A pilot study to examine the role of drug metabolizing enzymes in Parkinson's disease

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Submitted by

Sagar Verma

(DTU/13/M.TECH/393)

Delhi Technological University, Delhi, India

Under the supervision of

Dr. Pravir Kumar

Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road,
Delhi-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"A pilot study to examine the role of drug metabolizing enzymes in Parkinson's disease"**, submitted by **SAGAR VERMA (DTU/13/M.TECH/393)** in partial fulfilment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

Date: 14/07/2015

Dr. Pravir Kumar
Associate Professor
(Project Mentor)
Department of Bio-Technology
Delhi Technological University

Dr. D. Kumar
Professor, HOD
Department of Bio-Technology
Delhi Technological University

Sir Ganga Ram Hospital

CERTIFICATE OF EXTERNAL GUIDE

This is to certify that the thesis entitled "**A pilot study to examine the role of drug metabolizing enzymes in Parkinson's disease**" submitted by **Sagar Verma** to **Delhi Technology University**, Delhi, for the degree of **Master of Technology in Biomedical Engineering**, is his original work, based on the results of the experiments and investigations carried out by him at the Department of Research during the study period January to June 2015 under the co-guidance of Dr. Pravir Kumar and Dr. Vibha Taneja.

The above said work has not been previously submitted for the award of any degree, diploma or fellowship in any Indian or foreign University.

Date: 13 July 2015

Place: New Delhi

Vibha Taneja

Dr. Vibha Taneja
Consultant
Department of Research
Sir Ganga Ram Hospital
New Delhi – 110 060

Sir Ganga Ram Hospital Marg, Rajinder Nagar, New Delhi 110060, INDIA
Phones : +91-11-25750000, 42254000 Fax : +91-11-25861002 Email : gangaram@sgrh.com Website : www.sgrh.com

DECLARATION

I declare that my major project dissertation entitled "**A pilot study to examine the role of drug metabolizing enzymes in Parkinson's disease**" submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at "Department of Research, Sir Ganga Ram Hospital".

Date: 14/07/2015 SAGAR VERMA

Place: New Delhi

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LIST OF ABBREVIATIONS

3-MT: 3-Methoxytyramine

3-OMD: 3-O-Methyldopa

AADC: Aromatic amino acid decarboxylase

ATF6: Activating transcription factor 6

BP: Base pair

CBGD: Cortical-basal ganglionic degeneration

cDNA: Complementary DNA

CNS: Central nervous system

COMT: Catechol-o-methyl transferase

Ct: Threshold cycle

DA: Dopamine

DDC: Dopa decarboxylase

DLB: Dementia with lewy bodies

DNA: Deoxyribonucleic acid

DOPAC: 3, 4-dihydroxyphenylactic acid

ER: Endoplasmic reticulum

FP: Forward primer

Grp78: Glucose regulated protein 78

Hsc: Heat shock cognates

Hsp: Heat shock proteins

IRE1: Inositol requiring protein 1

L-DOPA: Levodopa

MAO B: Monoamine oxidase B

MB-COMT: Membrane bound Catechol-o-methyl transferase

MPTP: 1-methyl-4phenyl-1,2,3,6-tetrahydropyradine

MSA: Multiple system atrophy

NCBI: National centre for biotechnology information

NFW: Nuclease free water

NTC: No template control

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PD: Parkinson's disease

PERK: PKR-like endoplasmic reticulum kinase

PSP: Progressive supranuclear palsy

RNA: Ribonucleic acid

RP: Reverse Primer

RT: Reverse transcriptase

S-COMT: Soluble Catechol-o-methyl transferase

UPDRS: Unified Parkinson's disease rating scale

UPR: Unfolded protein response

A pilot study to examine the role of drug metabolizing enzymes in Parkinson's disease.

SAGAR VERMA

Delhi Technological University, Delhi, India

1. ABSTRACT

Parkinson's disease is a progressive neurodegenerative disorder which is characterized by loss of dopaminergic neurons in the brain. It affects more than 1% of the world's population. Levodopa, a precursor of dopamine (neurotransmitter responsible for movements) is the drug of choice for treatment of Parkinson's disease. Levodopa is metabolized to dopamine by Dopa decarboxylase enzyme. In addition, dopamine is metabolized to other non-functional forms by catechol-o-methyl transferase and monoamine oxidase enzymes. Hence, these three enzymes appear to be important for bioavailability of levodopa. In this study, we have examined the role of drug metabolizing enzymes in Parkinson's disease. In addition, pathogenesis of Parkinson's disease has been shown to be associated with protein aggregation. We also examined the role of two important molecular chaperones (Hsp70 and GrP78) implicated in Parkinson's disease. The expression levels of these enzymes and chaperones in patients were compared to heathy controls by real time PCR. Though the sample size (six patients and six healthy controls) was small, our results indicate that COMT and MAOB downregulated. However, no conclusive change was observed in molecular chaperones. The study needs to be extended to larger sample set to define a correlation of these enzymes with drug response in Parkinson's disease.

2. INTRODUCTION

Human body movements are controlled by extra pyramidal which either promote or inhibit movement. The extra pyramidal system includes sub thalamic nucleus, thalamus, basal ganglia and substantia nigra (SN). The basal ganglia consists of the putamen, caudate (which together forms striatum) and globus pallidus. The substantia nigra is a tiny structure located deep within the brain.
Dopamine (DA) is a neurotransmitter which is produced by darkly pigmented cells of substantia nigra and delivered to striatum. This motor pathway is called nigrostriatal pathway as it involves substantia nigra and striatum. Adequate levels of dopamine is responsible for normal movements.

Within the neuron, tyrosine is converted into levodopa with the help of tyrosine hydroxylase. Further, levodopa is converted into dopamine with the help of dopa-decarboxylase (DDC). This dopamine is stored in vesicles. When a signal impulse travel down the neuron it releases the dopamine into the synaptic cleft. This dopamine in the synaptic cleft binds to the dopamine receptors of the connecting neuron. Free floating dopamine in the synaptic cleft may be taken back into the neuron that released it and stored for future use. This process is called REUPTAKE. Free floating dopamine can also be converted into other substances by the action of enzyme COMT (Catechol-o-methyl transferase) and MAO-B (monoamine oxidase B) (Kuran 1995).

There is enormous degeneration of darkly pigmented cells of substantia nigra which produces dopamine. Due to this neurodegeneration, dopamine supply to the striatum is gradually reduced which causes a misbalance between acetylcholine (another neurotransmitter) and dopamine.

Extrapyramidal system loses its ability to promote or inhibit movement due to degeneration of more than 60% of dopamine producing cells in the substantia nigra which causes motor symptoms of Parkinson's disease (PD) such as rigidity, resting tremor, postural instability and bradykinesia.

Drug Metabolizing Enzymes

Severity of symptoms in Parkinson's disease depends on the availability of dopamine in the brain. There are various drug metabolizing enzymes such as Catechol-o-methyl transferase (COMT), Monoamine oxidase (MAO), Dopa decarboxylase (DDC) in the central nervous system which converts levodopa/ dopamine into other forms (Kuran 1995). Due to their enzymatic action, dopamine level in the CNS decreases. COMT converts levodopa and dopamine into 3-O-Methyldopa (3-OMD) and 3-Methoxytyramine (3-MT) respectively. MAO converts dopamine into3.4 dihydroxyphenylacetic acid (DOPAC) and 3-MT into homovanillic acid (HVA). A number of studies have shown that SNP's in these enzymes may play a role in PD motor symptoms (Kiyohara *et al.,* 2011; Hao *et al.,* 2011; Muellner *et al.,* 2015). Due to these polymorphisms patients may respond differently to levodopa therapy.

Molecular Chaperones

Molecular chaperones represents a group of proteins that are involved in cellular protein homeostasis of all organism. Molecular chaperones are involved in controlling the activity of regulatory proteins, folding and assemble of newly synthesized proteins, membrane translocation of secretory and organellar proteins, and refolding of misfolded and aggregated proteins. Heat shock cognates (HSCs) carry out these process under normal cellular conditions. But under stress conditions such as increased temperatures, heat shock proteins (HSPs), another set of molecular chaperones, are expressed. These heat chock proteins are crucial for survival of cells. Under cellular stress, two major families of HSPs are expressed, namely Hsp70 and Hsp90. Hsp27, Hsp40, Hsp60 and Hsp100 are other families of molecular chaperones. In many neurodegenerative disorders changes in protein metabolism are responsible for onset and progression of disease. Parkinson's disease pathology is characterized by the presence of intracellular inclusion bodies termed as Lewy bodies and progressive degeneration of neurons of substantia nigra. Lewy bodies have large amount of phosphorylated and ubiquitinated proteins. Studies across the globe have proved that these lewy bodies contain increased levels of a presynaptic protein, α-synuclein, or its aggregates are a hallmark of Parkinson's disease [Spillantini *et al.,* 1998].These protein aggregates of α-synuclein also play a role in other neurodegenerative disease such as Alzheimer's disease, multiple system atrophy (MSA) and dementia with lewy bodies (DLB) [Dickson. 2012; Serrano-pozo *et al.,* 2011; Irwin *et al.,* 2013*;* Halliday *et al*., 2011]. Numerous studies have shown the presence of HSPs in lewy bodies [Auluck *et al.,* 2002; Outeiro *et al.,* 2006]. This indicates the effort of molecular chaperones in regulating protein aggregation. A PD mouse model demonstrated up regulation of Hsp27, Hsp40 and Hsp70 in response to α-synuclein overexpression [St Martin *et al.,* 2007].

