insulin and amyloid beta are its substrates, it could be contributing to link the AD and T2DM. A transgenic AD mice which have been given a fat-rich nutrition, was lacking in insulin signalling resulting in decrease in levels of IDE and increase in levels of Aβ (Ho *et al.*, 2004; Zhao *et al.*, 2004). Loss-of-function mutations in the IDE induces T2DM and impairs the degradation of Aβ (Farris *et al.*, 2004). Even though procedures underlying T2DM outcome is not clear, it has been estimated that diminished role of IDE may promote hyperinsulinemia which could contribute to impaired glucose tolerance and insulin resistance in the long term. Pharmacological inhibition of the IDE decreases the level of insulin degradation and the degradation of islet amyloid peptide (Bennett *et al.*, 2003) and amyloid beta (Shiiki *et al.*, 2004) in the long term. An interesting point is the higher affinity of IDE for the insulin as substrate as compared to Aβ (Qiu *et al.*, 1998).

However, one hypothesis considering role of T2DM as a risk factor for AD is that T2DM has hyperinsulinemia characteristics resulting in reduced degradation of the amyloid beta by IDE which is leading to accumulation of Aβ. Because of insulin deficiency, reduced insulin signalling leads to IDE downregulation as well as higher Aβ accumulation and increased AD risk. On an interesting note, T2DM treatment may improve the neural functioning and/or slow down the AD pathogenesis e.g. with the improved control of diabetes, cognitive decline process got slowed down (Luchsinger *et al.*, 2011). Moreover, it is observed that the common medications used for T2DM treatment e.g. rosiglitazone, metformin can possibly decrease the AD-related cognitive decline as well as amyloid beta levels (Chen *et al.*, 2009; Watson *et al.*, 2005). Clinical trials of the intra-nasal insulin therapy have been performed in the early mild cognitive impairment (MCI) and AD patients, and initial results show that it slows down cognitive decline (Craft *et al.*, 2012).

Researches on the animal models are concordant with the observations performed clinically. Per study, in the 3xTg-AD mouse model, a drug named "pioglitazone" has enhanced plasticity, learning, also reduced Aβ plus tau pathologies (Searcy *et al.*, 2012), similarly "liraglutide" did

(Long-Smith *et al.*, 2013). Consistent to all this data is an observation that generation of hyperinsulinemia and insulin resistance by means of mutated insulin receptor, this remains unsuccessful in accelerating rates of Aβ accumulation along with cognitive decline in the transgenic mouse model of AD (Murakami *et al.*, 2011). Likewise, in the transgenic mice of AD, knockout of substrate 2 of insulin receptor predictably produces the significant metabolic dysfunction, but it reduces AD-related pathologies rather than accelerating them (Freude *et al.*, 2009; Killick *et al.*, 2009). Thus, other factors may be playing its role in these mechanisms due to which obesity as well as metabolic syndrome e.g. T2DM intensifies the likelihood of AD.

2.4.2 Inflammation

Pro-inflammatory pathways are contributing to the interactions between AD and T2DM. It is very well recognised that central obesity, metabolic syndrome and diabetes, all, contribute to chronic systemic inflammation (Lumeng and Saltiel, 2011). In T2DM, levels of certain proinflammatory cytokines increase (Lee and Pratley, 2005) and there are various anti-inflammatory drugs which reduce this effect (Esser *et al.*, 2015). When the individuals having metabolic syndrome have been observed, it is evidenced that those who were having more inflammation, were at higher risk to acquire impairment in the cognitive function as compared to ones having reduced levels of inflammation (Yaffe *et al.*, 2004). Due to obesity, the inflammatory cytokines residing in adipose tissues show increased levels (Wellen and Hotamisligil, 2003) as well as in nervous system. When the animal models were given diet that induced obesity, it has been observed that the inflammatory responses rise in several regions of brain e.g. cerebral cortex (Zhang *et al.*, 2005) hypothalamus etc. (Thaler *et al.*, 2012).

Current laboratory studies demonstrate elevated levels of proinflammatory cytokines i.e. IL-1β and TNF-α in cortex of the mice which were on fat-rich diet and in the primary glial cell cultures prepared from these mice. Also, it is observed that increased levels of proinflammatory factors in both central as well as peripheral nervous system lowers health of neurons. It is widely hypothesized that inflammatory pathways in AD directly take part in the disease initiation as well as growth (Viscogliosi and Marigliano, 2013). Recent GWAS analysis depict the numerous genes associated to the AD function of the innate immunity (Tanzi, 2012). It's not clear if the same polymorphism in genes is contributing to incidence of T2DM. Though, literature stays undecided but observational studies evidence that non-steroidal anti-inflammatory drugs (NSAIDs) use can possibly reduce risk of AD development (Côté *et al.*, 2012). From observing pro-inflammatory profiles of the obesity and the

metabolic disorder i.e. T2DM, the supposed part of all above mentioned pathways in the AD pathogenesis which is a key process involving in interaction across the diseases in inflammation.

2.4.3 Amyloidogenesis

Production of the amyloid peptides along with aggregation of the unusually folded protein are considered as communal pathological feature of both diabetes and the AD (Chiti and Dobson, 2006). The prominent pathological characteristic of AD is extracellular presence of amyloid plaques which are composed of the insoluble Aβ accumulation. Aβ can oligomerize and form large soluble assemblies, can arrange itself into the cross-β-sheet units by undergoing conformational changes and generate amyloid fibrils in senile plaques (Kirkitadze and Kowalska, 2005). As amyloid hypothesis believes that these assemblies of pathological Aβ are the initiating and central event in process of disease (Mohamed *et al.*, 2016). As previous studies had a focus on neurotoxicity effect of aggregated fibrillar Aβ while collected evidences have described the importance of Aβ assemblies in the neuropathy i.e. as happens in long-term potentiation impairment as well as death of neurons (Lambert *et al.*, 1998; Lesné *et al.*, 2006; Shankar *et al.*, 2008; Townsend *et al.*, 2006; Walsh *et al.*, 2002). However, soluble oligomers of Aβ appears to show better correlation to dementia and to synaptic loss as compared to accumulated Aβ in the plaques (Lue *et al.*, 1999; McLean *et al.*, 1999).

Islet amyloid deposits are the similar to the amyloid plaques, formed in the AD brain, in pancreas of diabetic individuals. About 90% of diabetic individuals contain pancreatic islet amyloid that in linked to reduced β-cell mass (Clark *et al.*, 1988).

2.4.4 Oxidative Stress

It happens when amount of the "free radicals" generated from the metabolic activity surpasses the antioxidant capability of the cell and results in attacking to nucleotides, proteins, lipids, also their impaired biological activities that eventually lead towards the death of cells (Sies, 1985). The excessive free radicals production is caused by hyperglycemia. (Vincent *et al.*, 2004). Substantial experimental and clinical data evidences that there is increased oxidative stress in diabetes (Rösen *et al.*, 2001) which mediate diabetic neuropathy (Vincent *et al.*, 2004). While enhanced oxidative stress is well-documented in the AD too (Lovell and Markesbery, 2001; Nourooz-Zadeh *et al.*, 1999; Nunomura *et al.*, 2004; Sayre *et al.*, 1997).

It is observed that the oxidative damage take place before the formation of amyloid beta plaques (Nunomura *et al.*, 2001; Praticò *et al.*, 2001) and an increase seen in reactive nitrogen species (RNS) which go along with onset of the deposition of Aβ; experimented in the transgenic mouse model of AD (Apelt *et al.*, 2004), it claims that oxidative stress can trigger the increased Aβ production. Ample experimental data suggests that oxidative stress is upregulating the expression of β-secretase as well as γ-secretase, promoting the phenomenon of amyloidogenic processing of the Aβ and APP (Jo *et al.*, 2010; Quiroz-Baez *et al.*, 2009; Tamagno *et al.*, 2008; Tong *et al.*, 2005). The oxidative stress boosts tau hyper phosphorylation along with tangle formation (Sultana *et al.*, 2006). Furthermore, oxidatively modified proteins which are taking part in the pathogenesis of AD, have been recognized in the AD brain through the redox proteomics (Butterfield *et al.*, 2006).

