Type 2 Diabetes and Alzheimer’s Disease: Investigation Into the Genetic Linkages of Shared Pathologic Molecular Mechanisms

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Type 2 Diabetes and Alzheimer’s Disease: Investigation into the Genetic Linkages of Shared Pathologic Molecular Mechanisms

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder which is characterized by elevated blood-glucose levels and it is increasing dramatically (Morris et al., 2012). More than 347 million people are affected by diabetes worldwide. The late recognition and insidious inception of this disease are the main cause of the large number of mortality and morbidity cases worldwide, especially in the developing countries like Pakistan (Azevedo et al., 2008). T2DM is linked with impairment of the cognitive function, and one of the adverse complications of T2DM is an enhanced possibility of developing Alzheimer’s disease (AD) (Peila et al., 2002; Arranitakis et al., 2004). AD a neurodegenerative disorder is common among elderly people. The AD patient suffers from memory loss and decline in intellectual functions (Baglietto-Vargas et al., 2016). Recent studies have shown shared links between these two disorders. However, the crosstalk between these factors and the mechanisms underlying the diabetes-related CNS complication is still elusive. Thus, the study was designed, and T2DM, AD, and T2DM/AD patients and healthy subjects (controls) blood samples were collected from different collaborating hospitals of Islamabad, Rawalpindi, Lahore, and Abbottabad.

A total number of 820 research participants: 250 controls, 450 T2DM, 100 AD, and 20 with both T2DM and AD were recruited and interviewed. Patient inclusion criteria: Patient Inclusion criteria include patients diagnosed with T2DM, AD and T2DM & AD both. Patient exclusion criteria: Patients who do not meet the above criteria were excluded from the study. Patients with type 1 diabetes, gestational diabetes and pregnant
women were also excluded. All the samples collected were transported to Dr. Ghosh’s Lab at the Department of Biology, Howard University, Washington DC with prior IRB approval by the Howard University Institutional Review Board (IRB-17-MED-43) and ASAB (NUST) (Ref. Ni. 28/IRB dated April 20, 2016 as well as material transfers’ agreements. The DNA samples were shipped through FedEx, and the RNA samples were shipped by a special logistics carrier (World Currier) with dry ice. The major goal of this study was to investigate shared genetic mechanisms associated with Type 2 Diabetes and Alzheimer’s disease in Pakistani Population. This will open the way to several lines of investigation and will help to understand disease pathogenesis.
Dedication

I dedicate this thesis project and the summation of the countless hours of dedicated research and lab work to my maternal grandmother Augustina Grace Egbejimba and to my paternal grandfather Thomas Nnanabu I.

It is with the utmost pride to be able to call you two my ancestors. My grandmother died from Alzheimer’s Disease and my grandfather passed away from complications of Type 2 Diabetes. It seemed like it was fate when I decided I would be focusing my thesis project around these two pathologies.

I will continue to aspire to make you two proud through my achievements and accomplishments during my life’s journey. Your memories are alive with me and your legacies will live on through me. Until we meet again.
Acknowledgments

This project would not have been possible without Dr. Ghosh, my thesis director, opening his lab at Howard University to me. With your patience, guidance, and flexibility I have been able to undergo this exciting yet challenging task of completing my thesis project while simultaneously getting my medical school education at Howard University College of Medicine. Your passion and love for research has inspired me and opened a world of research and the investigative sciences to me, to which I am extremely appreciative and thankful. Thank you for this educational experience.

Thank you to Dr. Morris, my research advisor, who has worked closely with me both on-site in Cambridge, MA to distantly when I moved to Washington, DC to start medical school. Dr. Morris has always been patient with me as my graduate school journey was quite unique. Dr. Morris, your belief in me truly inspired me to push through and finish my thesis project with pride.

To my loving family, my parents and my siblings, I came to you in 2015, following a brutal medical school rejection, with a detailed long-term plan that included me moving to Boston to take part in a rigorous master’s program. A program that I swore would be the catalyst that would get me into medical school. None of you ever doubted me, my potential, or my goals for a second and showed me the love I truly needed to reach this achievement.

Finally, and most importantly, thank you to Azuka, my fiancée who has been right by side day in and day out. You have seen every day of my struggle during these past 5 years and remained patient and hopeful. You saw potential in me that I never knew I had,
without you absolutely none of this would be possible. I thank you so much for your unwavering confidence, support, and love and I am honored to soon call you my wife. Love you.
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Chapter I.
Introduction

Type 2 diabetes Mellitus (T2DM) and Alzheimer’s Disease (AD) are two of the most prevalent diseases afflicting the elderly population worldwide (Chatterjee et al 2018). T2DM is a metabolic disorder characterized by hyperglycemia and insulin resistance, while pathologically AD presents with extracellular plaques of Aβ and intracellular neurofibrillary tangles of hyperphosphorylated tau. There is a growing consensus among epidemiological studies suggesting that individuals with T2DM are at a higher risk of developing AD. Insulin resistance, characteristic in T2DM, directly exacerbates Aβ and tau pathologies resulting in the pathophysiological traits of synaptic dysfunction, inflammation, and autophagic impairments that are common to both diseases and indirectly impact Aβ and tau functions in the neurons (Chatterjee et al 2018). This project was designed to highlight more possible genetic links between T2DM and AD.

Type 2 Diabetes

Diabetes mellitus is a chronic metabolic disorder. Its prevalence globally is increasing at an alarming rate and is expected to affect 552 million people worldwide by 2030 (American Diabetes Association, 2009). T2DM is the most prevalent subtype of diabetes. The most noteworthy features of T2DM are high levels of blood glucose (hyperglycemia), hyper-insulinemia, and insulin resistance (Taylor, 2012). Insulin resistance manifests through constantly decreased insulin sensitivity of muscle, liver and
fat cells to insulin. Another salient feature of T2DM is the formation of human islet amyloid polypeptide (IAPP), which ultimately causes pancreatic β-cell dysfunction (Marzban et al., 2003). Chronic hyperglycemia, one of the pathological hallmarks of T2DM, is the direct result of reduced uptake of circulating blood glucose for glycogenesis.

Alzheimer’s Disease

Diagnosed by Prof. Alois Alzheimer, a German psychiatrist and neuropathologist in 1906, Alzheimer’s disease (AD) is the most prevalent form of dementia in the aging population (Chatterjee et al 2018). Declared as the sixth major cause of death worldwide (Chatterjee et al 2018) those affected with AD suffer a gradual cognitive decline of abilities and memory functions till the disease renders them completely incapable of performing daily functions, ultimately leading to death (James et al., 2015). According to statistical data, over 30 million people are suffering from AD worldwide. This number has been predicted to double every 20 years to reach 66 million in 2030 and about 115 million by 2050 (Chatterjee et al 2018).

Clinically AD is classified into two subtypes. Around 95% of AD patients are aged 65 years or older and are diagnosed with “late-onset” or “sporadic AD” (sAD) while 5% of AD patients carry rare genetic mutations associated with “early-onset” or “familial AD” (fAD) that causes the onset of disease symptoms in a person’s life as early as thirties or forties (De Strooper, 2007).
Early-onset familial AD

In this subset of AD, the pathology is initiated by mutations in three known genes namely: amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2). (Chatterjee et al 2018).