In this study, we have focused on dopamine metabolizing enzymes including COMT, MAO, DDC and molecular chaperones (Hsp70 and Grp78). In review of literature, I have briefly described these proteins and evidences suggesting their role in Parkinson's disease.

3. REVIEW OF LITERATURE

3.1 Catechol-o-methyltransferase

Catechol-o-methyltransferase (COMT) was initially characterized and purified by Axelrod et al in 1958. It is located on chromosome 22, band q11.2. It is a cellular enzyme responsible for O-methylation of catecholamine neurotransmitters such as dopamine, epinephrine and norepinephrine. COMT inactivates catecholamine by transferring a methyl group from S-adenosyl-L-methionine (SAM) to the catecholamine [Männistö *et al.,* 1999]. COMT exists in two forms i.e. soluble form (S-COMT) and membrane-bound form (MB-COMT). Although both types of COMT are found in most human tissues, S-COMT is highly expressed in liver and kidney whereas MB-COMT is highly expressed in the brain [Tenhunen *et al.,* 1994; Karhunen *et al.,* 1994]. Substrates of COMT includes levodopa, catecholamine and its metabolites.

COMT plays a key role in dopamine metabolism in the periphery and central nervous system. COMT converts levodopa to 3-O-methyldopa (3-OMD) in the presence in peripheral decarboxylase inhibitor [Müller *et al.,* 2010]. In the CNS, COMT degrades dopamine to 3-methoxytyramine (3-MT) [Espinoza *et al.,* 2012]. Due to these actions of COMT, levodopa levels in the peripheral and dopamine levels in the brain get affected which increases the severity of Parkinson's disease. A recent study in Chinese population suggests that polymorphisms in COMT gene may be the risk factor of wearing-off in PD patients [Hao *et al.,* 2014].

3.2 Monoamine oxidase

Monoamine oxidase (MAO) is a mitochondrial membrane bound enzyme which generates hydrogen peroxide as a by-product by deaminating a number of hormones, monoamine neurotransmitters and dietary amines in the peripheral tissues and brain. MAO A and MAO B are two isoenzymes of monoamine oxidase. Both isoforms are located in the outer mitochondrial membrane and share 70% amino acid sequence identity. MAO A and MAO B are coded by two independent genes but they have similar exon-intron organization [Bach *et al.,* 1988; Grimsby *et al.,* 1991]. MAOB is predominantly found in glial cells, histaminergic and serotonergic neurons whereas MAO A is abundant in catecholaminergic neurons. In humans, MAO B oxidizes dopamine whereas in rodents, the same is done by MAO A [Shih *et al.,* 1999].

Numerous studies have shown that MAO B activity in brain increases with aging suggesting its role in aging process [Flower *et al.,* 1997; Arai *et al.,* 1988]. MAO B activates a proneurotoxin 1-methyl-4phenyl-1,2,3,6-tetrahydropyradine (MPTP) which is responsible for causing parkinsonian syndrome in rat models [Chiba *et al.,* 1984]. A recent study from China suggests that dyskinesia may be due to polymorphism in MAO B gene [Hao *et al.,* 2014]. Both Parkinson's disease and Alzheimer's disease are associated with elevated levels of MAO B in the CNS [Mallajosyula *et al.,* 2009]. In the CNS, MAO B converts dopamine into 3, 4-dihydroxyphenylactic acid (DOPAC) which decreases dopamine levels leading to increase in the severity of Parkinson's disease. However, the clear cut correlation between the levels of MAOs in the blood and Parkinson's disease has not been defined so far.

3.3 Dopa decarboxylase (DDC)

The gene encoding dopa decarboxylase (DDC) is located on position 12.2 on the short arm (p) of chromosome 7. DDC catalyses the decarboxylation of L-tryptophan to tryptamine, L-5-hydroxytryptophan to serotonin and L-3, 4-dihydroxyphenylalanine (L-DOPA) to dopamine. DDC is also called aromatic amino acid decarboxylase (AADC) as it decarboxylate 5-hydroxytryptophan as well as other aromatic amino acids. In the absence of DDC, COMT converts levodopa into 3-O-methyldopa. This leads to decrease in the levels of dopamine which increases the severity of symptoms in Parkinson's disease. A recent study indicates that polymorphisms in DDC gene affect the motor response to levodopa in Parkinson's disease [Devos *et al.,* 2014]. A study from Taiwan has observed elevated 3-O-methyldopa concentrations in dried blood spots in AADC deficient patients [Chen *et al.,* 2014].

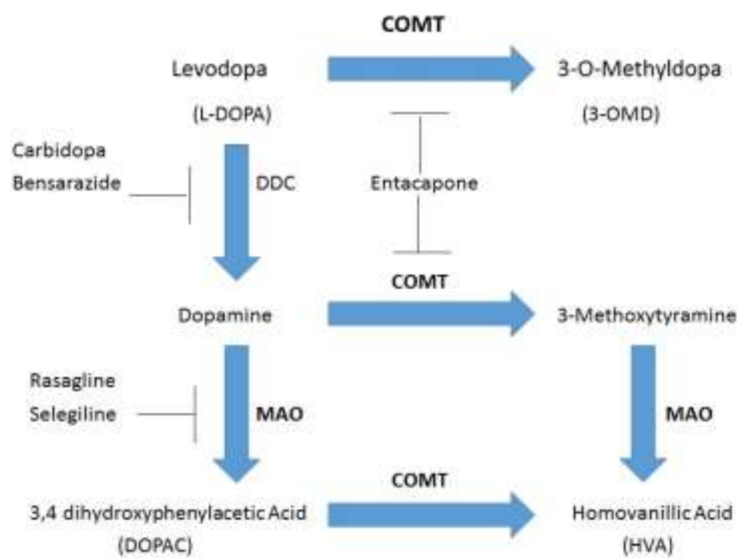

Figure1: Action of drug metabolizing enzymes and their inhibitors in dopamine synthesis pathway

3.4 Heat shock protein 70 (Hsp70)

Heat shock protein 70 (Hsp70), family of 70-kDa proteins, is a class of molecular chaperons which play an important role in folding processes including control of the activity of regulatory proteins, folding and assemble of newly synthesized proteins, membrane translocation of secretory and organellar proteins, and refolding of misfolded and aggregated proteins [Bakau *et al.* 2000; Hartl *et al.,* 2002; Young *et al.,* 2003, Neupert *et al.,* 2002]. Hsp70 is extensively studied molecular chaperon as it is majorly up-regulated in cellular stress. Both in vivo and in vitro studies have linked Hsp70 to α-synuclein and Parkinson's disease. Flower et al demonstrated the expression of Hsp70 to prevent α-synuclein-induced apoptosis in yeast exposed to heat shock [Flower *et al.,* 2005]. Another study using fluorescence imaging demonstrated that α-synuclein conformation and oligomerization was affected by Hsp70 [Klucken *et al.,* 2006]. An in vivo studies showed prevention of dopaminergic cell loss by direct targeting of Hsp70 in SNpc of MPTP- treated mice

[Dong *et al.,* 2005]. A recent study has shown Hsp70 reduces formation of α-synuclein oligomer and related toxicity in extracellular space [Danzer *et al.,* 2011]. Other than α-synuclein, Hsp70 also refold other misfolded proteins involved in PD such as parkin (PARK2) [Winklhofer *et al.,* 2003]. All these evidences indicates the disease modifying effect of Hsp70 on α-synuclein aggregation and related toxicity.

3.5 Glucose regulated protein 78 (Grp78)

Glucose regulated protein 78 (Grp78) is also known as BiP. This molecular chaperon is the endoplasmic reticulum (ER) homologue of Hsp70. In the endoplasmic reticulum, Grp78 prevents protein aggregation by controlling protein folding and regulates the signaling of unfolded protein response (UPR).

Many neurodegenerative disorders such as progressive retinal degeneration, Alzheimer's disease and Parkinson's disease are characterized by activation of UPR and intracellular or extracellular accumulation of aggregation of mutated gene products or misfolded proteins [Muchowski *et al.,* 2005; Hoozemans *et al.,* 2012; Nakanishi *et al.,* 2013]. Grp78 is associated with three stress sensor proteins namely PKR-like endoplasmic reticulum kinase (PERK) , Inositol requiring protein 1 (IREI1)and activating transcription factor 6 (ATF6)[Szegezdi *et al.,* 2009]. Within the ER, accumulation of unfolded proteins leads to dissociation of PERK, IRE1 and ATF6 from Grp78 thereby activating them. Upon activation, UPR proceeds which finally leads to apoptosis [Rutkowski *et al.,* 2004]. Accumulation of α-synuclein, hallmark of PD, leads to ER stress and activation of UPR. Data from a recent study suggests that Grp78 plays a neuroprotective in α-synuclein induced Parkinson. Two studies showed that BiP protein inducer X (BIX) induces BiP and prevents neuronal death by ER stress [Kudo *et al.,* 2008; Gorbatyuk *et al.,* 2013].

3.6 Peripheral Blood and Parkinson's disease

Most of the investigations related to neurodegenerative diseases are done using cerebrospinal fluid. However, it is difficult to get consent from patients for these invasive procedures and especially for the diseases like PD which have fatal outcome. Recently, several studies have focused on scanning the peripheral blood for transcriptional alterations to assess the pathogenesis of various neurodegenerative disorders including Alzheimer's disease and Parkinson's disease [Noelker *et al.,* 2011; Scherzer *et al.,* 2007; Buttarelli *et al.,* 2011]. Studies have shown that human peripheral leukocytes and T-lymphocytes express dopa decarboxylase gene, MAO and COMT [Kokkinou *et al.,* 2009]. Human peripheral blood mononuclear cell (PBMC) synthesizes catecholamines, including dopamine, and expresses dopamine receptors and the dopamine transporter on their cell membrane. Changes of the expression of dopamine receptors in PBMC have been reported in PD, as well as the reduction of the intracellular dopamine concentration and tyrosine-hydroxylase immunoreactivity [Barbanti *et al.,* 1999; Caronti *et al.,* 1999; Jiang *et al.,* 2006]. Studies have shown that analysis of dopamine receptors (DR) in lymphocytes of the human PBMC fraction is an attractive tool for evaluation of functional properties of dopaminergic function underlying variation in PD. PBL seems to be a promising tool to identify the dearrangement of dopamine transmission in PD.

