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Correlation of Serum TNF -ALPHA & Interferon - Gamma Level with H & Y Scale of Parkinson's Disease

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ABSTRACT

Aim: The aim of the present study was to examine the relationship of Serum TNF-α and Interferon- γ level in Parkinson disease and Disease severity.

Methods: This research was carried out among patients presented to Department of General Medicine, Vinayaka Mission's Medical College, Karaikal over an 18-month period. A total of 44 individuals were part of the study.

Results: Majority participants fall in the age group of 71-80. The age distribution range was 57-88, while the mean age of the participants was 73.16±8.80 years. The male: female ratio is approximately 2:1 in this study. Majority of participants belonged to moderate disability (65.9%) and minimum number of cases were in server or advanced disability (11.4%). Majority of participants belonged to grade 3(38.6%), which least number of participants belonged to grade 5(6.8%). 34 cases had hypertension, which is 77.3 % of the cases. 31 cases had DM-T2 which is 70.4 % of the cases.

Conclusion: We concluded in our study that gene expression of INF-γ and TNF-α were higher in patient with Severe form of PD. But the P-Value of these inflammatory markers were not statistically significant. A multi-centric study cross- sectional study should be done for further study.

Keywords: Serum TNF-α, Interferon- γ level, Parkinson disease, Disease severity

1. INTRODUCTION

Alzheimer's disease is the most prevalent followed by Parkinson's disease (PD).is the second most, prevalent neurodegenerative age-related disorder in this modern era.[1] PD was first described by James Parkinson.an English general physician by examining cardinal features based on aphysical examination of few handful of patients, in the year 1817.[1] Parkinson's disease (PD) is clinically diagnosed by resting tremors, tone abnormality (rigidity/stiffness), bradykinesia, and gait, Dysfunction with postural instability. Unfortunately, current treatments are still ineffective in alleviating dopaminergic neuron dysfunction, since the pathogenesis of PD remains unclear.[2] In recent years, there has been increasing evidence that neuroimmunity plays a vital role in the development of PD, and immune cell dysfunction, in particular, has received widespread attention.[3] Given the considerable bidirectional crosstalk between peripheral inflammation and neuroimmunity[4], the measurement of immune markers in peripheral blood may be an easier and less invasive way to monitor immune responses in PD.

A cluster of α-synuclein-specific T cells, which are activated by specific antigenic epitopes and release interferon (IFN)-γ, interleukin (IL)-4, and IL-10, are present prior to clinical diagnosis of PD.[5,6] Furthermore, naïve CD4+ T cells in patients with PD preferentially differentiate into Th1 cells in peripheral blood and secrete large amounts of IFN-γ and tumor necrosis factor (TNF)-α.[7,8] In vitro, experiments have shown that phosphorylated α-synuclein epitopes stimulate CD4+ T cells to upregulate IL-17A expression9. In addition, chemokines have been found to play a central role in the regulation of neuroinflammatory responses, mediating immune cell migration through the blood–brain barrier, which leads to inflammatory neural damage.[10] Cerebrospinal fluid (CSF) of patients with Lewy body dementia exhibits upregulated

expression of C-X-C motif chemokine receptor 4 (CXCR4), as well as the CXCR4 ligand, C-X-C motif chemokine ligand 12 (CXCL12), in CD4+ T cells, which are associated with neuroaxonal damage.[9] The role of various immune cells in PD is increasingly recognized, and screening for these cells and elucidating their function will contribute to our understanding of PD-specific immune status. Most studies have focused on CD4+ T cells, whereas the functions of CD8+ T cells and other immune cells, such as NK cells, require further elucidation.

The aim of the present study was to examine the relationship of Serum TNF-α and Interferon- γ level in Parkinson disease and Disease severity.

2. MATERIALS AND METHODS

This research was carried out among patients presented to Department of General Medicine, Vinayaka Mission's Medical College, Karaikal over an 18-month period. A total of 44 individuals were part of the study.