2.4.5 Mitochondria Dysfunction

Mitochondria plays role in the synthesis of ATP, Ca^{2+} homeostasis maintenance, antioxidant defence and redox signalling (Lin and Beal, 2006; Mattson *et al.*, 2008). Due to restricted glycolytic capacity, the neurons are relying remarkably on the synthesis of ATP by mitochondria, so, are practically prone to disruption of the mitochondrial function (Xiao-Jian *et al.*, 2011). Indeed, dysfunction of mitochondria has been anticipated as significant link between AD and diabetes (Moreira *et al.*, 2007). Abnormalities associated with the mitochondria i.e. increased intracellular levels of calcium, altered mitochondrial morphology, deficit antioxidant as well as bioenergetic capacity; are recognised in the diabetic patients (Anello *et al.*, 2005; Levy *et al.*, 1994) as well as in animal models (Edwards *et al.*, 2010; Fernyhough *et al.*, 2010; Mastrocola *et al.*, 2005; Moreira *et al.*, 2007; Moreira *et al.*, 2005). Moreover, AD cybrids (cell deprived of the endogenous but replaced with the platelet mitochondrial DNA extracted from the AD individuals) showed a spectrum of the mitochondrial dysfunction, decreased activity of cytochrome c oxidase as well as increased ROS generation (Swerdlow *et al.*, 1997). Recently, it has shown that mitochondrial proteome dysregulation in transgenic mice of AD before development of the significant neurofibrillary tangles and amyloid plaque, indicate that mitochondrial impairment may contribute to the AD pathology (Chou *et al.*, 2011). In an AD transgenic mouse model, when manganese superoxide dismutase has been knocked out which is a major antioxidant enzyme of mitochondria, it has been observed that it increases Aβ deposition levels along with the plaque pathology in the brain (Li *et al.*, 2004). In both AD patients and transgenic mice model, Aβ directly interacts

with the Aβ-binding alcohol dehydrogenase (ABAD); present in mitochondria, results in producing reactive oxygen species (ROS), dysfunctioning of mitochondria and the death of neurons (Lustbader *et al.*, 2004). Contrarily, inhibition of ABAD-Aβ interaction decreases accumulation of Aβ, preserves the mitochondrial function, also, make the spatial learning improved in the AD mouse model (Yao *et al.*, 2011). It is reported too that Aβ inhibits mitochondrial key enzymes e.g. α-ketoglutarate dehydrogenase, cytochrome-c oxidase, leading towards the mitochondrial impairment (Casley *et al.*, 2002).

2.5 Modifiers of Type 2 Diabetes Mellitus and Alzheimer's Disease

2.5.1 Endocrine Changes

2.5.1.1 Low Testosterone

In men, the age-related reduction of the testosterone commonly known as andropause which results in dysfunction as well as vulnerability towards the diseases in the androgen-responsive tissues which include muscles, adipose tissues, bones, also brain (Morley, 2000). Studies from past several years have identified the andropause as an important and significant risk factor of the AD (Overk *et al.*, 2013). Men having low testosterone either in the blood (Hogervorst *et al.*, 2001; Rasmuson *et al.*, 2002) or brain (Rosario *et al.*, 2011; Rosario *et al.*, 2004) are at higher risk for the development of AD.

It has been depicted from the studies, that there is a connection between low levels of testosterone and resistance to insulin in men (Grossmann *et al.*, 2008; Kapoor *et al.*, 2007). It has been depicted from longitudinal studies that, metabolic syndrome also results from low level of testosterone, and it appears 5 to 10 years before the appearance of cardiovascular and metabolic symptoms (Goncharov *et al.*, 2008; Hougaku *et al.*, 2006). Since metabolic syndrome is described as a precursor to T2DM development, it has been found that men suffering from T2DM, have potentially lower concentration of overall testosterone concentration. Thus, it is evidenced as shared risk factor of both T2DM and AD.

2.5.2 Genetic Factors

Studies suggest that there are common genetic predispositions underlying both the diseases T2DM and AD, having SNPs as common risk alleles contributing to shared pathogenic

pathways involving in enhancing the risk of developing AD eventually in diabetic patients. A GWAS study has been done to find out the shared genetic risk factors which are modifiers of the pathways underlying both the diseases, they identified 927 SNPs as shared risk alleles between T2DM and AD; out of which 395 are shared SNPs showing the same shared risk allele for both AD and T2DM known as consistent risk alleles. 532 are such SNPs which known as divergent risk alleles because they may contribute to any of the disease (T2DM or AD) without necessarily sharing to both diseases. Both are complex diseases so their genetic contributions consist of large number of variants and each contribute as small genetic risk. Some of the genes which are considered as risk factors for both diseases are enlisted (Table 2.1). (Hao *et al.*, 2015)

Table 2.1: Genes and no. of SNPs shared between T2DM and AD

Genes	No. of SNPs	Genes	No. of SNPs	Genes	No. of SNPs
PICALM	31	**APOE**	5	**GAB2**	1
MS4A4A/4E/6A/6E	28	**MTHFR**	1	**SORL**	5
CD2AP	24	**TOMM40**	23	**MS4A2**	15
APOC2/APOC4	12	**CLU**	3	**MARK4**	11
CD33	1	**MTHFD1L**	1	**BIN1**	7
CR1	7	**PVR**	2	**PTK2B**	3
EPHA1/EPHA1-AS1	7	**FERMT**	2	**TCF7L2**	66
HNF1B	6	**PROX1**	1	**FTO**	86
ABCC8	1	**TLE1**	1	**CDKN2B**	4

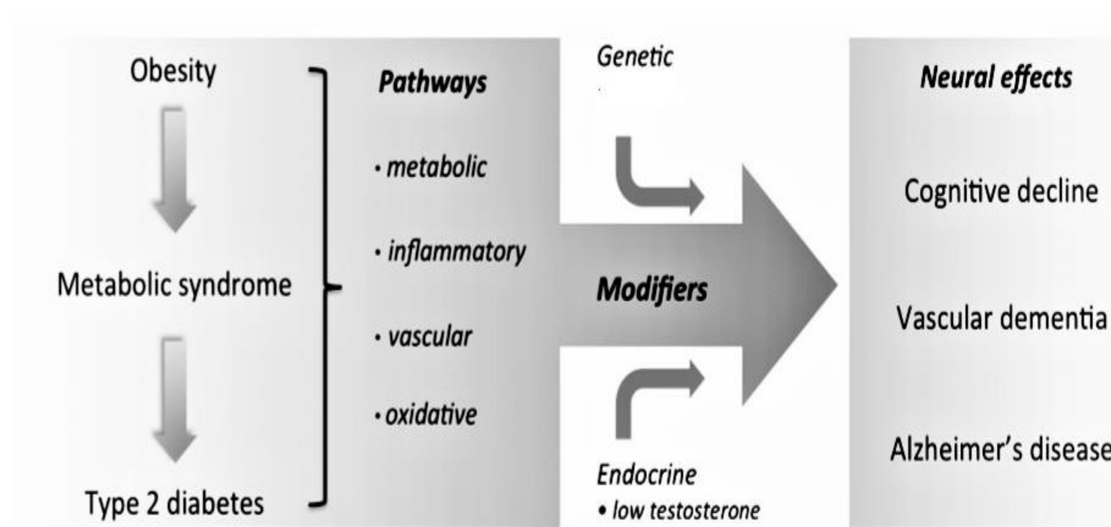


Figure 2.2 Pathways shared between T2DM and AD

2.5.2.1 Phosphatidylinositol Binding Clathrin Assembly Protein (PICALM)

A clathrin assembly protein is encoded by this gene, which recruits adaptor protein complex 2 (AP2) and clathrin towards plasma membranes at coated-pit formation sites and assembly of clathrin vessicle. By regulatin clathrin cage size the amount of membrane to be recycled can be determined. The protein is playing a role in AP2-dependent clathrin-mediated endocytosis. A chromosomal translocation at the position p13 or q14, results in fusion of the MLLT10 gene. Above mentioned gene plays role in acute myeloid leukemia, malignant lymphomas and acute lymphoblastic leukemia. The risk for Alzheimer's disease is related to the polymorphisms of this gene. Numerous transcripts which are alternatively spliced encode various isoforms, have been shown by this gene (Harold *et al.*, 2013).