4. METHODOLOGY

Inclusion Criteria

All patients suffering with Parkinson's disease (on treatment and drug naive) within the age group of 40-70yrs of both genders were included in this study.

Exclusion Criteria

All patients with Parkinson-plus syndrome such as progressive supranuclear palsy (PSP), multiple system atrophy (MSA), cortical-basal ganglionic degeneration (CBGD) and Dementia with Lewy bodies (DLB) were excluded from this study. Other chronic renal and liver diseases, malignancies etc. were also excluded.

Clinical assessment

The severity of Parkinson's patients was assessed by Part-III of Unified Parkinson's disease rating scale (UPDRS).

Blood Samples

Written informed consent was taken from the patients. Peripheral blood samples from patients were collected in EDTA vials at the first visit in the Neurology OPD. Sample from healthy controls were taken from blood donors visiting the blood bank. No additional invasive procedure was carried out to draw blood from these individuals.

Primer Designing

Consensus CDS sequence of the target genes were taken from NCBI database. This nucleotide sequence contains only exons. Primer3 website was used to design primers by using the primer conditions:

Table1: Primer picking conditions

	Min	**Opt**	**Max**
Primer Size	19	20	22
Primer Tm	57	60	62
Primer GC%	50	55	60

After selecting the primer picking conditions, Primer3 gave us the list of all possible primers for the given sequence. While designing the primers, following considerations were taken into account.

- Primer length should be 19-22 base pairs.
- GC content should be more than 50%.
- Tm difference should not be more than 2°C.
- Primers should not make hairpin or self-dimers.

All the set of primers were checked for specific amplification using in-silico PCR tool of UCSC genome browser. Three sets of primers per gene were procured and tested for amplification. From these three sets of primer, we picked the one which shows best amplification at 60°C in Real Time PCR. Only those real time PCR primers were selected which lie on exon boundary or included alternating exons. Given below is the CCDS sequence of target genes and location of primers in the sequence. Different colours are used to represent different sets of primers.

COMT CCDS SEQUENCE

ATGCCGGAGGCCCCGCCTCTGCTGTTGGCAGCTGTGTTGCTGGGCCTGGTGCTGCTG
GTGGTGCTGCTGCTGCTTCTGAGGCACTGGGGCTGGGGCCTGTGCCTTATCGGCTGG
AACGAGTTCATCCTGCAGCCCATCCACAACCTGCTCATGGGTGACACCAAGGAGCA
GCGCATCCTGAACCACGTGCTGCAGCATGCGGAGCCCGGGAACGCACAGAGCGTGC
TGGAGGCCATTGACACCTACTGCGAGCAGAAGGAGTGGGCCATGAACGTGGGCGAC
AAGAAAGGCAAGATCGTGGACGCCGTGATTCAGGAGCACCAGCCCTCCGTGCTGCT
GGAGCTGGGGGCCTACTGTGGCTACTCAGCTGTGCGCATGGCCCGCCTGCTGTCACC
AGGGGCGAGGCTCATCACCATCGAGATCAACCCCGACTGTGCCGCCATCACCCAGC
GGATGGTGGATTTCGCTGGCGTGAAGGACAAGGTCACCCTTGTGGTTGGAGCGTCCC
AGGACATCATCCCCCAGCTGAAGAAGAAGTATGATGTGGACACACTGGACATGGTC
TTCCTCGACCACTGGAAGGACCGGTACCTGCCGGACACGCTTCTCTTGGAGGAATGT
GGCCTGCTGCGGAAGGGGACAGTGCTACTGGCTGACAACGTGATCTGCCCAGGTGC
GCCAGACTTCCTAGCACACGTGCGCGGGAGCAGCTGCTTTGAGTGCACACACTACC
AATCGTTCCTGGAATACAGGGAGGTGGTGGACGGCCTGGAGAAGGCCATCTACAAG
GGCCCAGGCAGCGAAGCAGGGCCCTGA

MAOB CCDS SEQUENCE

ATGAGCAACAAATGCGACGTGGTCGTGGTGGGGGGCGGCATCTCAGGTATGGCAGC
AGCCAAACTTCTGCATGACTCTGGACTGAATGTGGTTGTTCTGGAAGCCCGGGACCG
TGTGGGAGGCAGGACTTACACTCTTAGGAACCAAAAGGTTAAATATGTGGACCTTG
GAGGATCCTATGTTGGACCAACCCAGAATCGTATCTTGAGATTAGCCAAGGAGCTA
GGATTGGAGACCTACAAAGTGAATGAGGTTGAGCGTCTGATCCACCATGTAAAGGG
CAAATCATACCCCTTCAGGGGGCCATTCCCACCTGTATGGAATCCAATTACCTACTT
AGATCATAACAACTTTTGGAGGACAATGGATGACATGGGGCGAGAGATTCCGAGTG
ATGCCCCATGGAAGGCTCCCCTTGCAGAAGAGTGGGACAACATGACAATGAAGGAG
CTACTGGACAAGCTCTGCTGGACTGAATCTGCAAAGCAGCTTGCCACTCTCTTTGTG
AACCTGTGTGTCACTGCAGAGACCCATGAGGTCTCTGCTCTCTGGTTCCTGTGGTAT
GTGAAGCAGTGTGGAGGCACAACAAGAATCATCTCGACAACAAATGGAGGACAGG
AGAGGAAATTTGTGGGCGGATCTGGTCAAGTGAGTGAGCGGATAATGGACCTCCTT
GGAGACCGAGTGAAGCTGGAGAGGCCTGTGATCTACATTGACCAGACAAGAGAAA
ATGTCCTTGTGGAGACCCTAAACCATGAGATGTATGAGGCTAAATATGTGATTAGTG
CTATTCCTCCTACTCTGGGCATGAAGATTCACTTCAATCCCCCTCTGCCAATGATGAG
AAACCAGATGATCACTCGTGTGCCTTTGGGTTCAGTCATCAAGTGTATAGTTTATTA
TAAAGAGCCTTTCTGGAGGAAAAAGGATTACTGTGGAACCATGATTATTGATGGAG
AAGAAGCTCCAGTTGCCTACACGTTGGATGATACCAAACCTGAAGGCAACTATGCT
GCCATAATGGGATTTATCCTGGCCCACAAAGCCAGAAAACTGGCACGTCTTACCAA

AGAGGAAAGGTTGAAGAAACTTTGTGAACTCTATGCCAAGGTTCTGGGTTCCCTAG
AAGCTCTGGAGCCAGTGCATTATGAAGAAAAGAACTGGTGTGAGGAGCAGTACTCT
GGGGGCTGCTACACAACTTATTTCCCCCTGGGATCCTGACTCAATATGGAAGGGTT
CTACGCCAGCCAGTGGACAGGATTTACTTTGCAGGCACCGAGACTGCCACACACTG
GAGCGGCTACATGGAGGGGGCTGTAGAGGCCGGGGAGAGAGCAGCCCGAGAGATC
CTGCATGCCATGGGGAAGATTCCAGAGGATGAAATCTGGCAGTCAGAACCAGAGTC
TGTGGATGTCCCTGCACAGCCCATCACCACCACCTTTTTGGAGAGACATTTGCCCTC
CGTGCCAGGCCTGCTCAGGCTGATTGGATTGACCACCATCTTTTCAGCAACGGCTCT
TGGCTTCCTGGCCCACAAAAGGGGGCTACTTGTGAGAGTCTAA

DDC CCDS SEQUENCE

ATGAACGCAAGTGAATTCCGAAGGAGAGGGAAGGAGATGGTGGATTACATGGCCA
ACTACATGGAAGGCATTGAGGGACGCCAGGTCTACCCTGACGTGGAGCCCGGGTAC
CTGCGGCCGCTGATCCCTGCCGCTGCCCCTCAGGAGCCAGACACGTTTGAGGACATC
ATCAACGACGTTGAGAAGATAATCATGCCTGGGGTGACGCACTGGCACAGCCCCTA
CTTCTTCGCCTACTTCCCCACTGCCAGCTCGTACCCGGCCATGCTTGCGGACATGCTG
TGCGGGGCCATTGGCTGCATCGGCTTCTCCTGGGCGGCAAGCCCAGCATGCACAGA
GCTGGAGACTGTGATGATGGACTGGCTCGGGAAGATGCTGGAACTACCAAAGGCAT
TTTTGAATGAGAAAGCTGGAGAAGGGGGAGGAGTGATCCAGGGAAGTGCCAGTGA
AGCCACCCTGGTGGCCCTGCTGGCCGCTCGGACCAAAGTGATCCATCGGCTGCAGG
CAGCGTCCCCAGAGCTCACACAGGCCGCTATCATGGAGAAGCTGGTGGCTTACTCAT
CCGATCAGGCACACTCCTCAGTGGAAAGAGCTGGGTTAATTGGTGGAGTGAAATTA
AAAGCCATCCCCTCAGATGGCAACTTCGCCATGCGTGCGTCTGCCCTGCAGGAAGCC
CTGGAGAGAGACAAAGCGGCTGGCCTGATTCCTTTCTTTATGGTTGCCACCCTGGGG
ACCACAACATGCTGCTCCTTTGACAATCTCTTAGAAGTCGGTCCTATCTGCAACAAG
GAAGACATATGGCTGCACGTTGATGCAGCCTACGCAGGCAGTGCATTCATCTGCCCT
GAGTTCCGGCACCTTCTGAATGGAGTGGAGTTTGCAGATTCATTCAACTTTAATCCC
CACAAATGGCTATTGGTGAATTTTGACTGTTCTGCCATGTGGGTGAAAAAGAGAACA
GACTTAACGGGAGCCTTTAGACTGGACCCCACTTACCTGAAGCACAGCCATCAGGA
TTCAGGGCTTATCACTGACTACCGGCATTGGCAGATACCACTGGGCAGAAGATTTCG
CTCTTTGAAAATGTGGTTTGTATTTAGGATGTATGGAGTCAAAGGACTGCAGGCTTA
TATCCGCAAGCATGTCCAGCTGTCCCATGAGTTTGAGTCACTGGTGCGCCAGGATCC
CCGCTTTGAAATCTGTGTGGAAGTCATTCTGGGGCTTGTCTGCTTTCGGCTAAAGGG
TTCCAACAAAGTGAATGAAGCTCTTCTGCAAAGAATAAACAGTGCCAAAAAAATCC
ACTTGGTTCCATGTCACCTCAGGGACAAGTTTGTCCTGCGCTTTGCCATCTGTTCTCG
CACGGTGGAATCTGCCCATGTGCAGCGGGCCTGGGAACACATCAAAGAGCTGGCGG
CCGACGTGCTGCGAGCAGAGAGGGAGTAG