Criteria for Inclusion:

Participants met the following criteria for inclusion:

- All patients with Parkinson's disease more than 18y/o.
- Patient willing to give consent

Criteria for Exclusion:

Participants were excluded if they met the following criteria:

- Age less than 18 years of age.
- Chronic inflammatory disease/ autoimmune diseases.
- Cerebro Vascular Accident.
- Malignancies.
- Alzheimer's disease
- Critically ill patients
- Patients on steroid therapy.
- Patients on immunosuppressive drugs/ Monoclonal antibody/Biological therapy.
- Pregnancy.
- Secondary Parkinson's disease/Wilsons Disease.
- Patients on Antipsychotic drugs.

1. Approval and Consent:

- Approval was obtained from the institute's ethics committee.
- All contestants provided informed consent.

2. Patient Background and Examination:

- A detailed medical history and comprehensive physical examinations were conducted for each participant.

3. Collection of Samples:

- Each participant provided 10 ml of blood.
- 3 ml of the sample was collected without anticoagulant for serum and biochemical analyses.
- The remaining 7 ml was used for Tumour necrosis alpha and Interferon gamma.

4. Isolation of RNA and Synthesis of cDNA:

Isolation of RNA and cDNA Synthesis

RNA Extraction:

The RNA extraction process began with the setup of RNase-free materials and tools to maintain RNA integrity. Key reagents included TRIzol, chloroform, isopropanol, ethanol, and RNase-free water. Equipment used consisted of a centrifuge, pipettes with RNase-free tips, and sterile microcentrifuge tubes.

The initial step in RNA isolation involved lysing the serum with lysing buffer. The cells were lysed in TRIzol reagent by pipetting until complete cellular lysis was achieved.

Phase separation was carried out by adding chloroform, followed by centrifuging the mixture to obtain distinct phases. The upper aqueous phase containing RNA was then isolated for further analysis. For RNA precipitation, 500 µl of isopropanol was mixed with the solution by slowly inverting the tube. The mixture was then left to incubate at room temperature for 15 minutes before being centrifuged at 12,000 x g for 10 minutes at 4°C, causing the RNA to form a pellet at the lowermost of the tube. This RNA pellet underwent a purification step with 1 ml of 70% ethanol, which was followed by vertexing and centrifugation at 7,500 x g for 5 minutes at 4°C. Once the supernatant was taken out, the RNA pellet was air-dried for 8-12 minutes to the appropriate moisture level.

Subsequently, the dried RNA pellet was liquified in 25-45 µl of RNase-free water through gentle pipetting. The quality and quantity of RNA were determined using a spectrophotometer or Nanodrop device to calculate the A260/A280 ratio. The secluded RNA was then kept at -80°C for future analysis.

Initiating cDNA Synthesis:

The cDNA synthesis process began with thawing the RNA samples on ice and centrifuging them briefly to collect any remaining material at the tube's lower part. The RNA's integrity was confirmed by agarose gel electrophoresis or a bioanalyzer to ensure it was suitable for reverse transcription. For cDNA synthesis, a standard reverse transcription kit like the High-Capacity cDNA Reverse Transcription Kit was used. The reaction mixture consisted of 2 µg of total RNA, along with specific capacities of RT buffer, dNTP mix, RT random primers, MultiScribe™ Reverse Transcriptase, and nuclease-free water, ensuring a total volume of 20 µl was achieved. This optimized combination facilitated precise cDNA synthesis. The reaction mixture was gently mixed, centrifuged briefly, and then placed in a thermal cycler to begin the reverse transcription program. This involved steps at 24°C for 12 minutes, 36°C for 120 minutes, and 85°C for 6 minutes to deactivate the enzyme. The cDNA was then kept at either -20°C or -80°C for future use. Validation and quality control of the cDNA involved assessing its concentration and purity using a spectrophotometer or Nanodrop device. In addition, the cDNA was confirmed through PCR using housekeeping gene primers to ensure successful reverse transcription and high-quality cDNA for subsequent real-time PCR and gene expression analysis. This thorough process guaranteed the production of dependable and top-notch cDNA for the research.