2.5.2.2 Clusterin (CLU)

This gene encodes such a protein which is "secreted chaperone" released under certain stress conditions. It may exist in the cytosol of cell. It has been found taking part in various fundamental biological events e.g. tumor progression, cell death, neurodegenerative disorders. Alternate splicing results present in both non-coding and coding variants. CLU (Clusterin) is Coding gene for Clusterin Protein. Disorders associated with CLU include Transient Neonatal Neutropenia and Follicular Dendritic Cell Sarcom. More of the functions are; ubiquitin protein ligase binding, chaperone binding etc. A significant paralog of CLU gene is CLUL1 (Jones and Jomary, 2002)

2.5.5 Apolipoprotein E (APOE)

This gene encodes apoprotein, main protein of chylomicron, binds to peripheral cell receptor and liver. It is important for catabolism of constituents of triglyceride-rich lipoproteins. This gene links to chromosome 19 in a cluster with associated apo lipoprotein C1 and C2 genes. Mutations may result in type III hyperlipoproteinemia (HLP III), familial dysbeta lipoproteinemia; where high levels of triglycerides and plasma cholesterol have been seen as the results of reduced clearance of the chylomicron as well as of the VLDL remnants. (Liu *et al.*, 2013)

2.6 Methylenetetrahydrofolate Reductase (MTHFR)

It is the main enzyme which catalyses reduction of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate that essentially takes part in the conversion of methionine synthase enzyme to the homocysteine (Hcy), then, to methionine; does remethylating the homocysteine (Mansouri *et al.*, 2013). It plays significant role in the modulation of folate metabolism, also affects the DNA methylation as well as synthesis of nucleic acids (Mansouri *et al.*, 2013; Nishiyama *et al.*, 2000).

It is positioned on the chromosome 1 i.e. 1p36.3, also, comprises of 11 exons (Goyette *et al.*, 1994). It is composed of two domains i.e. an N-terminal and a C-terminal, catalytic and regulatory respectively. It contains two promoters as well as two isoforms i.e. 77kDa and 70KDa (Tran *et al.*, 2002). With this gene, several DNA sequence variants are associated known as genetic polymorphisms. A report published in 2000 has shown that there are up to 24 polymorphisms associated with MTHFR (Sibani *et al.*, 2000). Most investigated SNPs are: C677T (rs1801133), A1298C (rs1801131).

Genetic variations in MTHFR gene influences the susceptibility to neural tube defects, vascular dementia, AD, occlusive vascular disease, acute leukaemia, colon cancer, metabolic disorders e.g. type 2 diabetes. Mutations in MTHFR gene are linked with the deficiency of methylenetetrahydrofolate reductase (E Trimmer, 2013; Födinger *et al.*, 2000).

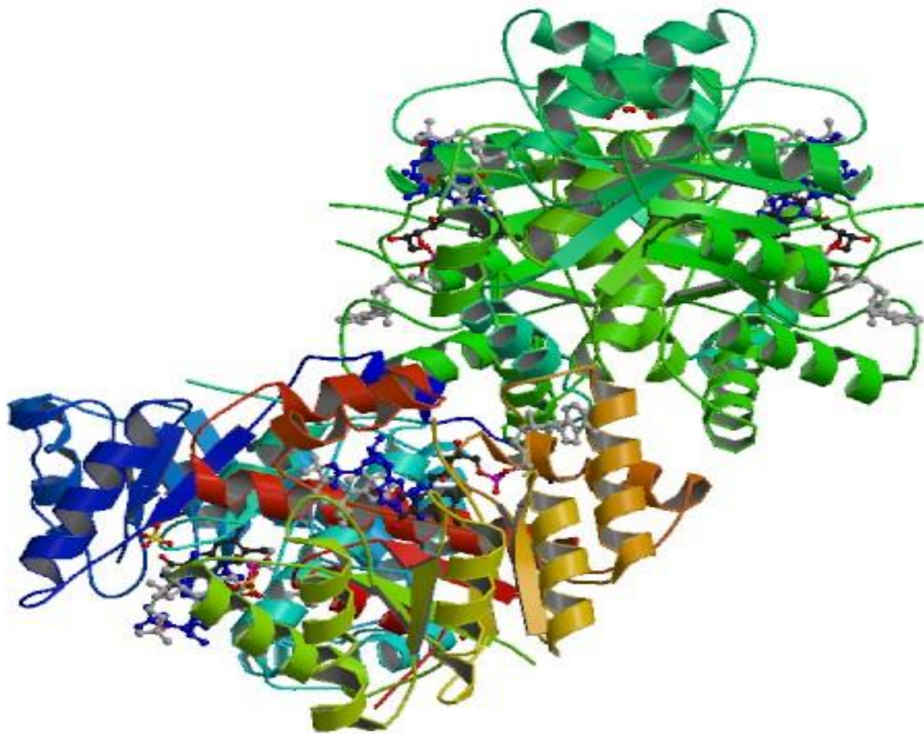


Figure 2.3: Structure of MTHFR gene

2.6.1 Role of MTHFR in both Type 2 Diabetes Mellitus and Alzheimer's Disease

There is a widely-studied mutation of MTHFR is missense mutation. It is C → T substitution residing at nucleotide number 677 which is responsible for the reduced activity of MTHFR as well as with moderate enhancement in concentration of plasma hyper homocysteinemia (Hcy) (Vijayan *et al.*, 2016). Deficiency of the Vitamin B12 or the folic acid and genetic factors such as MTHFR mutations trigger the mild to moderate conditions of Hcy. It has been studied worldwide in various populations but results found are widely controversial. A closer association of MTHFR C677T mutation with T2DM has been reported in a study and explained that this mutation causes decrease in the activity of enzyme by 70% as compared any other mutation or polymorphism (Murali, 2015). Indeed, reason of this increased decline of enzyme activity is the location of this polymorphism as this resides in exon 4 which codes for N-terminal consisting of catalytic domain of the MTHFR enzyme (AbdRaboh *et al.*, 2013).

This polymorphism of MTHFR may cause reduced availability of 5-mTHF which results in decline of enzyme activity. As result of this dysfunction, Hcy level increases in the plasma (Silva *et al.*, 2006). When plasma homocysteine increases, this results in producing cytotoxic effects (Andreassi *et al.*, 2003) and normal functioning of cell is compromised in different tissues (Mansouri *et al.*, 2013) such as neurodegenerative disorders e.g. AD.

A meta-analysis was done in China which discovered T allele of MTHFR or dominant model of the allele T i.e. CT+TT exhibits higher risk towards the development of AD (Hua *et al.*, 2011). A recent study has been done in Egypt and it has been observed that TT genotype of MTHFR and allele T was associated considerably with the severe condition of Alzheimer's disease (Elhawary *et al.*, 2013).

Raised levels of Hcy have been observed in the elderly people with the impairment in cognitive function in some of the studies but others show variation. Although it's been found that subjects having mutant allele of MTHFR, might have moderately high levels of plasma Hcy, Alanine (Ala) to the valine (Val) mutation in the MTHFR gene, but there is no such association with the AD (Chapman *et al.*, 1998). In this recent study, it has been found that polymorphisms of MTHFR were not showing association with VD or the LOAD; these common SNP polymorphisms might have the marginal role in pathogenesis of the dementia in older individuals (Zuliani *et al.*, 2001). Individuals having this mutation in homozygous form show strong association with vascular diseases as enzyme activity influences significantly. As decline in enzymatic activity is almost 70% for homozygous genotype while for heterozygous, it is almost 40% (van der Put *et al.*, 1995; Weisberg *et al.*, 2001). Among various ethnic groups, prevalence of this A677C polymorphism of MTHFR differs from 2% to 54.5% (Pepe *et al.*, 1998). It shows variations from east to west. There was highest frequency of the MTHFR T recorded in Mexico which is 57%, followed by Italy having 46%, China has 44.2% (Wilcken *et al.*, 2003). In India, it varies from 3% to 18% (Prasad and Wilkhoo, 2011; Somarajan *et al.*, 2011).