HSP70 CCDS SEQUENCE

ATGTCGGTGGTGGGCATAGACCTGGGCTTCCAGAGCTGCTACGTCGCTGTGGCCCGC
GCCGGCGGCATCGAGACTATCGCTAATGAGTATAGCGACCGCTGCACGCCGGCTTG
CATTTCTTTTGGTCCTAAGAATCGTTCAATTGGAGCAGCAGCTAAAAGCCAGGTAAT
TTCTAATGCAAAGAACACAGTCCAAGGATTTAAAAGATTCCATGGCCGAGCATTCTC
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GRP78 CCDS SEQUENCE

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CCTCCCCCAACTGGTGAAGAGGATACAGCAGAAAAAGATGAGTTGTAG

Table 2: List of primers

Gene		Sequence	Length	Tm	GC%	Hairpin	Size
COMT	FP	CGACAAGAAAGGCAAGATCGTG	22	60.16	50	0	150bp
	RP	GGGTTGATCTCGATGGTGATGA	22	59.9	50	0	
	FP	GACACGCTTCTCTTGGAGGAAT	22	60.35	50	0	194bp
	RP	CCCTTGTAGATGGCCTTCTCC	21	59.86	57.14	0	
	FP	TCATCACCATCGAGATCAACCC	22	59.9	50	0	213bp
	RP	ATTCCTCCAAGAGAAGCGTGTC	22	60.35	50	0	
MAOB	FP	TACTTTGCAGGCACCGAGAC	20	60.32	55	0	204bp
	RP	CACGGAGGGCAAATGTCTCT	20	60.04	55	0	
	FP	CTCCCCTTGCAGAAGAGTGG	20	59.96	55	0	152bp
	RP	CCACAGGAACCAGAGAGCAG	21	60.07	52.38	0	
	FP	AAGCTCCAGTTGCCTACACG	20	60.32	55	0	215bp
	RP	CTGCTCCTCACACCAGTTCT	20	59.32	55	0	
DDC	FP	ATGGAAGGCATTGAGGGACG	20	60.11	55	0	176bp
	RP	TAGGCGAAGAAGTAGGGGCT	20	60.03	55	0	
	FP	CTTCGCCTACTTCCCCACTG	20	60.11	60	0	221bp
	RP	CACTGGCACTTCCCTGGATC	20	60.39	60	0	
	FP	CATGTCCAGCTGTCCCATGA	20	59.75	55	0	184bp
	RP	CCCTGAGGTGACATGGAACC	20	60.04	60	0	
HSP70	FP	CTTCCAGAGCTGCTACGTCG	20	60.52	60	0	196bp
	RP	TGCTCGGCCATGGAATCTTT	20	60.03	50	0	
	FP	CTCCAGCTGAAGAAGGGTCA	20	60.98	55	0	201bp
	RP	TGGAGCCATCAGACTGAGGA	20	59.82	50	0	
	FP	ACAAGCAGAGTTTGACCATGGA	22	60.16	55	0	214bp
	RP	GTGTCTGTACCCTGCTCAGC	20	60.39	60	0	
GRP78	FP	GACAAGAAGGAGGACGTGGG	20	60.04	56	0	173bp
	RP	GCATCGCCAATCAGACGTTC	20	59.97	55	0	
	FP	CCCGTCCAGAAAGTGTTGGA	20	59.89	55	0	151bp
	RP	CTGGGTTTATGCCACGGGAT	20	60.11	55	0	
	FP	GCGGAACCTTCGATGTGTCT	20	60.39	55	0	158bp
	RP	CCTGACATCTTTGCCCGTCT	20	60.04	55	0	

PRIMERS RECONSTITUTION

For 100µM primers

UV cross linked nuclease free water was added to the primers as mentioned by the company to make 100µM stock. After adding nuclease free water, primers were vortexed for 15 minutes followed by short spin. An aliquot of 60µl of each primer was made. The primers were stored at -80°C.

For 10µM primers

100µM stock primer	= 10µl
NFW	= 90µl
Total	-=100µl

For 0.5µM Primers (Primer Pair)

Forward Primer (10µM)	= 2.5µl
Reverse Primer (10µM)	= 2.5µl
NFW	= 45µl
Total	= 50µl

Primers of 10µM and 0.5 µM were made for PCR and Real Time PCR respectively. Filtered tips were used in reconstituting the primers. All the pipettes, eppendorfs, tips, stands were UV cross-linked.

Checking the primers through PCR

All the primers were checked on conventional PCR. After running PCR, product amplification was checked through agarose gel electrophoresis. The constituents for one PCR reaction of 25 µl is as follows:

PCR Master Mix	=12.5µl
Forward Primer (FP)	=1µl
Reverse Primer (RP)	=1µl
cDNA	=1µl
Nuclease free water	=9.5µl
Total	=25µl

Checking primers through real time PCR

All the three sets of primers for each gene were checked through real time PCR. We selected the primer set which gave optimum Ct value and showed specific amplification in dissociation curve at 60°C.

RNA isolation using RNAzol

2ml blood collected in an EDTA vacutainer and was diluted with 2ml of PBS buffer and mixed by inverting the tube. Diluted blood was then gently overlaid onto a falcon (15 ml) containing Histopaque and centrifuged at 1200 rpm for 35 minutes at room temperature. The buffy coat/PBMC were separated and transferred to another falcon. Three volumes of PBS was added to the isolated PBMCs and mixed by tilting. Centrifugation was done at 2000 rpm for 10 minutes at room temperature. The supernatant was discarded; the pellet was re-suspended in 1 ml PBS (1x) and transferred into an eppendorf followed by centrifugation at 1500 rpm for 10 minutes at room temperature. The supernatant was removed completely and 1ml RNAzol was added to the pellet and rigorous pipetting was done to completely mix the pellet. Vortexing was done for 2minutes after adding 400 µl of nuclease free water. The eppendorf was then incubated at room temperature for 15 minutes. Centrifugation was done thereafter at 4°C at 12000 g for 15 minutes. The supernatant (600 µl) was transferred into 2 eppendorfs and equal amount of isopropanol was added and mixed with pipetting. Incubation was done for 15 minutes at room temperature and centrifuged at 12000 g for 10 minutes at room temperature. The supernatant was discarded from both the eppendorfs and 800 µl of 75% of ethanol was added to first eppendorf and the pellet along with the ethanol was transferred to the second eppendorf and centrifuged at 7500 g for 3 minutes at room temperature. Second ethanol (75%) wash was given after discarding the supernatant followed by centrifugation at 7500 g for 3 minutes at room temperature. Ethanol was removed and the pellet was air dried till it became transparent. Elution was done in 30 µl of nucleus free water. The RNA was then stored at -80°C till further use.

Quantification and checking the quality of RNA on agarose gel

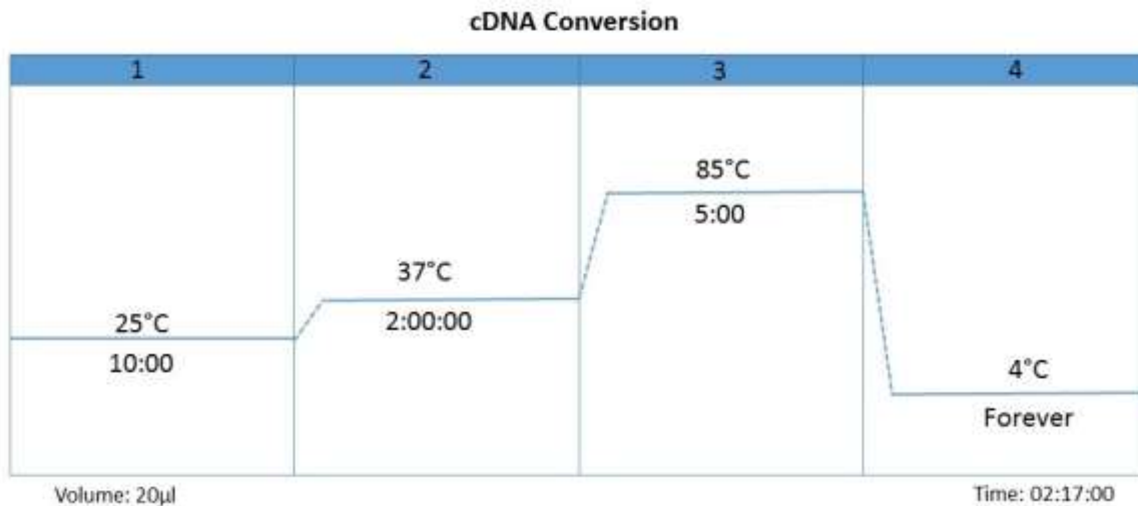
Gel electrophoresis was done to check the quality of RNA and ND1000 Nano Drop spectrophotometer was used to quantify the RNA concentration isolated from blood.