Late-onset Sporadic AD

The genetics of sAD are more complex. Excluding aging, the strongest risk factor for sAD, GWAS studies reveal that the epsilon four allele of the apolipoprotein E (ApoE4) gene is a significant risk factor for development of this disease. One copy of this allele enhances the risk of AD by 4-fold, while two copies of APoE4 gene increases the risk of AD by 12-fold (Bertram and Tanzi, 2009). However, according to (Li et al., 2015) only 50-60% of individuals are carriers of this gene. This fact suggests that there should be other factors that may also confer risk. Studies suggest that these other factors include cerebrovascular infarction, family history of diabetes, hypertension, and obesity.

Neuropathology

AD causes progressive loss of pyramidal cells of the hippocampus that are responsible for maintenance of higher cognitive function (Serrano-Pozo et al., 2011). Early symptoms of AD are characterized by synaptic dysfunction that disrupts connectivity between neural circuits, leading to gradual loss of memory. The pathology of AD is characterized by extracellular plaques of insoluble amyloid-β protein, and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein (Serrano-Pozo et al., 2011). Abnormal incomplete cleavage of Amyloid precursor protein (APP) which leads to the formation of insoluble amyloid-β protein, densely packed, form the core of the senile plaques (Chatterjee et al 2018). Normally in neurotypical brains, Tau
protein serves as a microtubule binding protein and plays an important role in axonal and vesicular transport (Chatterjee et al. 2018). Conversely, in a diseased state, tau protein is hyperphosphorylated and detached from the microtubules. This detachment and disruption of cytoskeletal integrity manifests in synaptic and behavioral impairments (Gilley et al., 2016). The formation of Aβ plaques occurs 15-20 years earlier before the cognitive functions decline, whereas the spatial and temporal spread of tau pathology correlates more strongly with the severity with disease progression (Serrano-Pozo et al., 2011). It has been hypothesized that various risk factors promote Aβ and tau-related pathological changes before the onset of visualized clinical symptoms. The objective of this project is to identify possible genetic risk factors responsible for alterations at the cellular and biochemical levels, and in doing so lead to possible early detection methods with goal of improving patient morbidity and mortality.

Pathophysiologic similarities Between AD and T2DM

Both T2DM and AD pathologies show marked impairment of glucose and energy metabolism, and amyloidogenesis remains a salient feature in both diseases. Extracellular β-amyloid plaques form one of the most notable features of AD, and deposits of amyloidogenic peptides are present in the pancreatic islets of Langerhans of T2DM patients (Chatterjee et al. 2018). The impact of brain insulin resistance present in T2DM plays a major role on the two hallmarks of AD, Aβ and tau accumulation. AD and T2DM pathologies are interconnected in the areas of synaptic dysfunction, inflammation, and autophagic impairment. (Chatterjee et al. 2018).
Insulin Resistance in the Brain: Alzheimer’s Disease

Insulin is released from the β cells of the pancreas and is transported to the brain and can cross the impenetrable blood brain barrier using a receptor-mediated mechanism (Chatterjee et al 2018). The crucial role of insulin in regulating blood glucose in the peripheral tissues is well documented, but the function of insulin the central nervous system is currently under investigation. The studies focusing on insulin role in the brain have been conflicting. There are reports about reduced insulin levels in the brains of AD patients; however, this finding was not significant when compared to age-matched controls (Chatterjee et al 2018) and (Stanley et al., 2016). Also, it has been hypothesized that there are reductions of insulin mRNA in AD, however the results of de novo insulin synthesis have been controversial (Chatterjee et al 2018) and (Blazquez et al., 2014). Several studies indicate widespread distribution of insulin receptors (IR) in the brain, particularly located in the olfactory bulb, hippocampus, and hypothalamus (Kim and Feldman 2015). The locations of these IRs may possibly demonstrate an intricate “neuroregulatory” role for insulin. IRs in the brain are densely concentrated at the synapses of neurons. The IRs of the brain are unique, in the fact that their function is not for glucose transport and metabolism but instead are diverse in functionality. Brain IRs are tasked to regulate brain homeostasis, modulation of synaptic plasticity, and neurotransmission and age-related neurodegeneration (Plum et al 2005).

Glucose transporter 4 (GLUT4) transports glucose into the brain. Insulin is required to activate GLUT4 gene expression and the translocation of the receptor from the cytoplasm to the plasma membrane to regulate glucose homeostasis and provide the energy required for a vast array of neuronal function. (Chatterjee et al 2018)
Figure 1. Pathways of neurodegeneration in the diabetic brain

Neurodegeneration in Alzheimer's disease is caused by the formation of neurofibrillary tangles and the deposition of extracellular β amyloid plaques. Both pathways are facilitated by insulin resistance, the major cause of type 2 diabetes. Chronic hyperglycemia-generated advanced glycation end products (AGEs), oxidative stress and neuroinflammation are also important causes of neurodegeneration, thus providing critical links between diabetes and AD. Reference: S. Pugazhenthi et al., Biochimica et Biophysica Acta 1863 (2017) 1037–1045.
Insulin Signaling in the Brain

There are plenty of recent studies that provide convincing evidence that issues with insulin signaling, due to insulin resistance, occurs in AD (Chatterjee et al. 2018) (Mullins et al. 2017). PET brain studies in patients with early stage AD clearly demonstrated significant lowered glucose uptake leading to the classification of AD as “Type 3 Diabetes (de la Monet and Wands 2008).

Insulin signaling mechanism are essential for maintaining synaptic plasticity and cognitive functions. When insulin binds to its IR, it is activated by auto-phosphorylation of tyrosine residues, which then activates insulin receptor substrates 1 (IRS-1,2) which leads to downstream signaling cascades through phosphatidylinositol-3-kinase (PI3K). PI3K activates AKT, which then phosphorylates GSK-3β, inhibiting its activity and resulting in glycogen synthesis (Avila et al. 2012). An impairment of GSK-3β is thought to be one of the leading key tau kinases in playing a central role in the AD pathogenesis (Chatterjee et al 2018). In a normal physiological state GSK-3β is involved in maintaining synaptic plasticity and regulates NMDA receptor-mediated long-term potentiation and depression effects at neuronal synapses (Bradley et al. 2012). In an diseased state however, GSK-3β is hyperactive and phosphorylates tau leading to aggregates of hyperphosphorylated tau forming neurofibrillary tangles (Avila et al 2010). Also, GSK-3β is a key mediator of apoptosis, suggesting that it may lead to excessive neuronal loss in degenerative diseases with increased production of Aβ (Qu et al 2014). The PI3K/AKT pathway also regulates synaptic plasticity by stimulating excitatory and inhibitory cell membranes, enhances neurotransmitter activities and increases glucose
metabolism in specific brain regions that are important for learning and memory
(Chatterjee et al 2018)

Insulin also activates the MAPK pathways leading to Ras activation at the plasma membrane and activation of Raf, MEK and ERK serine/threonine-specific protein kinases (Zhang et al. 2011). It is suspected that ERK plays a critical role in synapses formation and learning/memory functions and other neuroprotective functions. A defective ERK kinase may lead to AD pathology.