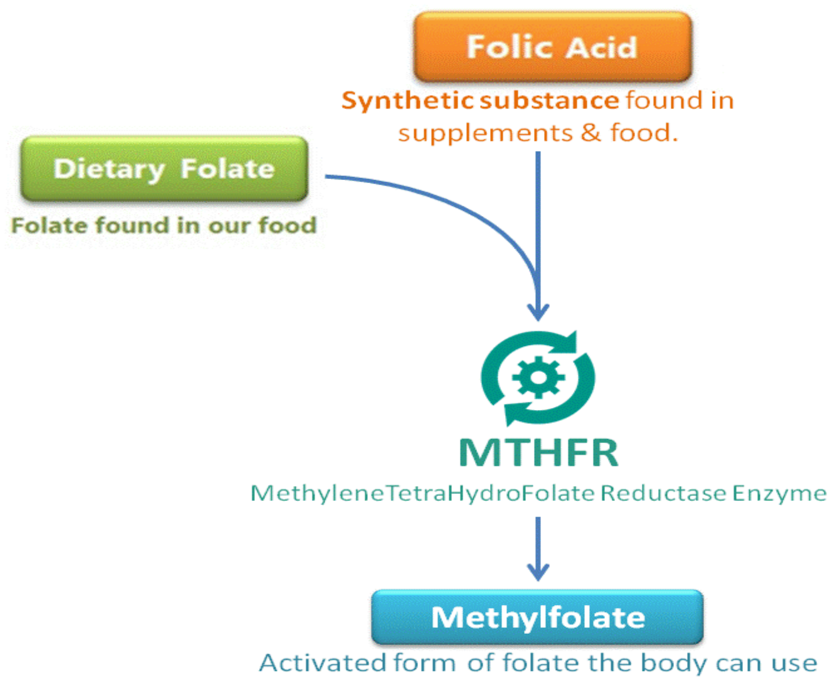


Figure 2.4: MTHFR role in folate cycle

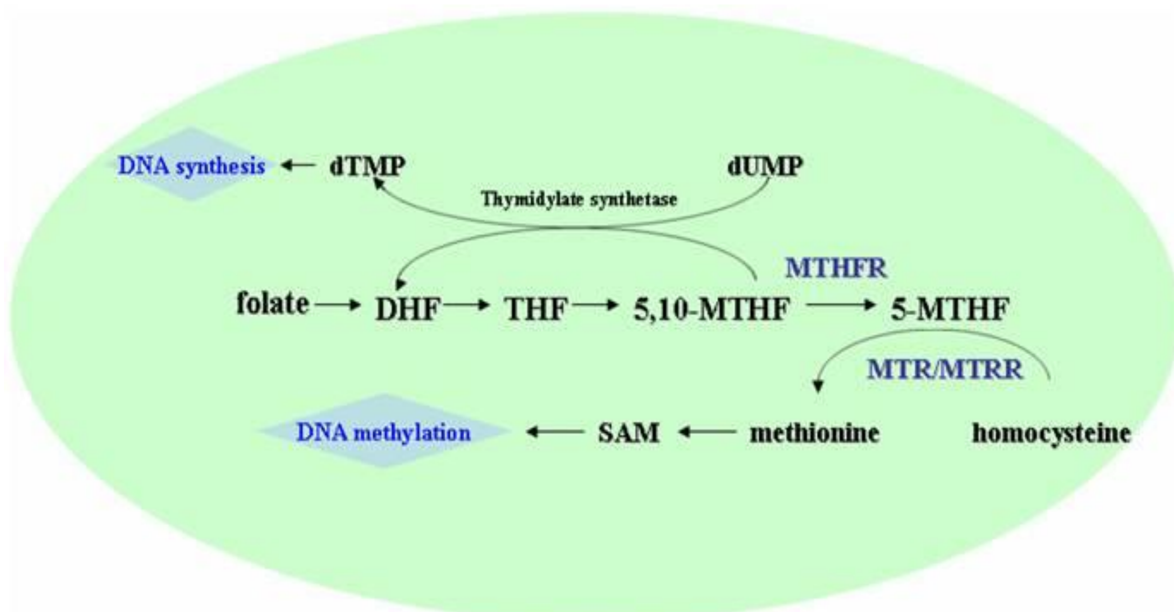


Figure 2.5: Role of MTHFR in DNA methylation and DNA synthesis

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Study Subjects

The study is outlined as a case control study which would include 200 human subjects, who will be classified into two subject groups. Total 100 cases are included in T2D group, 50 in AD group and 50 healthy volunteers in control group. The candidates will be selected after diagnosis of the mentioned disease by an expert physician. The subject groups will be selected from different infirmaries situated in Rawalpindi and Islamabad after obtaining written informed consent.

3.1.1 Inclusion criteria

Patients diagnosed with Type 2 Diabetes and Alzheimer's disease would be added in the case control study. Age factor is also considered for all study groups and human subjects who are ≥40s would be included in the study.

3.1.2 Exclusion criteria

Patients diagnosed with chronic disease conditions related to kidney, liver or mental disorders were excluded from the study. Women who are pregnant were excluded from the study. Patients who are smokers, alcohol consumers, or the patients who are taking non-steroidal anti-inflammatory drugs were excluded.

3.2 Sample Collection

The selected patients were required to give blood sample, taken using 5 ml syringes by a practiced phlebotomist in Purple capped K3 EDTA tubes. Written consent was taken before drawing blood. For handicapped participants, their consent was obtained from their guardians. These blood samples were then processed for genomic DNA and RNA extraction at Immunogenetics Lab (IGL) in Atta-ur-Rahman school of Applied sciences (ASAB), National University of Sciences and Technology (NUST), Islamabad.

3.3 Genomic DNA Extraction

Gnomic DNA was extracted using the standard phenol-chloroform method, using Proteinase-K digestion and SDS treatment. This method can take one or two days, depending on the time the samples are incubated for during the process. The first step was taking 750µL of a blood in a micro centrifuge tube and mix with equal volume of a solution A. The tubes were kept at room temperature for 15 minutes and the centrifuge for 1 minute at 13000 rpm. The resulting supernatant was discarded, leaving a pellet behind. The DNA pellet was re-suspended in of solution A, vortexed to mix properly and centrifuged again as before. After discarding the supernatant for the second time, 400µL of solution B 12µL of 20% SDS and 5µL of Proteinase-K was added to the pellet. The tubes were incubated at 65ºC for 3 hours or at 37ºC overnight.

After incubation, a fresh mixture of Solution C and Solution D was prepared and 500µL of this mixture was added to the tubes. The tubes were thoroughly inverted to mix the solutions and were the centrifuge for 10 minutes at 13000rpm. The resulting aqueous phase was collected in a newly labelled tube and 500µL of solution D was added to it. After inverting the tubes a few times, they were centrifuge again as before. The aqueous layer was separated in a new tube again and 55µL of chilled isopropanol or 1ml of 100% Ethanol was added to precipitate the DNA. At this stage, DNA strands are visible after inverting the tubes a few times. The tubes were then centrifuged for 10 minutes at 13000rpm and the supernatant was discarded. The tiny DNA pellet was seen as a white speck on the bottom or wall of the micro centrifuge tube. To the pellet, 200µL of 70% Ethanol was added and it was centrifuged at 13000 rpm for 7minutes. The Ethanol was discarded after the tubes were dried in an incubator at 37ºC for 30 minutes. DNA was dissolved in 150µL of TE buffer for storage and kept at 4ºC. (Table 3.1)

3.3.1 Agarose Gel Electrophoresis of DNA

DNA quality was checked by running it on 1% agarose gel. To make the gel. One gram of agarose powder was dissolved in 100ml of 1% TAE (tris-acetate and EDTA, pH 8) buffer. The mixture was heated until the agarose dissolved, upon which 5µL of Ethidium Bromide was added. Ethidium Bromide binds to the DNA and makes it visible under UV light. The mixture as poured in to the casting tray and left to set. After the gel had set, the 2 µl DNA was loaded with 3µl of loading dye and then run at 80V for 25 minutes in 1% Tris-Acetate EDTA buffer. The bands were observed under UV lights the single bright intact shows that the DNA is pure and in good quantity, whereas disintegrated bands or smears show protein contamination.

Table 3.1: Composition of solution used for DNA extraction

Solution A	Solution B	Solution C	Solution D
1. 0.32M Sucrose 2. 10mM Tris (pH 7.5) 3. 5mM $MgCl_2$ (Autoclave) 4. 1% Triton X 100(v/v)	1. 10mM Tris (pH 7.5) 2. 400mM NaCl 3. 2mM EDTA (pH 8)	Phenol	1. Chloroform 2. Isoamyl alcohol (24:1)

3.4 Genotyping

Genotyping is the process of determining differences in the genetic make-up (genotype) of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence.

3.4.1 Primer Designing

The SNP rs1801133 is selected for this study of methylenetetrahydrofolate reductase (MTHFR) gene. Primer's sequences were designed by taking the sequence from the ensemble and sequence from the NCBI. Forward and reverse primers were checked for complementarity using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Other properties were evaluated using Oligo calc, an online Oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). The primer sequences, Annealing temperatures and product sizes are given in Table 3.2.

3.4.2 Amplification Refractory Mutations System (ARMS) PCR

ARMS PCR is an efficient and sensitive method to detect the presence of single nucleotide polymorphisms (SNPs) and point mutations. For MTHFR gene SNP rs 1801133 was taken and PCR was carried out to amplify the specific region on the MTHFR gene. The reaction mixture having a total volume of 25ul was prepared in 0.2mL tubes (Biologix, USA). For PCR reaction mixture, 2.5µl of 10x Taq buffer containing $(NH_2)_2SO_4$, 3 µl of $MgCl_2$ (25mM), 0.5µl of 2mM dNTPs mix, 1µl of each forward and reverse primer, 50ng of template DNA, 0.25µlof Thermo Scientific TaqDNA polymerase (500U) were added, and PCR water was used to raise the volume up to 25µl of the total reaction.