RNA to cDNA conversion

RNA to cDNA conversion was done using High Capacity cDNA conversion kit from applied biosystems and 2 reactions of 20µl each of 1.5microgram cDNA was made. PCR cycle used for cDNA conversion is shown below. The constituents for one reaction is as follows:

RT Buffer	=2µl
Random Primers	=2µl
dNTPs	=0.8µl
RT Enzyme	=1µl
RNA	=1.5µg
Nuclease free water	=Remaining
Total	=20µl

Figure 2: PCR cycle of RNA to cDNA conversion

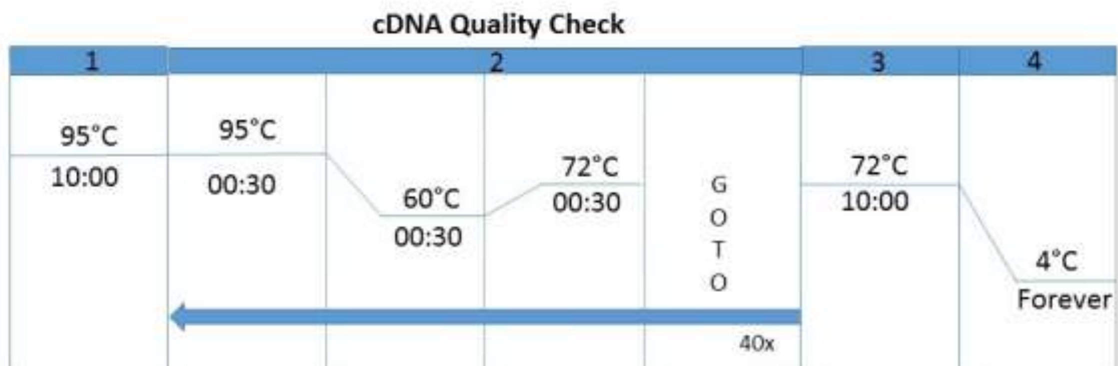


cDNA Quality Check

The quality of cDNA synthesized from cDNA conversion kit was checked through PCR using ACTB primers. The constituents for one reactions is as follows:

PCR Master Mix	= 12.5µl
Forward Primer (10µM)	= 1µl
Reverse Primer (10µM)	= 1µl
cDNA	= 1µl
NFW	= 9.5 µl
Total	= 25µl

Figure 3: PCR cycle for cDNA quality check



Real Time expression analysis of target genes

Real time expression was obtained by using Stratagene MX3005P real time PCR machine. SYBR Green dissociation curve was used to check real time expression. SYBR Green is an intercalating dye which binds to the minor grooves of any double stranded DNA. As SYBR Green binds to any amplified product, specific and nonspecific, only amplification plots will not assess specificity.

Dissociation curve were analysed to check whether SYBR Green has bind to specific or nonspecific product. ROX dye was selected as reference dye. Amplification plots and dissociation curves were observed from the experiment. The constituents for one reactions is as follows:

SYBR Green	= 5μl
Primer Pair (0.5μl)	= 1μl
cDNA	= 1μl
Nuclease free water	= 3μl
Total	= 10μl

Figure 4: Real Time PCR cycle

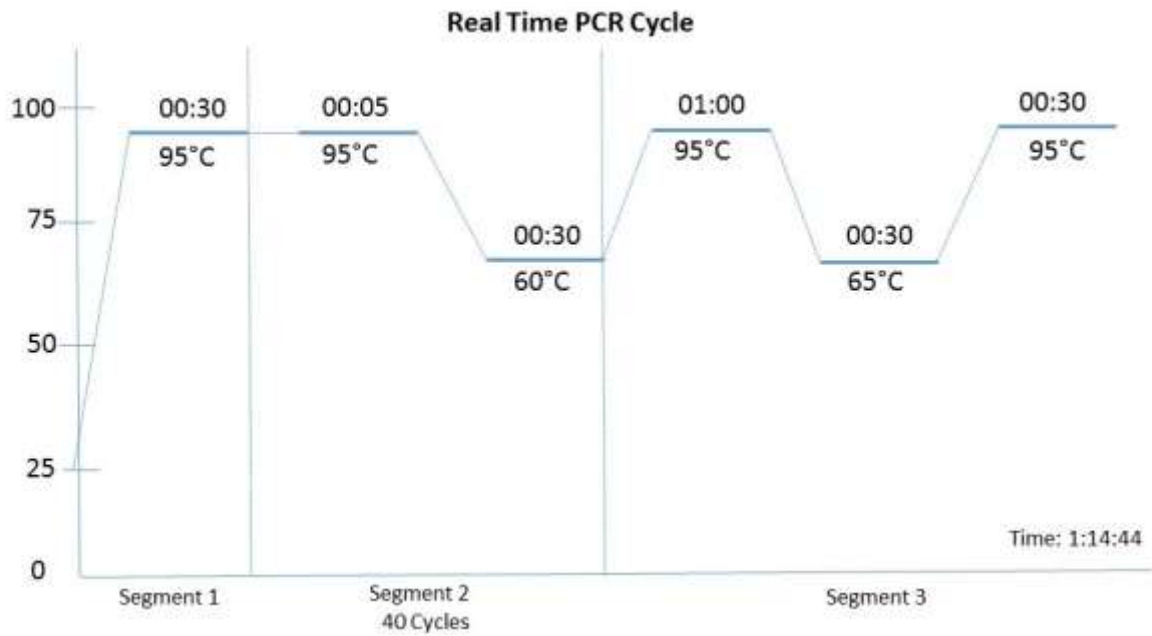
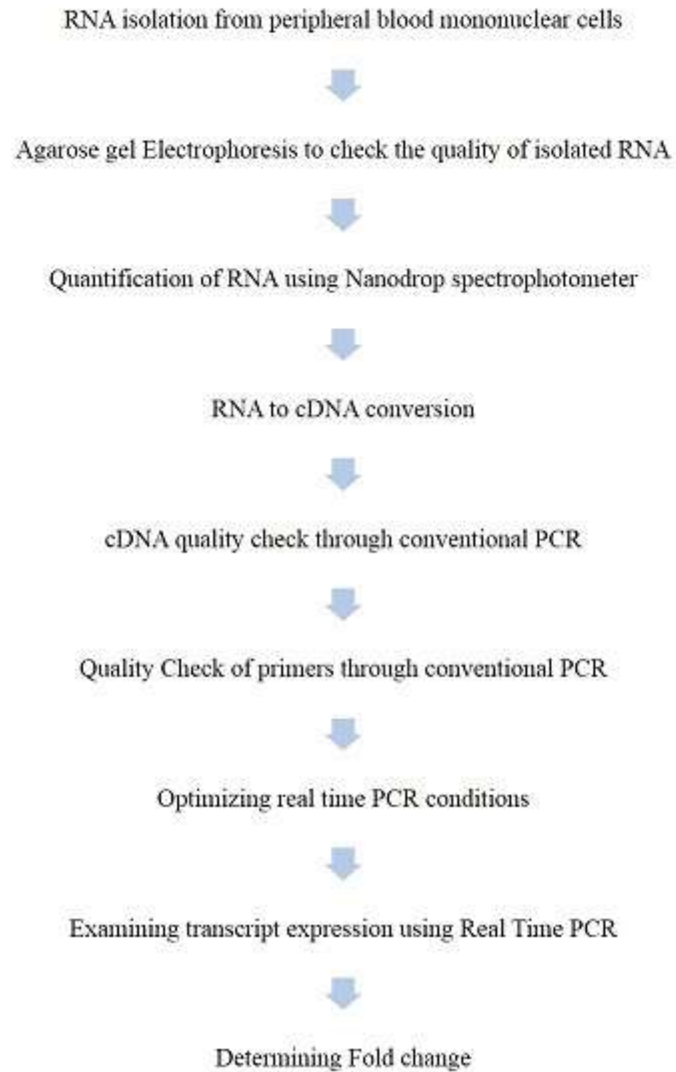


Figure 5: Workflow

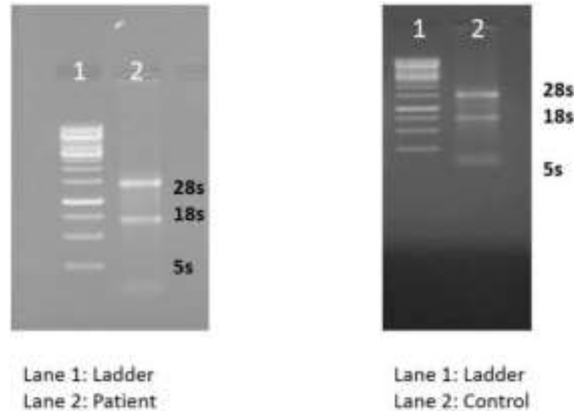


5. RESULTS

RNA extraction from peripheral blood mononuclear cells

Good quality RNA was extracted from peripheral blood mononuclear cells of six Parkinson's disease patients and age matched healthy controls. RNA was eluted in 25µl nuclease free water. RNA integrity was visualized through non denaturing agarose gel electrophoresis. As it can be seen from the pictures below 28s, 18s and 5s bands are clearly visible which shows that RNA is intact.