Mammalian target of rapamycin (mTOR) and its downstream components play a role in regulating neuronal survival and nutrient sensing and protein synthesis. In an insulin-resistant state, the compromised downstream signaling pathways lead to increased levels of Aβ oligomers and hyperphosphorylated tau. This then leads to impairment of autophagic clearance mechanisms. Autophagic dysfunction causes the progressive accumulation of toxic proteins and eventually causes neuronal death (Orr and Oddo, 2013). Insulin and downstream signaling pathways are evidently important for conservation and preservation of learning and memory processes which become compromised in Alzheimer’s patients. This evidence also emphasizes the depth of shared pathophysiology in both T2DM and AD.
Figure 2. Alzheimer’s Disease Pathogenesis

The overview of the diverse mechanisms by which Type 2 Diabetes can cause AD pathogenesis. Type 2 Diabetes accompanied by insulin resistance and hyperglycemia gives rise to metabolic problems in the brain and other target tissues that sets off a cascade of pathogenic processes such as oxidative stress, inflammatory responses, advanced glycation products and autophagic dysfunction. The reactive oxygen species generated by these pathways expedite the process of neuronal death. At the same time, the insulin resistance impairs the downstream signaling pathways and exacerbates the formation of Aβ oligomers and aggregates of hyperphosphorylated tau. The cumulative effect of all these factors expose the neurons to a range of assaults and gradually result in the loss of synapses and neuronal death. (Ref.: Chatterjee & Mudher, Front Neurosci. 2018;12:38)

Amyloid plaques

A hallmark pathology in AD is the formation of extracellular amyloid plaques composed of insoluble deposits of amyloid-β (Aβ) protein aggregates. Aβ is generated from proteolytic cleavage of amyloid precursor protein (APP) (Chatterjee et al 2018). At lower concentrations, Aβ is soluble and is cleared and degraded easily. While higher concentrations aggregate into insoluble plaques that are resistant to degradation, an increase in accumulation of insoluble Aβ plaques arise due to impaired clearance of
Aβ protein. Studies suggest that elevated plasma glucose levels, a common pathological feature of T2DM, can initiate the formation of Aβ plaques (Chatterjee et al 2018).

Impact of Dyslipidemia on Aβ

Insulin plays an essential role in lipid metabolism; another role is facilitating the entry of glucose into adipocytes. Insulin also inhibits breakdown of fat in adipose tissue through the inhibition of intracellular lipases that hydrolyze triglycerides and releases fatty acids. With impairments in insulin signaling leads to an increase of lipolysis leading to elevated synthesis of free fatty acids. The human brain produces around 30% of total body cholesterol, hence slight alterations in lipid metabolism can have profound effects on cognitive function (Chatterjee et al 2018). Overall, there is a strong impact of insulin resistance, hyperglycemia, dyslipidemia and other salient features of T2DM that directly influence the pathological features of Aβ amyloidogenesis as observed in AD.

Influence of T2DM and Tau Pathology in AD

The physiological function of tau protein promotes the assembly and stabilization of microtubules (Sotiropoulos et al. 2017). In the neuro-typical brain, tau protein usually contains 2-3 moles of phosphate/mole per tau protein, compared to AD brains tau becomes hyperphosphorylated with 6-9 moles of phosphate/mole of tau and aggregates to form intracellular neurofibrillary tangles, another pathological hallmark of AD (Chatterjee et al 2018). Studies have shown that learning and memory defects were causally related to the higher accumulation of neurofibrillary tangles in the temporal lobe which is the brain region associated with learning and memory. The post translational modification of tau (hyperphosphorylation) trigger cellular and biochemical changes
causing abnormal structural alterations which become harder to degrade. Two hallmark features of T2DM, insulin resistance and hyperglycemia, influence post translational modification of tau, as in hyperphosphorylation, leading to an exacerbation of AD. There is a complex relationship between tau aggregation and insulin resistance. Figure 2 depicts how insulin resistance leads to impaired formation of autophagosomes and disrupts lysosomal function, which results in an accumulation of Aβ and tau protein aggregates.

Figure 3. T2DM and AD impairs the formation of autophagosomes

*T2DM and AD impairs the formation of autophagosomes leading to impaired clearance of toxic aggregates (Chatterjee et al 2018)*
Chapter II.

Methods

The work schedule and experiments were planned accordingly, and all the bench work was carried out in Dr. Ghosh’s Lab at the Department of Biology, Howard University, Washington DC, USA. The qualitative and quantitative analysis of DNA and RNA was carried out. The cleanups of all RNA samples were done before proceeding with the Taqman Low Density Array (TLDA) gene expression experiments. Microarray gene expression analysis experiment was also done from selected RNA samples.

Ingenuity Pathway Analysis IPA® determining the commonality and differences in the molecular mechanisms underlying Type 2 Diabetes and Alzheimer’s disease were carried out.

RNA isolation by PAXgene blood RNA Isolation procedure

PAXgene Blood RNA Tubes were centrifuged for 10 minutes at 3000–5000 x g using a swing-out rotor

The supernatant was removed by decanting or pipetting. Added 4 ml RNase-free water to the pellet, and closed the tube using a fresh secondary Hemogard closure

Vortexed the pellet is until visibly dissolved and centrifuged for 10 minutes at 3000–5000 x g using a swing-out rotor. Removed and discarded the entire supernatant. Added 350 μl Buffer BR1, and vortexed until the pellet is visibly dissolved
Pipetted the sample into a 1.5 ml microcentrifuge tube. Added 300 μl Buffer BR2 and 40 μl proteinase K. Mixed by vortexing for 5 seconds and incubated for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm.

(After incubation, set the temperature of the shaker–incubator at 65°C (for step 20)

**Do not mix Buffer BR2 and Proteinase K Together**

Pipetted the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube and centrifuged for 3 minutes at maximum speed (14000 x g, but not to exceed 20,000 x g). Carefully transferred the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube

Added 350 μl ethanol (96–100%, purity grade p.a.). Mixed by vortexing and centrifuged briefly.

(1–2 seconds at 500–1000 x g) to remove drops from the inside of the tube lid

(Note: The length of the centrifugation must not exceed 1–2 seconds, as this may result in pelleting of nucleic acids and reduced yields of total RNA)

Pipetted 700 μl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube, and centrifuged for 1 minute at 8000–20,000 x g. Placed the spin column
in a new 2 ml processing tube, and discarded the old processing tube containing flow-through

Pipetted the remaining sample into the PAXgene RNA spin column and centrifuged for 1 minute at 8000–20,000 x g. Placed the spin column in a new 2 ml processing tube, and discarded the old processing tube containing flow-through.

(Note: Carefully pipet the sample into the spin column and visually check that the sample is completely transferred to the spin column)

Pipetted 350 μl Buffer BR3 into the PAXgene RNA spin column. Centrifuged for 1 minute at 8000–20,000 x g. Placed the spin column in a new 2 ml processing tube, and discarded the old processing tube containing flow-through.

Added 10 μl DNase I stock solution to 70 μl Buffer RDD in a 1.5 ml microcentrifuge tube. Mixed by gently flicking the tube, and centrifuged briefly to collect residual liquid from the sides of the tube.