Amplification profile consisted of initial denaturation at 95ºC for 2 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, amplification at 58ºC for 30 seconds, extension at 72ºC for 1 minute and a final extension of 72ºC for 2 minutes. The PCR product was then confirmed using 2% agarose gel made by 1g of agarose in 50ml of 1X TAE buffer.

3.4.3 Analysis of PCR products on Agarose Gel

Agarose gel electrophoresis was used to determine the presence/absence of the allele in the PCR products, using 2% agarose gel. Gel was prepared as mentioned above for DNA

quantification, except that 2g of agarose powder was added to 100ml of TAE buffer. The PCR products were run at 120V for 35 minutes. DNA ladder of 100bp was also run to confirm the size of band. The results were analysed through Gel Documentation system (Dolphin Doc).

Table3.2. PCR Primers and Amplification Conditions

SNP of MTHFR Gene	Primers	Sequences (5'→3')	Annealing Temperature (˚C*)	Product Size (bp*)
rs1801133	Forward Primer 1	TGAAGAAGGTGTCT GCGGGAGGAGC	58	236
	Forward Primer 2	TGAAGAAGGTGTCT GCGGGAGGAGT		
	Reverse Primer	GAACTCAGCACTCC ACCCAGA		

bp=Base pair, (˚C) =Degree Celsius

3.3 EXPRESSION PROFILING

3.3.1 RNA Extraction

Cellular RNA was extracted using the TRizol reagent (Catalog NO 15596-026, invitrogen). The method included the steps of blood cell lysis, phase separation, RNA precipitation washing and then solubilization of separated RNA in a buffer for long term storage and stability.

Process was started using by transferring 250µl fresh blood to a 1.5ml microfuge tube already containing chilled 750µl of trizol and vortexed vigorously for a few seconds for thorough homogenization. About 20µl of freshly prepared 5N Glacial acetic acid was then added to the microfuge tube and mixed vigorously by hand for 15-20 seconds to lyse the cells and then was left to stay for more 5 minutes at ice. To filter out the debris, the mixture was then centrifuged at 13000 rpm for 15 minutes at 4ºC. Supernatant was collected in a new tube.

For phase separation 200µl of chloroform was added and mixed by hand by shaking vigorously for 15-20 seconds. Shaking has shown to result in better emulsification and higher volume of aqueous phase later.

The mixture was left to incubate on ice for 15 minutes and then spun at 13000rpm for 10 minutes at 4ºC to complete the phase separation step. Two layers were obtained of which the upper transparent layer was transferred to a new 1.5ml microfuge tube and the red pink layer was discarded. 500µl of chilled isopropanol was added to the microfuge tube and centrifuge at 13000 rpm for 10 minutes at 4ºC to allow for RNA precipitation. The insolubility of RNA in Isopropanal resulted in a palette formation at the bottom of the tube after centrifugation of the mixture. The supernatant was then discarded and the pallet was washed with chilled 75% ethanol in Diethylpyrocarbonate (DEPC) treated water by centrifugation at 18000rpm for 6 minutes at 4ºC.

The supernatant was now decanted and the pellet was left to air dry on ice in a laminar air flow to prevent any contaminations for 2-5 minutes. RNA pellet was re-suspended in 20µl of DEPC-treated water and incubated for 10 minutes at 65ºC temperature. RNA was then stored at -80ºC ultra-low temperature freezer for long term storage.

3.3.2 Quantitative and Qualitative Analysis

The dissolved RNA was taken from -80ºC and was immediately transferred to ice. Quantification was done by using Thermo Scientific™ NanoDrop 2000 and 2000c, and also assessed the purity of RNA by considering its 260/280 absorbance value. For further qualitative analysis, 3µl RNA was loaded on 1% agarose gel.

3.3.3 Complementary DNA (cDNA) Synthesis

The extracted RNA was converted to complementary DNA. For each reaction, RNA having a concentration of 500ng was added to PCR tube along with 1µl oligo (dT) 18 (Life technologies #SO131) primer, 1µ1 of revertAid Reverse Transcriptase (Thermo scientific #EP0442), 1µl of RiboLock RNase inhibitor, 4µl of RevertAid Reverse Transcriptase Buffer, 2µl of 10mM dNTPs mix and the total volume was raised to 20µl final reaction by adding DEPC-treated water for volume makeup.

All the micro centrifuge tubes and pipette tips used in the process of cDNA conversion were either marked RNases free by the manufacturers or were made so by treating them with 1% DEPC-treated water and then autoclaving them

Contents of the tube were gently mixed, centrifuged briefly and then transferred to 2720 thermal Cycler by Applied Biosciences. The program was set to incubate the mixture at 42ºC for 60 minutes to allow for the enzyme to work at its optimum to form cDNA from RNA strands. Then resulting mixture contained cDNA and was stored at -20º.

3.3.4 Semi-Quantitative PCR

For the confirmation of cDNA synthesis 2µl of cDNA, 4µl Taq buffer, 2µl $MgCl_2$, 1µl of 2mM dNTPs mix, 1µl forward primer for GAPDH, 1µl reverse primer for GAPDH and 0.5µl of Thermo Scientific Taq DNA Polymerase was added to PCR tube. Final volume was raised to 15µl by adding nuclease free water. The reaction mixture was slightly spin and loaded to thermocycler. The reaction conditions for GAPDH were 95ºC for 10 minutes followed by 35 cycles of denaturation at 95ºC for 45 seconds, annealing at 58ºC for 45 seconds and extension at 72ºC for 10 minutes. Final extension was given at 72ºC for 7 minutes and the reaction product was kept at 4ºC.

3.3.5 Gel Electrophoresis

To confirm the amplification of GAPDH specific region, PCR product was run on 2% agarose gel. For the preparation of 2% agarose gel, 1g agarose was dissolved in 50ml water and stained with 3µl ethidium bromides (0.5µg/ml). 5µl of PCR product was loaded along with 3µl of loading dye. The gel was analysed on Dolphin-Doc plus gel documentation system (wealtech). Length of the product was determined by comparing the product size with Thermo Scientific GeneRuler 100bp DNA ladder.

3.3.6 Designing Primers for Quantitative Analysis

Primers for GAPDH were already reported in literature. They were checked for self-complementarity and other properties by Oligo Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html).

Primers for MTHFR gene were designed using Primer3web (version 0.4.0) software. Its mRNA sequence was taken from NCBI. Primers were tested for self-complementarity and other properties by Oligo Calc: Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html). Primers were also checked for non-specific binding using UCSC In-Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr). Primers were ordered from Gene Link (http://www.genelink.com/). Sequences of primers are given in Table 3.3.

3.3.7 Conventional PCR for Optimization of q-PCR Primers

Several PCR reactions were run to optimize the primers for GAPDH and MTHFR genes for their PCR profile and amplification conditions on a conventional PCR. Concentration of template cDNA was kept constant for each reaction at 500ng. Primers have 10µM concentration. For MTHFR rection mixture, 2.5µl of 10x Taq buffer containing $(NH_2)_2SO_4$, 2µl of $MgCl_2$ (25mM), 1.75µl of 2mM dNTPs mix, 1µl of each forward and reverse primer, 500ng of template cDNA, 0.25µl of Thermo Scientific Taq DNA polymerase (500U) were added, and Nuclease free water was used to raise the volume up to 25µl of the total reaction.

Amplification profile consisted of initial denaturation at 95ºC for 2 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, amplification at 55.9ºC for 30 seconds, extension at 72ºC for 1 minute and a final extension of 72ºC for 2 minutes.

The PCR product was confirmed using 2% agarose gel made by 1g of agarose in 50ml of 1X TAE buffer. Before the gel was poured into the caster the solidification, 5µl of ethidium bromide (o.5µg/ml) was dissolved into it. 3µl of each PCR product was stained with 2µl of loading dye (0.25% Bromophenol blue in 40% sucrose solution) and loaded into wells. For each gel, the thermo scientific GeneRuler 100bp DNA ladder was loaded alongside per products. The gel was then run at 70V for 45 minutes by power station 300 (Lab net) power apply unit. Later for analyzing the gel, Dolphin Doe Plus gel documentation system (Weltech) was utilized and the length of the product was determined by comparison to the ladder.