Figure 6: RNA bands of patient and control



RNA quantification was done using ND1000 Nanodrop spectrophotometer. RNA concentration varied from 250ng/µl to 300ng/µl. $A_{260/280}$ and $A_{260/230}$ ratio were good which ensured good quality RNA was extracted and its free from contamination.

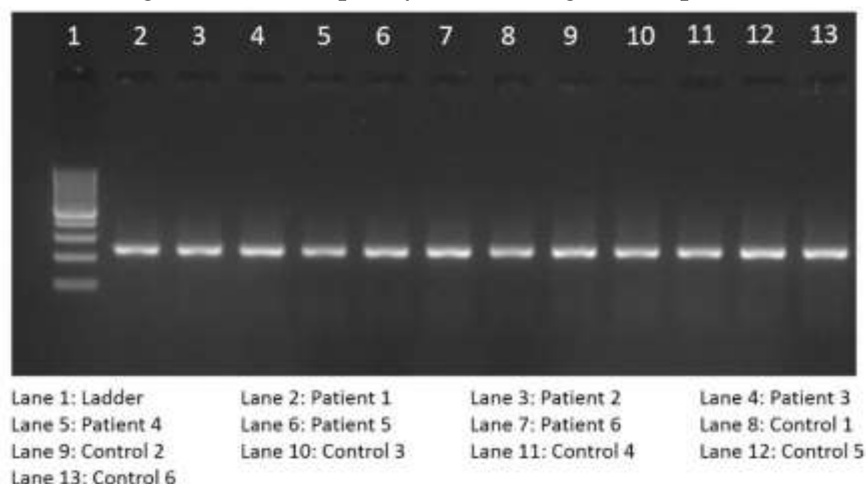
RNA to cDNA conversion

RNA was converted to cDNA by using high capacity reverse transcriptase kit from applied biosystems. Two 20µl reactions were made which contained 1500ng of RNA each.

cDNA quality check

cDNA synthesized from RNA was checked for its quality on conventional PCR. cDNA of six PD patients and six healthy controls were checked used ACTB primers. PCR products were visualized on agarose gel electrophoresis. This quality check was done to assure that cDNA has been synthesized and it is good to use in real time PCR. The image below shows that all the products were equally amplified and cDNA can be used in real time PCR.

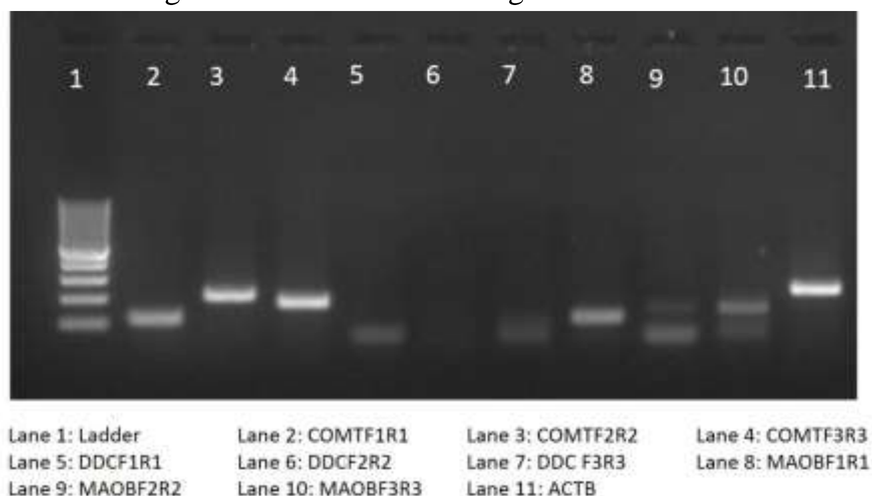
Figure 7: cDNA quality check using ACTB primers



Primer check using conventional PCR

All the sets of primer were checked using conventional PCR. The product were visualized through agarose gel electrophoresis with a 100bp ladder. All primers of COMT gave good amplification whereas no amplification was observed for DDC gene. MAOB F1R1 showed significant amplification as compared to other two sets of MAOB.

Figure 8: Primer check through conventional PCR



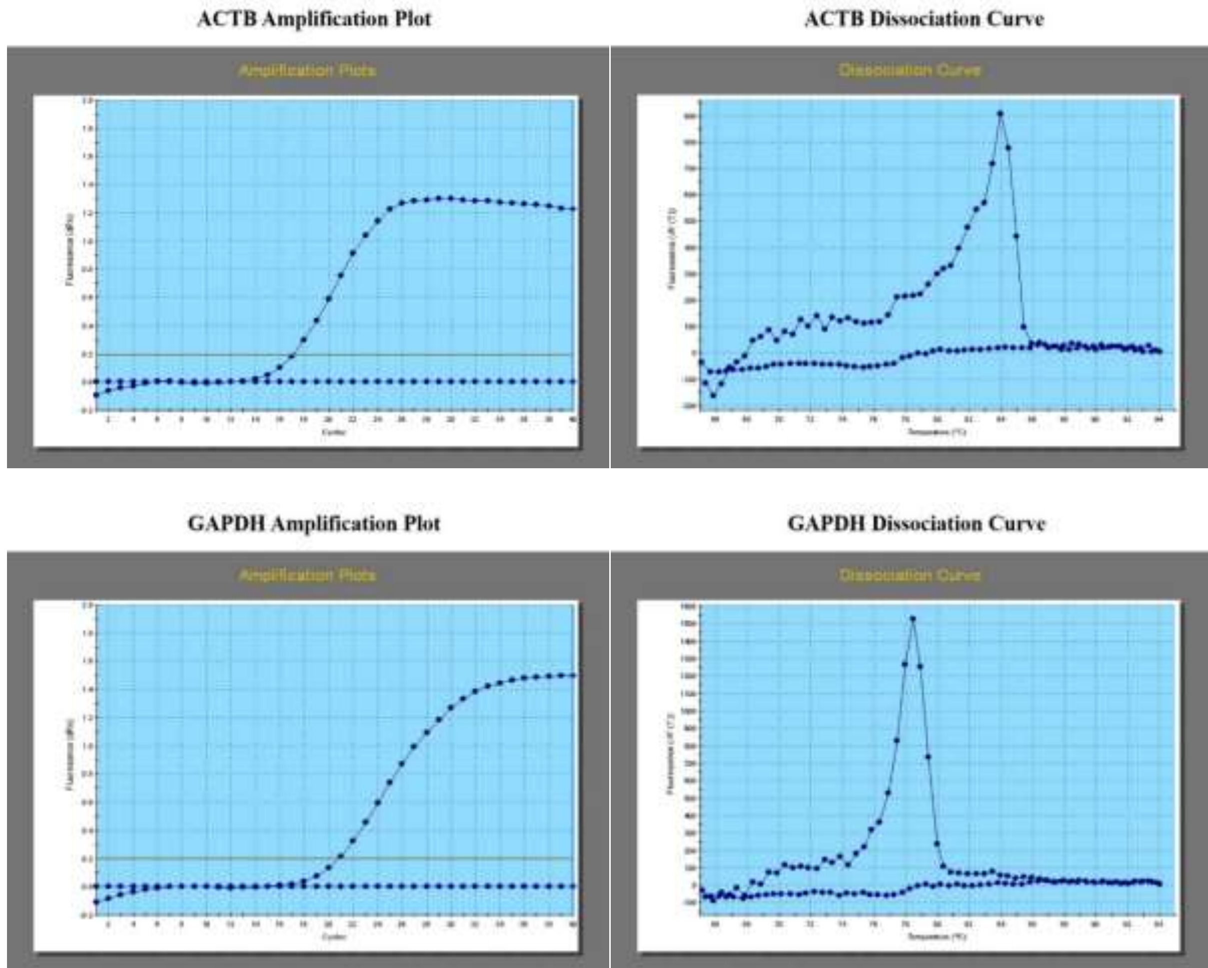
Primers check using real time PCR

After checking primers on conventional PCR, all primers were checked on real time PCR. Real time PCR results showed that COMT F2R2 gave the optimum Ct at 60°C whereas no Ct was observed in DDC primers. MAOB F1R1 showed the optimum Ct. Dissociation curve of COMT F2R2 and MAOB F1R1 showed single peak indicating specific amplification. Two peaks were observed in MAOB F2R2 and MAOB F3R3 which meant two products were amplified i.e. nonspecific amplification. Similarly HSP70 F1R1 and GRP78 F3R3 showed specific amplification and optimum Ct. COMT F2R2, MAOB F1R1, HSP70 F1R1 and GRP78 F3R3 were chosen for the study.

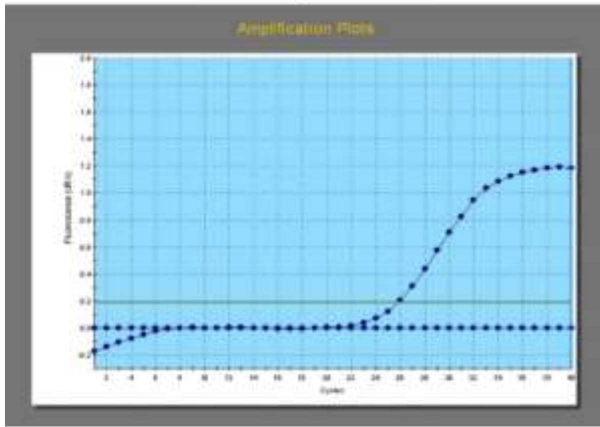
Optimization of Real time PCR

SYBR Green dissociation curve was used to check the real time expression. The plate was setup for two PD patients and two healthy age matched controls for six genes. ACTB and GAPDH were used as internal controls. ACTB and GAPDH were in triplicates whereas target genes were in duplicates. Tight Ct was observed in all the replicates. A no template control (NTC) was used for each gene. No Ct was observed in NTCs. A representative amplification plot and dissociation curve is shown below for each of the genes to demonstrate the specificity.