(During the process in preparing 10 samples, add 100 μl DNase I stock solution to 700 μl Buffer RDD. Use the 1.5 ml microcentrifuge tubes supplied with the kit. Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube not by vortexing)
Pipetted the DNase I incubation mix (80 μl) directly onto the PAXgene RNA spin column membrane, and placed on the benchtop (20–30°C) for 15 minutes

(Note: Ensure that the DNase I incubation mix is placed directly onto the membrane. DNase digestion will be incomplete if part of the mix was applied to and remained on the walls or the O ring of the spin column)

Pipetted 350 μl Buffer BR3 into the PAXgene RNA spin column, and centrifuged for 1 minute at 8000–20,000 x g. Placed the spin column in a new 2 ml processing tube, and discarded the old processing tube containing flow-through

Pipetted 500 μl Buffer BR4 to the PAXgene RNA spin column, and centrifuged for 1 minute at 8000–20,000 x g. Placed the spin column in a new 2 ml processing tube, and discarded the old processing tube containing flow-through

(Note: Buffer BR4 is supplied as a concentrate. Ensure that ethanol is added to Buffer BR4 before use)

Added another 500 μl Buffer BR4 to the PAXgene RNA spin column. Centrifuged for 3 minutes at 8000–20,000 x g

Discarded the tube containing the flow-through and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuged for 1 minute at 8000–20,000 x g

Discarded the tube containing the flow-through. Placed the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube and pipetted 40 μl Buffer BR5 directly onto the
PAXgene RNA spin column membrane. Centrifuged for 1 minute at 8000–20,000 x g and eluted the RNA

(It is important to wet the entire membrane with Buffer BR5 in order to achieve maximum elution efficiency)

Repeated the elution step (step 18) as described, using 40 μl Buffer BR5 and the same microcentrifuge tubes. Incubated the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chilled immediately on ice

(Note: This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature)

If the RNA samples are not being used immediately, store at −20°C or −70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C. Used the RNA samples in a diagnostic assay, followed the instructions supplied by the manufacturer

(Note: For quantification in Tris buffer, use the relationship A260 = 1 = 44 μg/ml)

[FOR 16 samples, it was a 4-hour straight job and had to pipetted over 400 times]

DNA & RNA Isolation from Blood (Quick-DNA/RNA Blood™ Tube Kit-Zymo Research Kit)

All centrifugation steps were performed at 10,000-16,000 x g for 30 seconds unless specified with the following protocol sequentially. Transferred the contents of the DNA/RNA Shield™ - Blood Collection Tube to a 50 ml tube.

Added 120 μl Proteinase K to the tube and mixed by vortexing. Incubated at room temp (20-30°C) for 30 mins. Added 9 ml isopropanol and mix by vortexing. Transferred 700 μl
sample to the column and centrifuge. Discarded the flow-through. Reloaded until all liquid is passed through. Transferred the column into a new Collection Tube. Added 400 μl DNA/RNA Prep Buffer to the column and centrifuge. Discarded the flow-through. Transferred into a clean micro centrifuge tube (not provided). Added 200 μl RNA Recovery Buffer directly to the column matrix, let stand 5 minutes and then centrifuged. Added 100 μl DNase/RNase-Free Water directly to the column matrix, did not touch for 5 minutes, and then centrifuged to elute DNA/RNA from the respective column.

<table>
<thead>
<tr>
<th>DNA Purification</th>
<th>RNA Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(DNA is bound to the column)</strong></td>
<td><strong>(RNA is in the flow through)</strong></td>
</tr>
<tr>
<td>8. Transfer the Zymo spin column into a new collection tube</td>
<td>8. At this point, RNA samples can be in-column DNase I treated.</td>
</tr>
<tr>
<td>9. Add 700 μl DNA/RNA wash buffer to the column and centrifuge. Discard the flow through</td>
<td>9. Add 400 μl DNA/RNA Prep Buffer to the column and centrifuge. Discard the flow-through</td>
</tr>
<tr>
<td>10. Add 400 μl DNA/RNA wash buffer to the column and centrifuge. Discard the flow through</td>
<td>10. Add 700 μl DNA/RNA Wash Buffer to the column and centrifuge. Discard the flow-through.</td>
</tr>
<tr>
<td>11. Add 200 μl DNA Recovery Buffer and centrifuge the column for 2 mins. Transfer the column in to new micro centrifuge tube.</td>
<td>11. Add 400 μl DNA/RNA Wash Buffer and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Carefully transfer the column into a clean <strong>microcentrifuge tube</strong>.</td>
</tr>
</tbody>
</table>

Figure 4. DNA purification and RNA purification
Tempus™ Blood RNA Tube and Tempus™ Spin RNA Isolation Kit Protocol

Processing Stabilized Blood Before Purification

1. The sample was frozen, so I thawed the sample in the Tempus tube at room temperature (18 to 25 °C).

2. Removed the cap from the Tempus tube, then poured the contents of the tube into a clean 50-mL tube (conical tube).

3. Pipetted 3 mL of 1× PBS (Ca2+/Mg2+-free) into the tube to bring the total volume to 12 mL.

   ![Diagram](image)

   NOTE: IMPORTANT! If the initial blood sample was less than 3 mL, make up the difference by adding enough 1× PBS bringing the total volume to 12 mL. Otherwise, RNA yield will be decreased significantly.

Replaced the cap on the tube, then vortexed the tube vigorously (at maximum vortex speed) for 30 seconds ensured proper mixing of the contents.

   ![Vortex](image)

   IMPORTANT! Vortexed the diluted sample for at least 30 seconds; vortexing for less than 30 seconds may cause clogging of the purification consumable. Note: Frothing of the sample after vortexing is normal.
Centrifuge the tube at 4 °C at 3,000 x g (rcf) for 30 minutes.

Carefully pour off the supernatant. Left the tube inverted on absorbent paper for 1 to 2 minutes. Blotted the remaining drops of liquid off the rim of the tube with clean absorbent paper.

Note: The RNA pellet was transparent and invisible. IMPORTANT! Handled the tube carefully, did not shake the RNA pellet off the bottom of the tube. Pipetted 400 μL of RNA Purification Re-suspension Solution into the tube, then vortexed briefly and re-suspended the RNA pellet.

IMPORTANT! To prevent washing any blood residue down the inside of the tube, the pipet tip was inserted into the tube and added the re-suspension solution to the bottom of the tube. The re-suspended RNA was be kept on ice while the next steps were prepared.
Performing the Purification Run

Labeled the RNA purification filter, then inserted the filter into a waste collection tube

1. Pre-wetted the filtration membrane with RNA Purification Wash Solution (100 µl) into the purification filter.
2. Pipetted the re-suspended RNA (400 µl) into the purification filter, then centrifuged @ 16000g for 30 Sec.
3. Removed the purification filter, discarded the liquid waste collected in the waste tube, then re-inserted the purification filter into the waste tube.
   Pipetted RNA Purification Wash Solution 1 (500 µl) into the purification filter, then centrifuged @ 16000g for 30 Sec.
4. Removed the purification filter, discarded the liquid waste collected in the waste tube, then re-inserted the purification filter into the waste tube.
5. Pipetted RNA Purification Wash Solution 2 (500 µl) into the purification filter, then centrifuged @ 16000g for 30 Sec.
6. (NO DNAsé Treatment at this point)
7. Removed the purification filter, discarded the liquid waste collected in the waste tube, then re-inserted the purification filter into the waste tube.
8. Centrifuged to dry the membrane @ 16000g for 30 Sec
9. Transferred the purification filter to a new, labeled collection tube to collected the eluate.

10. Pipetted Nucleic Acid Purification Elution Solution (100 µl) into the purification filter, closed the cap, incubated the entire tube for 2 minutes at 70 °C, then centrifuged @ 16000g for 30 Sec.

11. Pipetted the collected RNA eluate back into the purification filter, then centrifuged @ 16000g for 2 Min.

12. Discarded the purification filter, then transferred approximately 90 µL of the RNA eluate to a new, labeled collection tube.

13. IMPORTANT! When transferring the RNA eluate, carefully pipetted the liquid out of the collection tube starting from the top of the liquid and ensured that the pelleted particulates are not disturbed.

RNA Quality Control Results and Discussion

The RNAs of the samples were measured by Nanodrop 2000 according to manufacturer’s protocol (http://www.nanodrop.com/Productnd2000overview.aspx)

A total 1150 number of RNA and DNA samples quantification was measured by Nanodrop 1000 according to the manufacturers, protocol. (https://tools.thermofisher.com/content/sfs/manuals/nd-1000-v3.8-users-manual-8%205x11.pdf).