3.3.8 Real Time PCR

Next step after the primers on conventional PCR was to apply them on Applied Bio system 7300 Real-Time PCR system. A prepared master mix reaction by Maxima called SYBR Green/ROX qPCR Master Mix (2X) (Catalog #K0221, ThermoFisher Scientific) was used to test those conditions

The reaction mixture was prepared according to the instruction provided in the leaflet. First 12µl of 2X qPCR Master mix was added to PCR tube. 1µl of primers, forward and reverse each was added next followed by 500ng of template cDNA and the reaction volume was raised to 25µl by addition of the Nuclease-free water. The reaction profile was set as 95ºC for 10 minutes as the initial denaturation followed by 40 cycles by amplification at 95ºC for 15 seconds, 55ºC for 1 minute and 72ºC for 45 seconds. The final extension phase was omitted instead the dissociation stage was carried for melt-curve analysis which helps in identifying the presence of primer dimmers or any nonspecific amplification. It consisted of a single hold of 72ºC for 1 min and then 95ºC for 15 seconds, 60ºC for 1 minute, 95ºC for 15 sec. Data acquisition was performed during the annealing and the extension step.

Reaction of each sample was run as duplicates. The amplified product of real time PCR was run on 2% agarose gel for 40 minutes at 85 V. This was to ensure that the product is specified to primers being used. Gel was made by weighing 1g of agarose mixed in to 50ml of 1X TAE buffer. After being heated and dissolution, 5µl of ethidium bromide (0.5µg/ml) was added and the gel was poured into the caster to solidify at room temperature. 5µl of the product was then stained with 2µl of loading dye (0.25% Bromophenol blue in 40%sucrose solution) and loaded into wells. Thermo Scientific GeneRuler ladder of 100bp DNA was run along the products to

measure the product size. Results were than analyzed on Dolphin Doc Plus documentation system (Weltech).

3.3.9 Data Analysis

Ct values obtained from Real Time PCR of MTHFR gene and GAPDH gene provided the data to analyse the number of fold change in the expression of our gene of interest in both T2DM and AD. The calculations were done as follows:

- Ct value of GAPDH in Control was labelled X1
- Ct value of MTHFR in Control was labelled as X2
- Ct value of GAPDH in Experimental group of T2DM was labelled as Y1
- Ct value of MTHFR in Experimental group of T2DM was labelled as Y2
- Ct value of GAPDH in Experimental group of AD was labelled as Z1
- Ct value of MTHFR in Experimental group of AD was labelled as Z2

$$\Delta Ct\ Control = (Ct\ of\ MTHFR\ in\ Control) - (Ct\ of\ GAPDH\ in\ Control)$$

$$= X2 - X1 = \Delta Ct\ Control$$

$$\Delta Ct\ Exp.\ (T2DM) = (Ct\ of\ MTHFR\ in\ Exp.\ (T2DM)) - (Ct\ of\ GAPDH\ in\ Exp.\ (T2DM))$$

$$= Y2 - Y1 = \Delta Ct\ Exp.\ (T2DM)$$

$$\Delta Ct\ Exp.\ (AD) = (Ct\ of\ MTHFR\ in\ Exp.\ (AD)) - (Ct\ of\ GAPDH\ in\ Exp.\ (AD))$$

$$= Z2 - Z1 = \Delta Ct\ Exp.\ (AD)$$

Calculate $2^{-\Delta ct}$ for each experimental control group

To calculate the fold change between both experimental groups of T2DM and AD, and Control groups; we applied the following formula:

$$Fold\ Change = 2^{-\Delta\Delta ct} = 2^{-\Delta ct}\ for\ Experimental\ group - 2^{-\Delta ct}\ for\ Control$$

Table 3.3 Sequences of Primers

Sr. No.	Code	Primer Sequence (5' → 3')	Length	Product size (bp*)	%GC Content	Annealing Temperature
1	MTHFR-FRT	TCCAGGGAGA ACCACTTGTC	20	236	55	55ºC
2	MTHFR-RRT	GAGGAAACAG CAGCCTCAAC	20	236	55	55ºC
3	GAPDH-FRT	CCTGCACCACCA ACTGCTTA	20	74	55	58ºC
4	GAPDH-RRT	CATGAGTCCTTC CACGATAC	20	74	55	58ºC

*bp = Base Pair

RESULTS

4.1 Study Subjects

In the current study, blood samples had been collected from 200 total individuals in the Pakistani Population. Out of them, 100 were patients of type 2 diabetes mellitus, 50 were of Alzheimer's disease and 50 were controls.

4.2 Physical Parameters

4.2.1 Age and Gender

As literature depicts that there is great influence of age as well as gender in the development of T2DM. Generally, T2DM targets the persons of high age. Similarly, Alzheimer's disease has its late onset i.e. LOAD affecting persons of age ≥65. Out of 100 diabetic individuals, 50 were males and 50 were females. Out of 50 AD, 25 were males and 25 were females. (Figure 4.1)

4.2.2 Concurrent Ailments

Most of the individuals which were included in study were either pure diabetic having no other ailments or suffering from hypertension and heart problems. The individuals having any microbial diseases or serious kidney disorders were excluded from this study.

4.3 Expression analysis of methylenetetrahydrofolate reductase (MTHFR) gene by Real Time PCR

This study had been designed to evaluate the expression level of mRNA of Methylenetetrahydrofolate reductase (MTHFR) gene and to find out the correlation of MTHFR polymorphism C677T among Diabetic and Alzheimer patient as compared to control.

4.4 Quantitative PCR

Real Time PCR (qPCR) was performed for amplification of coding regions of methylene-tetrahydrofolate reductase gene using the SYBR green technology. We performed the experiments in triplicates. cDNA dilutions of 500ng were used and the relative expression of MTHFR had been detected in patients' groups i.e. T2DM group, AD group and control group. Amplification plot and dissociation curve has been shown in Figure 4.2 and 4.3 respectively.

Ct values of all study groups had been recorded. GAPDH was used for being as internal control so data could be normalized with this. The Ct values of MTHFR in T2DM patients were higher as compared to Ct values of other two groups i.e. AD and controls.

4.5 Expression Analysis

For calculation of difference in the gene expression in the study groups, we performed the following calculations:

- As qPCR had been performed in triplicates, so means of every Ct value of all samples were calculated for gene MTHFR and GAPDH i.e. mean of Ct values of triplicate runs for MTHFR gene as well as for GAPDH gene.
- From the Ct values, ΔCt for each sample had been calculated.
- ΔCt = Mean Ct value of MTHFR – Mean Ct value of GAPDH
- From ΔCt values, we calculated $2^{-\Delta Ct}$ for every sample according to guidelines provided in "Nature Protocols" (Schmittgen and Livak, 2008).
- The ΔΔCt value of all groups was calculated as first we took mean of the ΔCt of controls, and then subtracted them from ΔCt values of other groups.
- For groups of T2DM, AD and controls, $2^{-\Delta\Delta Ct}$ had been calculated and plotted by using the GraphPad Prism (Figure 4.4)
- Then "one-way ANOVA" had been applied with the "Bonferroni's posttest" via GraphPad Prism software Version 5.00.

4.6 Statistical Analysis

For the compilation of our data, we used worksheets of Microsoft Office Excel. For the statis analysis, evaluation as well as graph generation, we used GraphPad Prism. Statistical Analysis had been carried out using one-way ANOVA when we were analysing three groups. We applied Bonferroni posttest after the one-way ANOVA. The results were considered as significant at the p value less than 0.05.

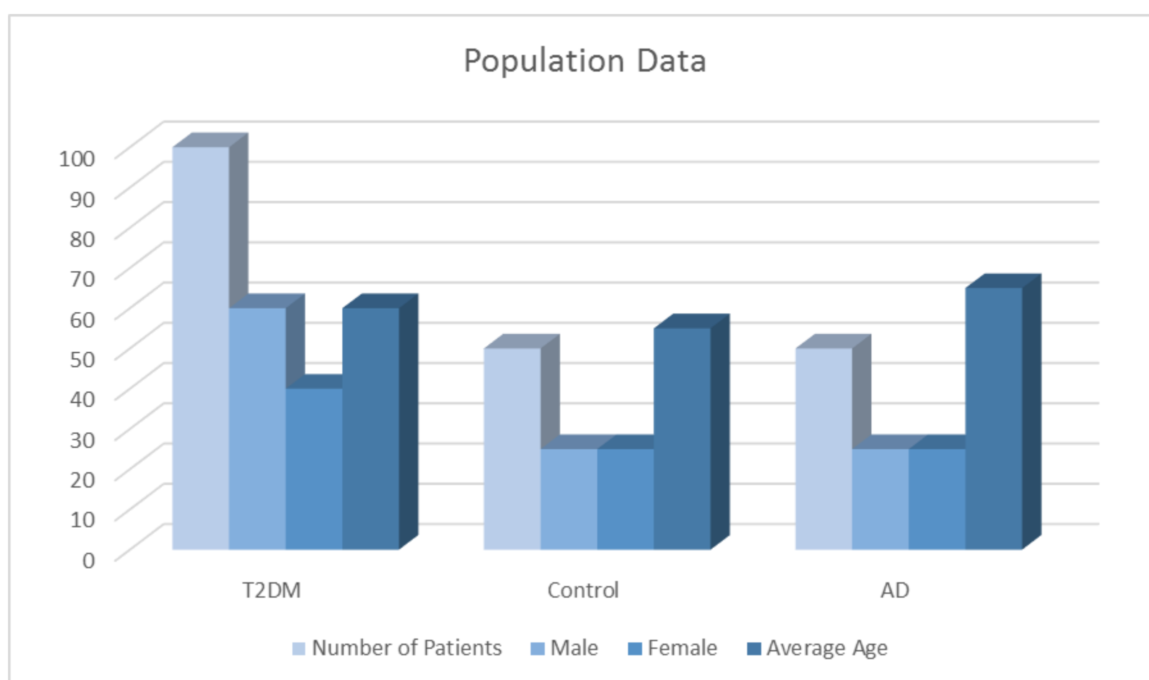


Figure 4.1 Average number of individuals of study groups i.e. control, T2DM and AD has been shown on x-axis while the patients' profile has been shown on y-axis including their gender and ages