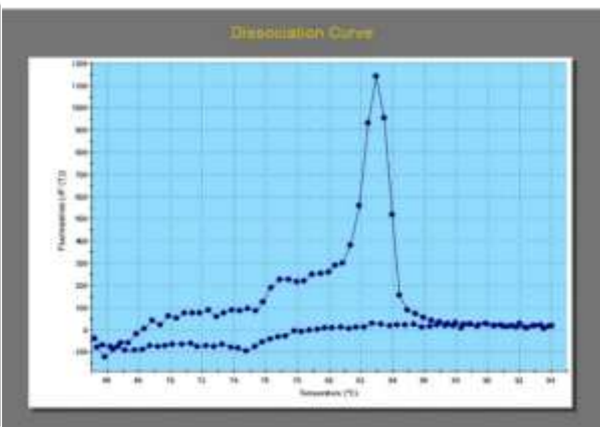
Figure 9: Amplification plots and dissociation curves of study genes



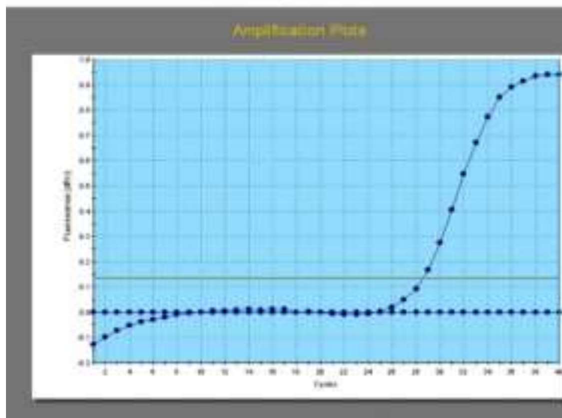
COMT Amplification Plot



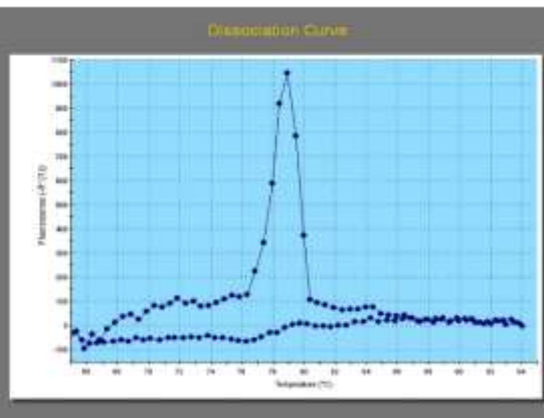
COMT Dissociation Curve



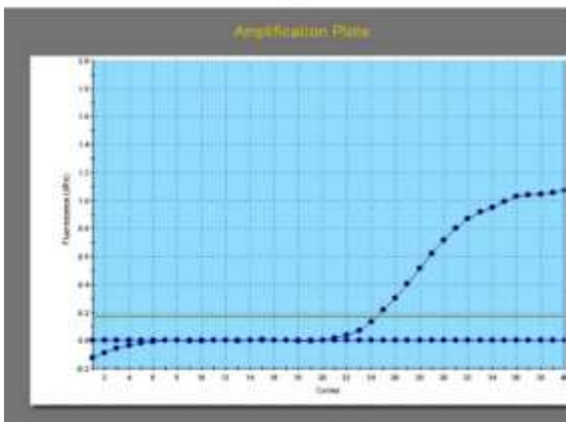
MAOB Amplification Plot



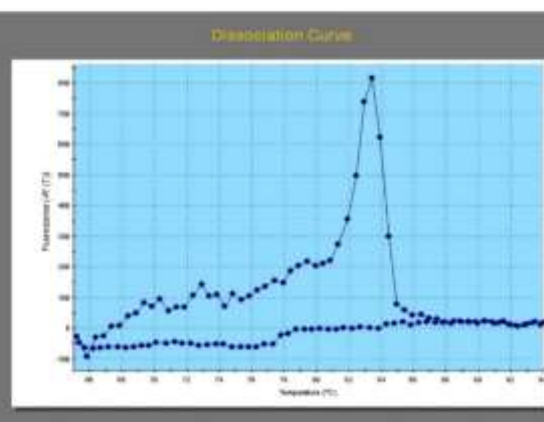
MAOB Dissociation Curve



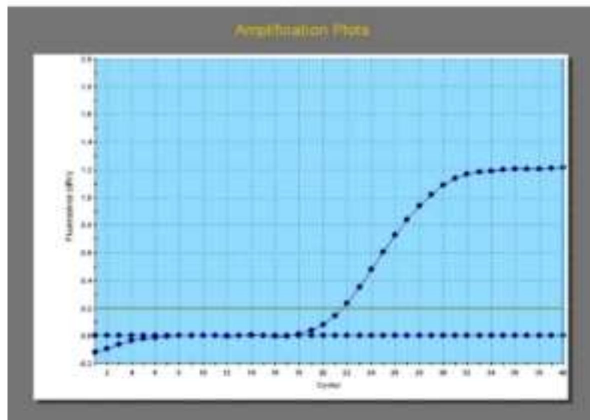
HSP70 Amplification Plot

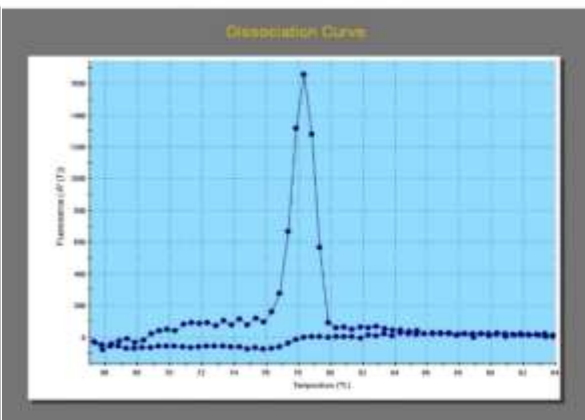


HSP 70 Dissociation Curve



GRP78 Amplification Plot **GRP 78 Dissociation Curve**





Calculation of fold change with an example

There are two methods of calculating fold change. In method I, we subtracted controls average individual fold change value from patients individual fold change average value for a specific gene. In method II, we subtracted average controls average individual fold change value from individual patient's fold change value. Both the methods are explained below:

Method I

Table 3: Individual fold change of two PD patients and controls

Well	Well Name	Ct (dRn)	Average	Delta Ct	Individual fold change
A1	ACTB PD1	16.88			
A2	ACTB PD1	16.63			
A3	ACTB PD1	16.38	16.63		
A7	COMT PD1	25.96			
A8	COMT PD1	25.44	25.7	9.07	0.001860621
C1	ACTB PD2	16.03			
C2	ACTB PD2	16.83			
C3	ACTB PD2	16.38	16.41333333		
C7	COMT PD2	26.96			
C8	COMT PD2	26.86	26.91	10.49666667	0.000692131
E1	ACTB C1	17.24			
E2	ACTB C1	17.04			
E3	ACTB C1	16.65	16.97666667		
E7	COMT C1	26.1			
E8	COMT C1	26.15	26.125	9.148333333	0.00176229
G1	ACTB C2	16.9			
G2	ACTB C2	16.74			
G3	ACTB C2	16.28	16.64		
G7	COMT C2	26.77			
G8	COMT C2	26.68	26.725	10.085	0.000920688

Average Ct value was calculated for every replicate as shown in the table above. Formulas used are explained below:

Delta Ct = Ct (target) – Ct (endogenous)

Individual fold change = $2^{-\text{delta Ct}}$

$$\text{Fold change} = \frac{\text{Individual Patient Individual fold change}}{\text{Controls Individual fold change average}}$$

Table 4: Patient and Control's average individual fold change

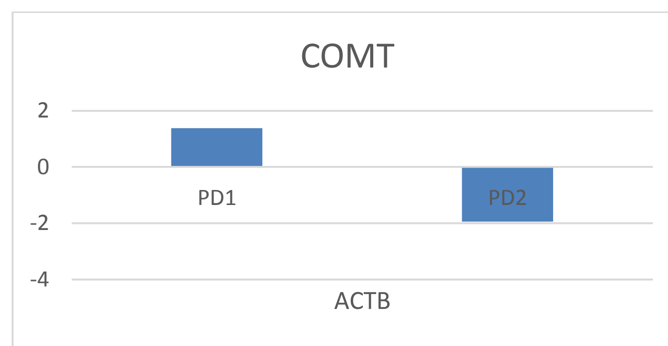
	Patient individual fold change		Control Individual fold change		
	PD1	PD2	C1	C2	Control Average
COMT	0.001860621	0.000692131	0.00176229	0.000920688	0.001341489

	PD1	Control Average	Fold Change	
COMT	0.001860621	0.001341489	1.386982236	
	PD2	**Control Average**	**Fold Change**	**Less than 1**
COMT	0.000692131	0.001341489	0.51594266	-1.938199877

Upregulation is shown in +Y axis and downregulation is shown in –Y axis. Fold change values which are less than 1 shows downregulation. In order to show down regulation in –Y axis, we use the formula:

Down Regulation = -1/value less than 1

Figure 10: Fold change of PD1 and PD2 with Control average (Method I)