The qualitative analysis was also carried out for all RNA samples. All the gel images were incorporated in the Lab logbook as well as in research work records. After the quality analysis was done for all RNA samples, best RNA samples were shortlisted
and undergone genomic DNA clean up followed by cDNA synthesis. The RNA quality analysis was carried by gel electrophoresis on 1% Agarose gel.

High-Throughput Quantitative Real Time PCR (qRT-PCR)

TaqMan Low Density Array (TLDA) is a medium-throughput innovative microfluidic design based on probe-based qPCR chemistry offering an increase in the number of RNAs analyzed on one plate TLDA involves the use of a predefined pool of RT-primers that are used to synthesize the cDNA. These cDNAs are then spun into a customized 384 well micro-fluidic card to determine the expression of multiple RNAs using qPCR (here by ABI 7900HT Fast Real Time PCR System. 


Each well of the card contains dried primers and probes to amplify specific RNA(s). Therefore, up to 384 reactions can be processed in single TLDA card.

Sample Preparation for TLDA & ABI HT-7900 PCR System (With Applied Biosystem Reagents)

Materials used:
1. RNAs from the specified samples
2. Genomic DNA Cleanup Kit DNA Free KIT™ (ABI) (Part # AM1906)
3. DEPC Treated Water
4. High Capacity RNA-to-cDNA Kit (Part # 4387951)
5. TaqMan® Universal Master Mix II, no UNG (Part # 4440040)
I started by selecting the number of runs wish to perform per subjects/experiental samples (Recommended RNAs required is 1000ng/sample, scale up accordingly, if you need more) Our starting materials is about 2000ng and calculations thereoff.

Genomic DNA Clean up

Using *DNA Free KIT™* (ABI) (Part # AM1906)

Pipetted required amount of RNA according to Stock RNA Conc. (to 1000/2000ng)

1. Made up volume with H₂O to 16µl
2. Added DNAse Buffer 2 µl + DNAse 2 µl (Now Total volume = 20 µl)
3. Incubated at 37 ⁰C for 30 minutes
4. Added DNAse Inactivation Reagent 4 µl
5. Incubated and mixed occasionally for 2 minutes
6. Centrifuged at 10,000 x g for 1.5 min and transferred the RNA (in supernatant) to a fresh tube (Stored at -20 ⁰C, for downstream applications)

cDNA Synthesis

Using the High Capacity RNA-to-cDNA Kit (Part # 4387951)

Preparing the RT Reaction

Allowed the kit components to thaw on ice. (Note: Prepare the RT reaction on ice)

Calculated the volume of components needed to prepare the required number of reactions
(for my case I used 18 µl of cleaned RNA for duplicate study) (9 µl for each reaction recommended)

Added 20 µl of 2x RT Buffer + 2 µl of 20x RT Enzyme mix = Total Volume 40 µl

Mix gently and place the reaction mixture on ice

Performing Reverse Transcription (RT)
Aliquoted the RT Reactions in individual Tubes (40 µl)
(Briefly centrifuged the plate or tubes and spun down the contents and eliminated air bubbles)
Programmed the thermal cycler conditions using the following conditions to run the RT
STEP 1: 37 °C for 60 min; STEP 2: 95 °C for 5 min; and then to 4 °C

Preparing Sample for TLDA Plate
1. With TaqMan® Universal Master Mix II, no UNG (Part # 4440040)
2. Took 20 µl of Reverse Transcribed Sample + added 30 µl of H20 in a fresh tube
   = 50µl
3. Added 50µl of Master Mix to the Sample = 100 µl (Mixed well)
4. Pipetted the whole 100µl sample and followed the instructions below
Fill Port of the Card with reaction Master mix

Figure 5. Card with reaction Master mix

TLDA Plate Preparation –

Centrifuging the TaqMan Array Card

Figure 6. Centrifuging the TaqMan Array Card
Sealing Plates

Figure 7. Sealing Plates

Cutting and Loading into the Instrument

Figure 8. Cutting and Loading into the Instrument
Running TLDA Card on ABI HT 7900

**Workflow**

1. **Prepare your samples**
   - Isolate the total RNA
   - Evaluate the RNA
   - Perform reverse transcription

2. **Prepare the card**
   - Prepare the PCR reaction mix
   - Add the reaction mix to the card
   - Centrifuge the card
   - Seal the card

3. **Run the card**
   - Set up the experiment (SDS Software)
   - Perform the run
   - Configure the analysis settings (RQ Study Manager or ΔΔCt Study)
   - Review the results
   - (Optional) Perform downstream analysis

**10 minutes**

- For example, TaqMan® Gene Expression Cells-to-Ct™ Kit
- High Capacity RNA-to-cDNA Kit
- High Capacity cDNA Reverse Transcription Kit
- A TaqMan® master mix
- TaqMan® Array Micro Fluidic Card
- System software and information CD
- 7900HT Fast Real-Time PCR System with a TaqMan® Array Micro Fluidic Thermal Cycling Block
- System software
TaqMan Low Density Array (TLDA) is a medium-throughput innovative microfluidic design based on probe-based qPCR chemistry offering an increase in the number of RNAs analyzed on one plate. TLDA involves the use of a predefined pool of RT-primers that are used to synthesize the cDNA. These cDNAs are then spun into a customized 384 well micro-fluidic card to determine the expression of multiple RNAs using qRT-PCR (ABI 7900HT Fast Real Time PCR System) (See workflow above). Each well of the card contains dried primers and probes to amplify specific RNA(s), therefore,
up to 384 reactions were processed in single TLDA card. TaqMan® Gene Expression Assays consist of a pair of unlabeled PCR primers, and a TaqMan® probe with a FAM™ or VIC® dye label on the 5’ end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3’ end, and coupled to Applied Biosystems® instruments (7900HT Real-Time PCR system) and software (TaqMan® RQ Software, Ver. 1.2.1) (Wong et al. 2015)

The custom designed microfluidic card was used in a 384 well format that included 18sRNA (Manufacturing Control), GAPDH, (as endogenous control). The level of expression of each gene (g) was calculated as Relative Quantities (RQ) using the equation RQ = 2^(-Ct), where Ct = −[Ctg− (Ct18s + CtACTB + CtGAPDH + Ct β2 + Ct HPRT /5], and Delta Ct values were averaged for triplicates. The 16 Format TLDA Custom Array genes list for 2 plates includes 18s, ACTBL2, APC, ARNT, ATP1B1, BCL2, CCK, CD3G, CTP1A2, CYP2D6, ENTPD3, LEPR, LRP12, MYC, RRAD, AND TRAP2 (PLATE-1) and 18s, AMACR, APOC1, APOC2, ATF5, CLCNKB, CYP1B1, EGFR, GAPDH, JUB, MGP, PRL, RARB, SLC2A13, SLC33A1, UGT1A4; UGT1A7; UGT1A10; UGT16 (PLATE-2). The cDNA samples were mixed with Taqman Universal Master Mix and made the volume up to 100 ul. The final volume of 8 samples (100 µl each) was pipetted into 8 respective ports. The plate was centrifuged and sealed till end position followed by cutting and loading the plate into the instrument (Ghosh et al., 2015) with the designated Amplification (Thermal) Cycle.Probe-based Real-time PCR Approaches for Quantitative Measurement of microRNAs. Journal of Visualized Experiments. 2015. 10.3791/52586.
Custom Array Plate Design (TLDA) with 28 Gene Transcripts

Custom Array Plate #1 & #2 with Gene Transcripts, where 18S RNA was designated as the manufacturing control and GAPDH was internal control. The 16 Format Taqman Low Density Array (TLDA) Custom Array Plate design is shown below including complete list of genes in Panel-A. The Panel-B shows the image of Original Plate (Figure 9).
The quality control summary and results were analyzed for the TLDA experimental run with ABI RQ Manager Software (V 1.2.1) for the post run analysis. Data analysis will be done through DataAssist™ and StatMiner®.