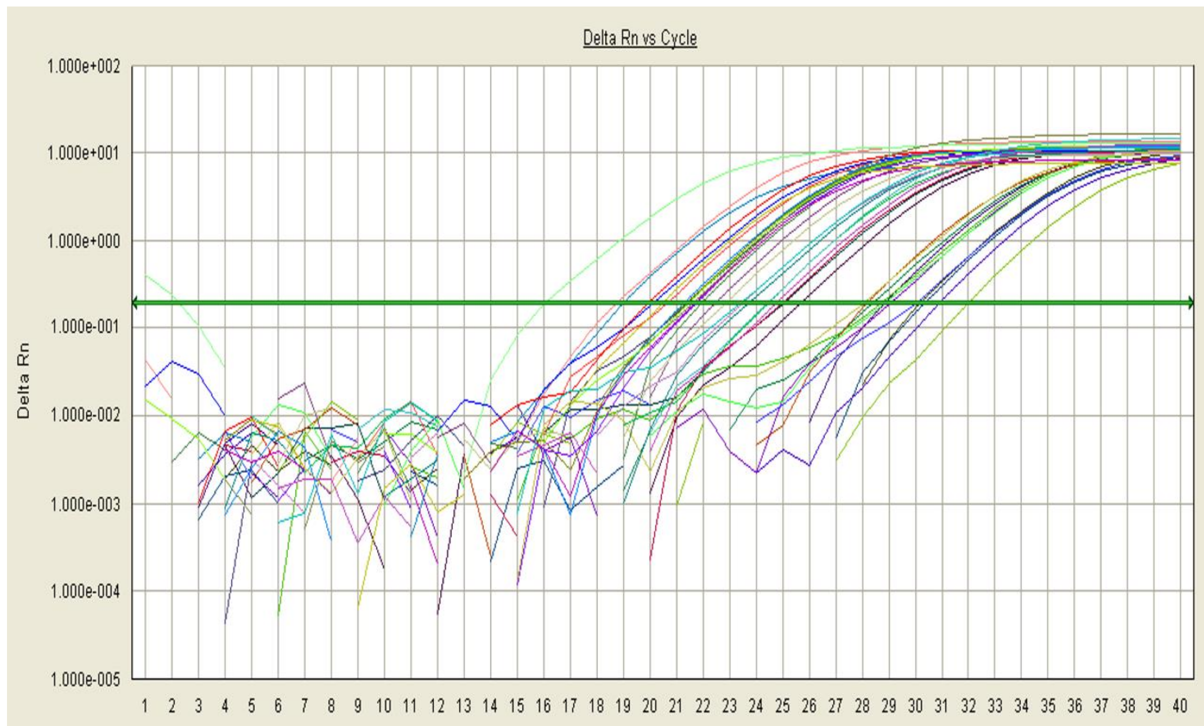


Figure 4.2: Amplification plot of MTHFR gene. Cycle number on x-axis while delta Rn on y-axis