Method 2

Table 5: Calculating patient and control individual fold change average

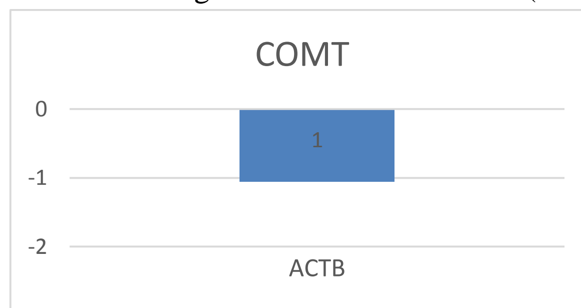
	PD1	PD2	Patient Average	C1	C2	Control Average
COMT	0.001860621	0.000692131	0.001276376	0.00176229	0.000920688	0.001341489

$$\text{Fold change} = \frac{\text{Patients Individual fold change average}}{\text{Controls Individual fold change average}}$$

Table 6: Method 2 for calculating fold change

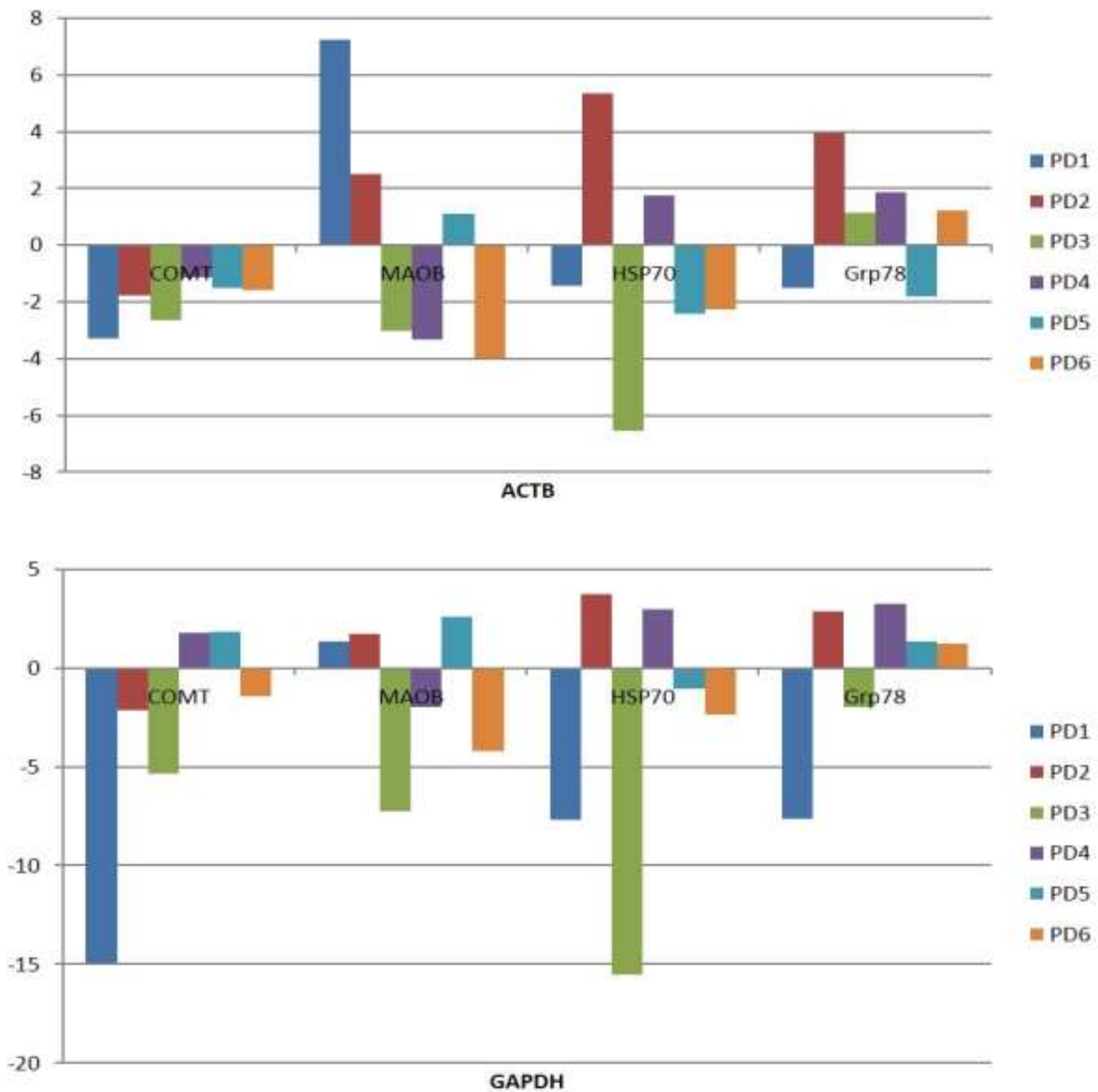
	Patient Average	Control Average	Fold Change	Less than 1
COMT	0.001276376	0.001341489	0.951462448	-1.051013629

Figure 11: Fold change of COMT with ACTB (Method 2)



Real time PCR results

Figure 12: Fold change of study genes with ACTB and GAPDH (method II)



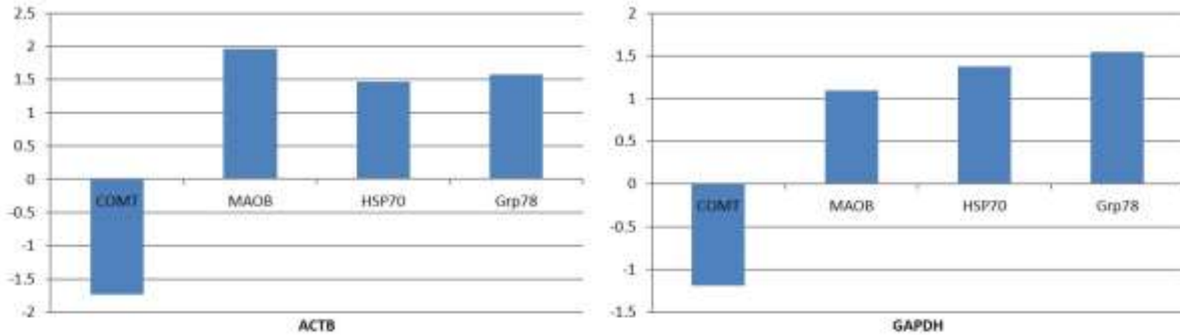
The transcript levels of all the genes were analyzed by normalizing separately with ACTB and GAPDH as endogenous controls.

On normalizing with ACTB, all six patients showed down regulation of COMT. Levels in PD1 and PD3 are more than 2 fold downregulated. PD2, PD5 and PD6 showed more than 1.5 fold down regulation. In MAOB PD3, PD4, PD6 shows downregulation of 3 folds whereas PD1 and PD2 showed upregulation. No fold change was observed in PD5.
Levels of Hsp70 were down regulated in 4 PD patients (PD1, PD3, PD5, PD6). PD2 showed 5 fold upregulation and PD4 showed no change. In Grp78 2 PD pateints showed downregulation (PD1, PD5), 2 PD pateints showed upregulation (PD2, PD4) and 2 PD pateints (PD3, PD6) showed no change in their expression levels.

On normalizing with GAPDH, 4 patients (PD1, PD2, PD3, PD6) showed downregulation of COMT whereas no change of COMT was ovserved in PD4 and PD5. In MAOB PD3, PD4 and PD6 showed downregulation whereas PD5 showed upregulation. PD1 and PD2 showed no significant change in the expression level. In molecular chaperone Hsp70, 4 PD pateints (PD1, PD3, PD5, PD6) showed downregulation. Out of these 4 patients, PD1 and PD3 showed more than 7 fold change. Mixed resulted were seen in Grp78. PD2 and PD4 showed upregulation, PD1 and PD3 showed downregulation and PD5 and PD6 showed no change.

The average transcript levels for all six patients:

Figure 13: Fold change of study genes with ACTB and GAPDH (method 2)



On averaging the levels of the patients as described above, COMT levels are down regulated with both ACTB and GAPDH as the endogenous control. However, MAOB, HSP70 and Grp78 show upregulation with ACTB as well as GAPDH.

Table 7: Patient details

	Age	Gender	Age of onset	Family history	UPDRS score*
PD1	48	Female	ND	ND	ND
PD2	51	Male	3 years	Not genetic	ND
PD3	71	Male	6 moths	Not genetic	14
PD4	63	Male	8 years	Not genetic	14
PD5	58	Male	4 years	Not genetic	51
PD6	56	Male	6 months	Not genetic	24

*Part III of the UPDRS was used to assess to severity of patients

6. DISCUSSION AND FUTURE PERSPECTIVE

COMT and MAOB are dopamine metabolizing enzymes which convert dopamine/levodopa into 3-OMD, 3MT and DOPAC. Downregulation of these enzymes indicate increased bioavailability of levodopa in the system. However the downregulation COMT and MAOB in our patient set could be probably due to the inhibitors of these enzymes given along with levodopa therapy given throughout the treatment. MAOB inhibitors are mostly given as they are thought to prevent progression of disease and COMT inhibitors are given late in the disease to decrease dose of levodopa as side due to increased side effects. It is important to include drug naïve patients in the study and follow them up.

This is an ongoing study and as the sample size increases, one can correlate the levels of these enzymes with the response to levodopa treatment. In addition, it will be interesting to correlate the levels with other demographic features and clinical manifestation.

The discrepancy in fold regulation between individual patients (down regulation compared to controls) and the average fold change (up regulation) for MAO is due to very small sample size at present.

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