Our Genes of Interest and Their Relative Expressions

Table 1. Genes of Interest & Their Relative Expressions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOC1</td>
<td>Plays a central role in high density Lipoprotein (HDL) and very low-density lipoprotein (VLDL) metabolism: Late onset of Alzheimer’s Disease/Breast Cancer/ Metabolic Dysfunction</td>
</tr>
<tr>
<td>APOC2</td>
<td>Cause hyperlipoproteinemia type 1B, characterized by hypertriglyceridemia, xanthomas. Increased risk of pancreatitis/Early atherosclerosis</td>
</tr>
<tr>
<td>RRAD</td>
<td>Diminished insulin-stimulated glucose uptake: Metabolic Dysfunction/Diabetes</td>
</tr>
<tr>
<td>ARNT</td>
<td>Insulin secretion and glucose tolerance linked to Type 2 Diabetes</td>
</tr>
<tr>
<td>LEPR</td>
<td>Involved in the regulation of fat metabolism: Obesity / Pituitary Dysfunction</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Catalyzation of reactions involving drug metabolism and synthesis of cholesterol, steroids, and various lipids: Neurobehavioral/Alzheimer’s Disease</td>
</tr>
<tr>
<td>SLC2A13</td>
<td>Required for transport of myoinositols: Cancer/ Parkinson Disease</td>
</tr>
<tr>
<td>TP53</td>
<td>Provides instructions for a tumor suppressant protein: Cancer</td>
</tr>
</tbody>
</table>

*Table describes gene of interest and the role of that genetic expression*
Differential Gene Expression by 96-Well Plate (FAST) for Alzheimer’s Gene Panel

In addition to TLDA gene expression studies, experiments by using conventional 96-well plate (Clear) over the ABI 7900HT- Fast Real time PCR system was done for relative quantification. The following is protocol that has been optimized for generating first strand cDNA using High-Capacity RNA-cDNA Synthesis Kit (Cat # 4387406, Applied Biosystem, Santa Clara, CA, USA) for the use in downstream quantitative real-time RT-PCR.

First strand cDNA Synthesis:

1. Used up to 2 μg of total RNA per 20-μL reaction.
2. Allowed the kit components to thaw on ice.
3. Referring to the table below, calculated the volume of components needed to prepare the required number of reactions.
4. Aliquoted the RT reaction mix into a plate/tube.
5. Sealed the plates/tubes with the appropriate seal or caps.

Table 2. cDNA Synthesis Materials & Quantities

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+RT reaction</td>
</tr>
<tr>
<td>2X RT Buffer Mix</td>
<td>10.0 μL</td>
</tr>
<tr>
<td>20X RT Enzyme Mix</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>RNA sample</td>
<td>up to 9 μL</td>
</tr>
<tr>
<td>Nuclease-free H2O</td>
<td>0.5(1) to 20 μL</td>
</tr>
<tr>
<td>Total per reaction</td>
<td>20.0 μL</td>
</tr>
</tbody>
</table>

*Table describes components and volume per reaction of cDNA synthesis*
6. Briefly centrifuged the plate/tubes to spin down the contents and eliminated any air bubbles.

7. Placed the plate or tubes on ice until you are ready to start the reverse transcription reaction.

8. Incubated the reaction for 37°C for 60 minutes. Stopped the reaction by heating to 95°C for 5 minutes and held at 4°C. For convenience, the incubation can be performed in a thermal cycler.

9. The cDNA was ready for use in real-time PCR applications or for long-term storage in a freezer (−25°C to −15°C).

Preparation of the PCR reaction mix

1. Combined the following components for the number of reactions required, plus 10% overage.

Table 3. Preparation of the PCR Reaction Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>384-well plate</td>
<td>96- or 48-well plates[1]</td>
</tr>
<tr>
<td>TaqMan® Fast Advanced Master Mix [2X]</td>
<td>5.0 µL</td>
<td>10.0 µL</td>
</tr>
<tr>
<td>TaqMan® Assay (20X)</td>
<td>0.5 µL</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Nuclease-Free Water[2]</td>
<td>3.5 µL</td>
<td>7.0 µL</td>
</tr>
<tr>
<td>Total volume per reaction</td>
<td>9.0 µL</td>
<td>18.0 µL</td>
</tr>
</tbody>
</table>

[1] Standard and Fast
[2] Adjust the volume of Nuclease-Free Water for a larger volume of cDNA.

2. Vortexed briefly to mix.

3. Centrifuged briefly to bring the reaction mix to the bottom of the tube and eliminated air bubbles.
Preparation of the PCR reaction plate

1. Transferred the appropriate volume of PCR reaction mix to each well of an optical reaction plate.

2. Added cDNA template (1 pg to 100 ng in Nuclease-Free Water), or Nuclease-Free Water for NTC, to each well.

3. 1.0 μL for a 384-well plate

4. 2.0 μL for 96- and 48-well plates (Standard and Fast)

5. Note: Made sure to adjust the volume of Nuclease-Free Water for the PCR reaction mix for a larger volume of cDNA.

6. Sealed the reaction plate with optical adhesive film, then centrifuged briefly bringing the PCR reaction mix to the bottom of the well and eliminated air bubbles.

7. Applied a compression pad to the plate, required by the real-time PCR system.
Chapter III.

Results

Following are the expression status (in fold change, up, down-regulated) of different disease specific (Alzheimer’s Disease) genes in T2DM and AD in Pakistani population based on different selection criteria (Overall, Male, Female, smokers, non-smokers) with relative comparison with our previous studies (Slovak Population) (Figures 10 -13)

Figure 10. Differential expression of Alzheimer’s Disease panel genes

Differential expression of Alzheimer’s Disease panel genes through TLDA in Pakistani female diagnosed with Alzheimer’s Disease.
Figure 11. Differential expression of Alzheimer’s Disease panel genes

Differential expression of Alzheimer’s Disease panel genes through TLDA in Pakistani female with Type 2 Diabetes.
Figure 12. Differential expression of Alzheimer’s Disease panel genes

*Differential expression of Alzheimer’s Disease panel genes through TLDA in Pakistani male diagnosed with Alzheimer’s Disease.*

Figure 13. Differential expression of Alzheimer’s Disease panel

*Differential expression of Alzheimer’s Disease panel genes through TLDA in Pakistani male with Type 2 Diabetes.*
Figure 14. Quantitative Real-time PCR (qRT-PCR) validation of the selected 96 gene panel (Alzheimer’s Disease) of interest

Taqman Low Density Array (TLDA) in ABI platform after analysis by SDS RQ Manager Version 1.2.1(ΔΔCt) in contrast to calibrator samples. A – Alzheimer’s Female; B - Alzheimer’s Male; C- T2DM Female; D – T2DM Male.
Results from 96-Well Fast Plate Alzheimer’s Disease Panel Genes (TLDA)

We used the panel of assays (for details visit: https://www.thermofisher.com/order/catalog/product/4414070#/) in this pre-made plate which was based on the "amyloid hypothesis" that includes 92 genes that are involved in APP processes that generate beta-amyloid and included genes implicated in multiple secondary steps of beta-amyloid aggregation, tau hyperphosphorylation, excitotoxicity, inflammation, oxidation, and microglial activation. It also includes assays for genes involved in cholesterol biosynthesis due to the correlation between high cholesterol and increased risk of Alzheimer's. Genes associated with Alzheimer's disease pathology, biochemistry and genetics are also included. Gene Signature Plates are 96-well plates that are pre-configured with the most appropriate TaqMan® Gene Expression Assays for a specific biological process, pathway, or disease state. Each plate contains predefined assays and endogenous controls dried down in the wells, ready for accurate assessment of an entire gene signature in one simple experiment.