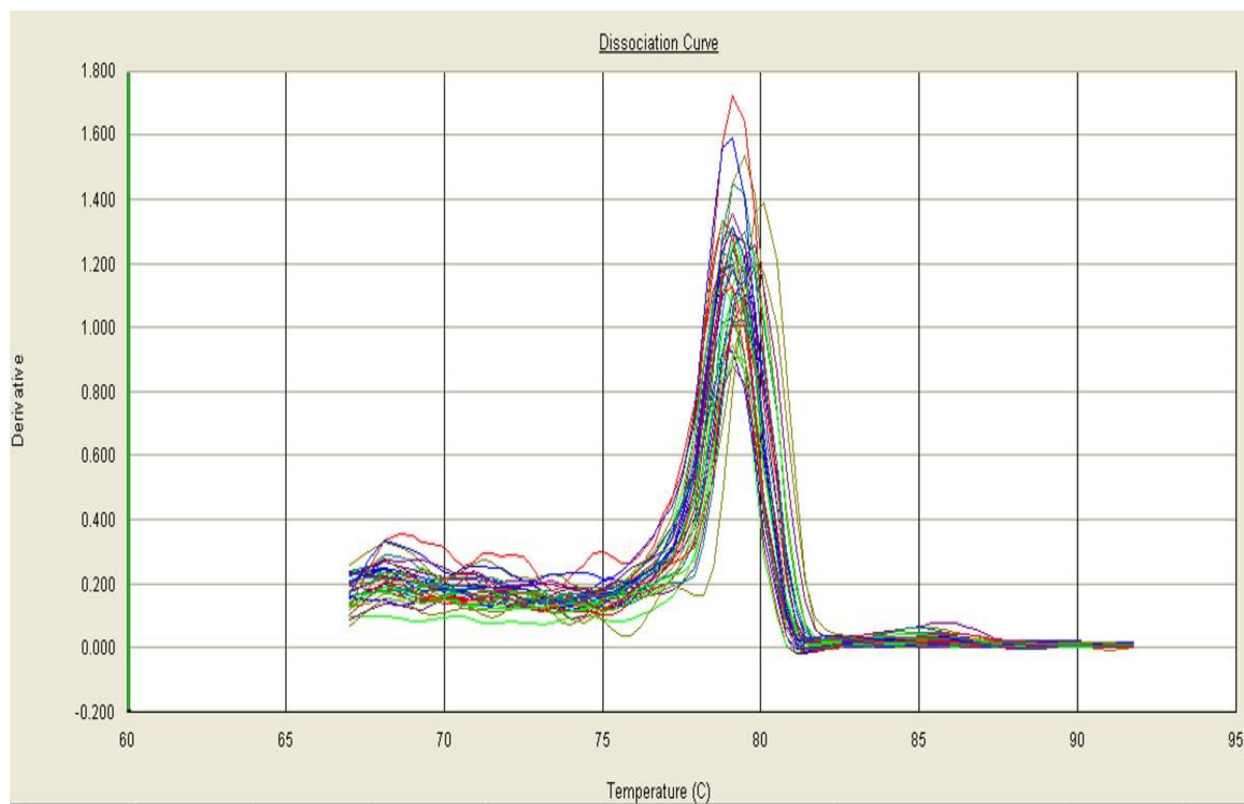


Figure 4.3: Dissociation curve of MTHFR gene

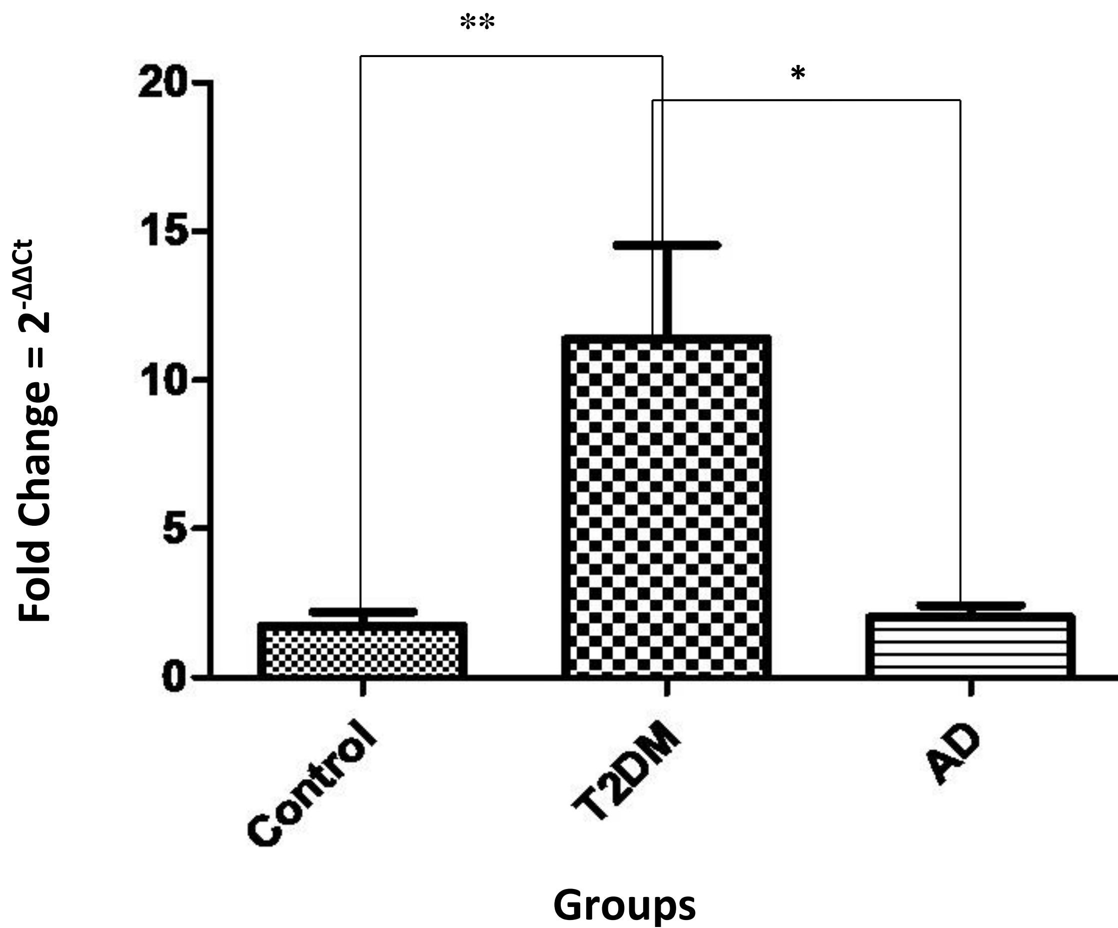


Figure 4.4: Fold change of MTHFR gene expression between T2DM and AD groups in comparison with healthy controls. Error bars represent the SEM (standard error of the mean). There is reduced expression of MTHFR in AD, while a significant increase in the expression of MTHFR in type 2 Diabetes Mellitus

Table 4.1: One way ANOVA results of the Mean Fold change in the expression of MTHFR in Control, T2DM and AD samples

P value	**<0.0001**				
P value summary	***				
Do the means differ significantly? (P < 0.05)	Yes				
ANOVA Table	SS		df		MS
Treatment (between columns)	2243		2		1121
Residual (within columns)	16010		100		160.1
Total	18250		102		
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant	Summary	95% CI of diff.
Control vs T2DM	-9.642	3.542	Yes	**	-16.27 to -3.014
Control vs AD	-0.2734	0.07466	No	ns	-9.191 to 8.644
T2DM vs AD	9.369	2.503	Yes	*	0.2552 to 18.48

CHAPTER 5

DISCUSSIONS

5.1 Discussions

Type 2 diabetes mellitus, considered as a multifactorial metabolic disorder. There are many factors which perform different roles in the disease development i.e. age, genetics, ethnicity, weight, environment and lifestyle (Fonseca, 2009). Environment has an influence on genetics as it can cause alteration in its DNA methylation which could lead towards the type 2 diabetes disorder (Barrès *et al.*, 2009). In its pathogenesis, the peripheral insulin resistance takes place. To compensate this condition, β-cell produces more insulin which cause hyperinsulinemia. And as a result of decline of β cell function, it leads to hypoinsulinemia (Fonseca, 2009). It has worldwide incidence and affects many organs including the brain. It has many microvascular as well as macrovascular complications including polyuria, polydipsia, ketoacidosis or even coma.

Studies provide evidences that type 2 diabetes mellitus has been strongly associated with cognitive impairment resulting into Alzheimer's disease (Cukierman *et al.*, 2005; Stewart and Liolitsa, 1999; Strachan *et al.*, 1997). This study has also been focused on the correlation of both the diseases as there are many genetic risk factors involved in the onset of both the complications e.g. PICALM, CLU, APOE, TOMM40, HMHA1, MTHFR (Peila *et al.*, 2002). Alzheimer's disease (AD) considered as the most prevalent neurodegenerative problem characterised via neural loss along with progressive memory deficit. Pathophysiological features of the AD consist of intracellular neurofibrillary tangles (NFTs) as well as extracellular amyloid beta protein plaques (Glenner, 1990).

This study focuses on one of the risk gene which is involved in the manifestation of both the diseases (T2DM and AD) which is methylenetetrahydrofolate reductase (MTHFR) and its most common polymorphism C677T. MTHFR is involved in folate metabolism and affect homocysteine levels. The C →T transition residing at nucleotide position 677 that leads to substitution of valine with the highly-conserved alanine into the mature protein. Thus, genetic variant that contains polymorphism C677T is associated with the reduced activity as well as increased thermolability of enzyme (Frosst *et al.*, 1995; Jacques *et al.*, 1996). There are many

studies to find out the association of MTHFR C677T with the T2DM and AD but results are yet controversial.

According to a meta-analysis performed in 2014 (Zhu *et al.*, 2014), it has been evidenced that there is significant relationship found of the MTHFR C677T polymorphism with the T2DM in population of china and it is assumed as the MTHFR C677T allele may be the risk genetic aspect for T2DM. Another study performed (Wang *et al.*, 2005) Chinese population and they found a strong association of MTHFR C677T with the onset of AD depicting it as a plausible risk gene for AD. There was significant difference of both T-allele and TT- genotype frequencies among AD and control individuals.

Results from the Mendelian randomization method reinforced this hypothesis that the increased Hcy has been causally linked to elevated risk of the T2DM (Huang *et al.*, 2013). A study has been performed on more than 105 patients having T2DM along with proper renal function; results showed the levels of Hcy were 35% lower in patients having T2DM as compared to healthy persons (Mazza *et al.*, 2005). This reduced Hcy level could possibly be explained as hyperglycemia can influence the acceleration in hepatic transsulfuration path because of disorder of insuln (Mazza *et al.*, 2005), later, higher glucocorticoids reduced Hcy level (Ratnam *et al.*, 2002). Cell culture as well as animal studies have also shown that diabetic status may be reduced instead of increasing the level of circulating Hcy because of higher Hcy catabolism (Dicker-Brown *et al.*, 2001; Ratnam *et al.*, 2002). However, this data from animal studies doesn't provide significant association results of Hcy level with elevated diabetes status but many studies have found this significant association results showing that MTHFR which is key enzyme for Hcy metabolism is potential risk factor for enhanced diabetes risk.

This case-control study which aims on finding the expression levels of MTHFR (C677T) in both the diseases T2DM as well as AD. We took 100 T2DM patients and 50 AD patients along with 50 controls, and we performed real time PCR to find out the relative levels of expression of the MTHFR gene in all these samples. Results have shown an increased expression level of MTHFR in both the T2DM as well as AD patients as compared to controls, although the expression level of gene is not significantly increased in AD patients whereas in T2DM, there was significant elevated level of MTHFR gene observed. It shows that MTHFR is post-transcriptionally expressing at higher level in T2DM as compared to AD. Because a significant decrease in the expression level of MTHFR gene has been observed between T2DM and AD.

Results are suggesting that expression of MTHFR elevates which causes the incidence of T2DM and AD. It contradicts the earlier studies (Zhong *et al.*, 2013) which lacks any such association of this polymorphism with the occurrence of diseases.

This study is first such study performed on Pakistani population for finding the association of this genetic risk factor with the development of T2DM and AD and it suggests MTHFR (C677T) as a potential genetic risk factor for the incidence of both diseases T2DM and AD.

5.2 Conclusion

Results have manifested that MTHFR C677T polymorphism contributes towards the susceptibility of T2DM as well as AD, and have promoted the hypothesis which claimed that increased Hcy is associated with the expanded risk of T2DM and AD. However, presence of the interactions of gene with environment perhaps can describe inconsistency of the results found from the individual studies related to genetic association. So, the case–control studies which show the gene-environment interactions could support the more elucidation of the T2DM genetics as well as AD genetics.

5.3 Future Prospects:

Such study should be carried out on large population size, different ethnic groups to better elucidate the effect of this gene in the onset of T2DM and AD. Moreover, it should be extended to other polymorphisms of this gene so that better understanding could be developed and it could possibly be used for developing better therapeutics to treat both the ailments.

CHAPTER 6

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