Representative subjects (one each male and female) from each disease conditions (T2DM and AD) were investigated to see their relative gene expression status. The results showed different gene-expression pattern in those selected subjects. A significant differential change was observed among T2DM male and Female, whereas the AD subjects also showed different gene expression pattern among male and female (Figure 15). The detailed and further investigations are underway.
Figure 15. Male and Female ALZ Gene panel

Differential expression of Alzheimer’s Disease panel genes through TLDA in Pakistani Male & Female diagnosed with Alzheimer’s Disease.
Figure 16. Stacked Bar-Chart with the Number of Genes Involved in AD Subjects

Bar graph shows up/down-regulated expression of genes involved in AD subjects. Of note is the upregulation of Apoptosis signaling and downregulation of IL-10 signaling (involved in healing and stopping inflammatory pathway).

Our Genes of Interest and Their Relative Expressions from Validation Studies (PAK-A) and with Other Population (PAK/AA/SLOVAK-B)

The following figures depict the genes of interest in this project and their relative expressions (up/down-regulation) in comparison to gene expressions from previous projects studying other populations (Slovakian, African Americans and Pakistani)
<table>
<thead>
<tr>
<th>GENE</th>
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</tr>
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<tbody>
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</tr>
<tr>
<td></td>
<td>Mixed-regulation - Female/Non-smokers</td>
<td>lipoprotein (VLDL) metabolism; Late onset of Alzheimer’s Disease / Breast Cancer / Metabolic Dysfunction</td>
</tr>
<tr>
<td>APOC2</td>
<td>Up-regulated - In All Categories</td>
<td>Cause hyperlipoproteinemia type IB, characterized by hypertriglyceridemia, xanthomas. Increased risk of pancreatitis / Early atherosclerosis.</td>
</tr>
<tr>
<td>RRAD</td>
<td>Up-regulated - Male/Smokers</td>
<td>Diminished insulin-stimulated glucose uptake: Metabolic Dysfunction/Diabetes</td>
</tr>
<tr>
<td></td>
<td>Down-regulated - Female/Non-smokers</td>
<td></td>
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<td>Down-regulated - Female/Non-smokers</td>
<td></td>
</tr>
<tr>
<td>SLC2A13</td>
<td>Up-regulated - In Most categories</td>
<td>Required for transport of myoinositol: Cancer/Parkinson Disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP53</td>
<td>Up-regulated - Male/Smokers</td>
<td>Provides instructions for a tumor suppressant protein: Cancer</td>
</tr>
<tr>
<td></td>
<td>Down-regulated - Others</td>
<td></td>
</tr>
</tbody>
</table>

Figure 17. Our Genes of Interest and Their Relative Expressions from Validation Studies (PAK-A) and with Other Population (PAK/AA/SLOVAK-B)
Metabolic Dysfunctions (ARNT) Gene Expression in the Studied Subjects

Figure 18. Metabolic Dysfunctions (ARNT) Gene Expression

ARNT gene expression was up-regulated in African American and PAK-nonsmokers. ARNT was down-regulated in Slovak and PAK-Smokers population. ARNT is responsible for insulin secretion and glucose tolerance.
Cancer Related (\textit{MYC}) Gene Expression in the Studied Subjects

\textit{MYC} in SLOVAK population

\textit{MYC} in AA Participants

\textit{MYC} in PAK Participants

\textit{MYC} in PAK Participants (Smokers)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure19.png}
\caption{Cancer Related (\textit{MYC}) Gene expression}
\end{figure}

\textit{A proto-oncogene and encodes a nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. \textit{MYC} gene regulation up-regulated in PAK and Slovak populations and down-regulated in PAK-smokers.}
Figure 20. Other Gene Expressions of Our Genes of Interest

*Other gene expressions in the studied subjects (Neurobehavioral/Alzheimer’s/Cancer).*

Ingenuity Pathway Analysis (IPA®)

Overall Approach for Differential Gene Expression (Microarray) and with combined “Signature Pathways” by IPA. Since gene expression levels reflect the joint effect of several underlying biological functions, disease specific biomarkers may be involved in distinct biological functions. However, a single gene can be involved in multiple distinct biological processes. One solution to this problem is to first infer gene regulatory networks (Pathways) that appear to control or regulate phenotypically relevant biological functions, and then to extract the most biologically and statistically relevant
biomarkers. Through our current gene expression studies with Pakistani population, we have been able to identify some specific disease (T2DM & AD) pathway related genes (gene panel per se) along with the database using Ingenuity Pathway Analysis (IPA, Ingenuity Systems: http://www.ingenuity.com). In this current project, subjects were selected (n=2 in each group; case (T2DM and AD) versus control (Healthy, non-diseased) from our recruited subjects to complete the microarray gene expression analysis coupled with IPA analysis for the significant pathways, with special emphasis on endocrine system disorders, insulin receptor signaling, Type I & II Diabetes Mellitus Signaling, beta-cell receptor signaling, and glucocorticoid receptor signaling. Combined with our previous results, we were able to identify a subset of genes in these respective pathways, that could usefully classify the T2DM and AD cases, according to their P values, highest fold change, highest expression levels, and consistency of fold change in each individual sample.

Pathway Identification

We constructed the probable pathways with the significantly induced genes (filtering through fold change > 2.0 and P value <0.05) to identify the specific pathways associated with respective diseases (T2DM & AD). Using Ingenuity Pathway software to examine any functional correlations between the pathologies. Data sets containing gene identifiers and corresponding expression values will be uploaded into an application and mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Genes differentially expressed with p<0.05 were overlaid onto global molecular networks, developed from information contained in the knowledge base. Networks were then algorithmically generated based on their connectivity. Canonical pathway analysis
identified the function specific genes that significantly present within the networks. Biofunction analysis identifies the associations between the data set and the information in the IPA library. This associations were calculated from the ratio of the number of genes from the data set that are associated with the biofunctions divided by the total number of molecules that are associated with the function. The probability that each biological function and/or disease assigned to that data set was due to chance alone, and were calculated using a right-tailed Fischer’s Exact Test. Over-representation of the molecules in each process will be statistically significant when $P < 0.05$. The over-represented functional or pathway processes are those which have more focus molecules than expected by chance. Using a pathway approach, we capitalized on the prior biological knowledge about genes and pathways, and therefore, we may have a better chance to identify the genes and mechanisms that are involved in Type 2 diabetes and Alzheimer’s disease.
Figure 21. Pathways with ALZ Gene Panel (96 genes) Expression Information

Networks AD-Female

IPA analysis reflecting the top gene connections of genes and canonical pathways derived from AD - female participant.
Figure 22. Pathways with ALZ Gene Panel (96 genes) Expression Information Networks AD-Male

IPA analysis reflecting the top gene connections of genes and canonical pathways derived from AD - male participant
Figure 23. Pathways with ALZ Gene Panel (96 genes) Expression Information

Networks T2D – female

IPA analysis reflecting the top gene connections of genes and canonical pathways derived from T2D – female
Figure 24. Pathways with ALZ Gene Panel (96 genes) Expression Information

Networks T2D – Male

IPA analysis reflecting the top gene connections of genes and canonical pathways derived from T2D – Male
Figure 25. IPA Analysis Reflecting the Top Network of the genes and Canonical Pathways derived from Pakistani AD participants.
Chapter IV.

Discussion

The major goal of this study was to investigate shared genetic mechanisms associated with Type 2 Diabetes and Alzheimer’s disease in Pakistani Population. Understanding the gene expression (up-regulation and down-regulation) of our specific genes of interest will give us a better understanding on the why certain pathologies manifest themselves and could offer an explanation of the shared co-morbidities shared by patients with Type 2 Diabetes and Alzheimer’s disease.

Important Differential Expression of Genes and their Functions related to Alzheimer’s Disease

DOWN-REGULATED Genes and Functions

- **CSNK1A1**: Casein kinase-1 isoforms differentially associate with neurofibrillary and granulovacuolar degeneration lesions.
- **CSNK1D**: Cholesterol Biosynthesis.
- **CYP45A1**: Cholesterol Biosynthesis.
- **GAP 43**: Growth Associated Protein-43. Responsible for synaptic damage/dysfunction leads to cognitive impairment.
- **APOE**: Responsible for major cholesterol carrier and lipid transport (Lipid Biosynthesis).
- **GJB1**: Gap junction protein. Involves in cellular function and communication.
- **GRIN2A & 2B**: Chemical message to brain. Speech and language.
GSK3B: Negative regulation of glucose homeostasis and energy metabolism.

Defect of this gene function have been associated with Parkinson’s and Alzheimer’s disease.

Further research into the normal physiological functions of these genes while attempting to comprehend the resulting downstream effects of the downregulation of these specific genes, allows us to postulate and present reasoning as to why certain pathologies may present themselves in the patient populations. For example, with the downregulation of genes responsible for cholesterol biosynthesis (CYP45A1 and CSNK1D), a person’s body which is unable to create cholesterol for physiological use might respond by delaying the breakdown and clearance of cholesterol which can possibly lead up to the buildup of cholesterol into plaque which interrupts with neuronal signaling. The downregulation of APOE also can be held responsible for the buildup of lipid plaques.

The downregulation of genes specifically linked to brain function can have deleterious effects on physical impairment. Genes involved in cellular function and communication through the actions of the transmembrane gap junction protein regulates and controls the transfer of communication signals across cell membranes of the peripheral nervous system. Complications include the demyelination of oligodendrocytes and Schwann cells, which results in delayed transmission rates of nerve communication in the peripheral nervous system. This condition, most commonly will lead to muscle weakness and sensory problems in the outer extremities of the limbs, resulting in muscle atrophy and soft tissue injuries.
Glutamate [NMDA] receptor subunit epsilon-2A/B (GRIN2AB) functioning is involved in long-term potentiation, an activity-dependent increase in the efficiency of synaptic transmission thought to underlie certain kinds of memory and learning (Chatterjee et al 2018). It can be hypothesized that a lower expression of this protein is responsible for the failure to maintain developed cortical circuits.

Glycogen synthase kinase 3 beta is an enzyme in humans that is encoded by the GSK3B gene and is identified as a phosphorylating and an inactivating agent of glycogen synthase. Downregulation of this gene results in negative regulation of glucose homeostasis and energy metabolism.

**UP-REGULATED Genes and Functions**

SLC30A3: Zinc Transport/homeostasis, normally up-regulated in brains, an inflammatory markers for obese women, also an association with serious psychiatric illness, associated with an increasing rate of suicide.

SLC18A3: Responsible for transport of Acetylcholine. Short and long-term recognition memory.

CDC2: Cell Cycle related.

APLP1: Amyloid beta protein precursors in modification of glucose and insulin homeostasis.

APBB2: Amyloid beta protein precursors and related to Alzheimer’s Disease.

IL1A: Immune response.

The upregulation of certain genes may accelerate the pathological cellular processes. This may be accomplished by a ramped up, unregulated immune response in which the body’s uncontrollable immune response will cause more harm than good. For example, an upregulation of Interleukin 1 alpha (IL-1A), will lead to an uncontrolled immune response. In addition, IL-1A plays a role in insulin resistance, this protein is an important modulator of glucose and insulin homeostasis (Chatterjee et al 2018).

Amyloid beta is a hallmark pathology of Alzheimer’s disease. Amyloid beta is cleaved from amyloid precursor protein and is the main component of the amyloid plaques found in the brains of Alzheimer’s patients (Chatterjee et al 2018). An upregulation in the expression of amyloid genes (APLP1, APBB2 and NAE1) create an abundance of Aβ that can form membrane ion channels allowing the unregulated calcium influx into neurons (Chatterjee et al 2018). This disrupts the calcium ion homeostasis and leads to apoptosis seen in neuronal cells in Alzheimer's disease patients. Other suggested mechanisms in which amyloid beta may damage and cause neuronal death include the generation of reactive oxygen species during the process of its self-aggregation, this process causes lipid peroxidation and the generation of a toxic aldehyde called 4-hydroxynonenal which, in turn, impairs the function of ion-motive ATPases, glucose transporters and glutamate transporters (Chatterjee et al 2018). As a result, amyloid beta promotes depolarization of the synaptic membrane, excessive calcium influx and mitochondrial impairment, and there is some evidence suggesting that misfolded Aβ can induce tau to misfold (Chatterjee et al 2018).
Research Limitations

The current research is a pilot information that how this two-disease pathophysiology are interlinked with the genomic classifier with limited number of participants out of the total cohort. To correlate this disease progression in any population, the gene expression along with the other social and environmental information is necessary. Current study did not have any scope with the limited time and resources but is underway in the mentor’s lab to unveil the true connection between these two disease pathways.

Future Research Directions

Once these genes (signature biomarkers) are validated in large-scale population-based studies (undergoing), it will help to elucidate the mechanisms of action, in which, researchers and clinicians can then be enabled to devise the ways for better therapeutic management to prevent future disease and disorder development.

Our future goal is to complete large-scale population-based studies for the candidate disease marker specificities, which may have the capabilities to address the health disparity diseases (Type II Diabetes here) that particularly affects minority populations.

Conclusion

In this investigational pilot project, two genes, i.e., ARNT and CYP2D6, both downregulated, showed the possibility of becoming putative biomarkers for metabolic disorders and neurobehavioral disease, respectively.
We have been able to observe the expression status of some putative signature biomarker genes through our gene expression studies in connection with the clinical outcomes, e.g., Metabolic Disorders (Diabetes/Obesity), Neurobehavioral development (Alzheimer’s disease), and Cancer.

In the studied subjects, gender and smoking were somewhat decisive factors (risk factors) in the disease-specific gene expressions. Genes: APOC1, RRAD, ARNT, CYP2D6, and TP53 showed up-regulation in males and smokers, but down-regulated among females and non-smokers. Exposure, ethnicity, gender, age, smoking, and disease condition may exert important underlying roles on their relative gene expressions. Utilizing Pathway Analysis over IPA revealed distinct gene networks and the respective canonical pathways that were carried out both in T2DM and AD participants.

While examining Alzheimer’s disease gene panel (Custom Disease panel), significant differential changes were observed among T2DM male and female, whereas the AD subjects also showed a different gene expression pattern between male and female.
References