Real-Time PCR Setup:

Preparation and Arrangement

The real-time PCR setup consisted of several crucial steps to accurately quantify mRNA levels of TNF-α, and IFN γ. This process included designing primers, setting up reactions, amplification, and analysing data.

Primer Designing

Unique primers were designed for the target genes (TNF-α, and IFN γ) and a reference gene (e.g., GAPDH) to normalize the data. The primers were designed with specific characteristics:

Length: 18-24 nucleotides

GC Content: 50-60%

Melting Temperature (Tm): 56-60°C

Amplicon Size: 100-200 base pairs Here are the primer sequences:

Setting Up Reactions

The qRT-PCR reactions were arranged in a 96-well plate for efficient highthroughput analysis. The total volume for each reaction was 20 µl. All reactions were done in triplicates to ensure consistency and precision.

PCR Amplification Process:

The qRT-PCR was carried out using a real-time PCR machine (e.g., Applied Biosystems 7500 Real-Time PCR System) with the following thermal cycling conditions:

Initial Denaturation:

95°C for 10 minutes

4.8.2 Cycling (40 cycles):

Denaturation: 95°C for 15 seconds

Annealing: 60°C for 30 seconds

Extension: 72°C for 30 seconds

Melt Curve Analysis (to confirm amplification specificity):

95°C for 15 seconds

60°C for 1 minute

Gradual increase to 95°C with continuous fluorescence monitoring

Data Analysis:

After amplification, the threshold cycle (Ct) values were determined for each reaction. The Ct value represents the cycle number where the fluorescence signal surpasses the background threshold, indicating amplified product presence.

The levels of target gene expression were determined using the 2^(-ΔΔCt) method, where:

ΔCt = Ct value of the target gene minus Ct value of the housekeeping gene.

ΔΔCt = ΔCt value of the sample minus ΔCt value of the control.

The change in expression of TNF-α, and IFN γ in Parkinson's patients were measured. This comprehensive real-time PCR amplification method ensured precise and dependable quantification of mRNA expression levels of TNF-α, and IFN γ, aiding in the examination of their connection to Parkinson's Disease.

Data Analysis:

- Analysed the gene expression of TNF-α, andIFN γin Parkinson's cases.
- Conducted statistical analysis to pinpoint significant variations in gene expression levels between different severity or stages of Parkinson's Disease.

3. RESULTS

Table 1: Baseline characteristics

Age group	**No.**	**%**
Up to 60 years	5	11.4
61-70 years	13	29.5
71-80 years	15	34.1
81-90 years	11	25.0
Gender		
Female	15	34.1
Male	29	65.9
WRS score		
Early illness	10	22.7
Moderate	29	65.9
Severe or advanced	5	11.4
H & Y scale grading		
2	12	27.3

3	17	38.6
4	12	27.3
5	3	6.8
Comorbidity		
Hypertension	34	77.3
DM-T2	31	70.4

Out of these participants 5 fall in the age category of up to 60 years, which is 11.4 % of the total participants. 13 participants fall in the category of 61-70 years, which is 29.5 % of the total participants. 15 participants fall in the category of 71-80 years, which is 34.1 % of the total participants. And 11 participants fall in the category of 81-90 years, which is 25 % of the total participants. 15 participants were female, which is 34.1 % of the total participants. The remaining 29 participants were Male, which is 65.9 % of the participants. The severity of Parkinson's disease as per Webster rating scale which is interpretated as Early illness (score: 1-10), moderate disability (score: 11-20) and Severe or advanced disability(score:21-30). Out of the 44 Participants 10 belong to early illness, which is 22.7% of the total participants. 29 participants belong to moderate disability, which is 65.9 % of the participants. 5 participants belong to severe or advanced disability, which is 11.4 % of participants. Out of 44 cases 12 belonged to grade 2, which is 27.3% of the total participants. 17 belonged to grade 3, which is 38.6% of the total participants. 12 belonged to grade 4, which is 27.3 % of the total participants. 3 cases belonged to grade 5, which is 6.8 % of the total participants. 34 cases had hypertension, which is 77.3 % of the cases. 31 cases had DM-T2 which is 70.4 % of the cases.

Table 2: TNF- α and INF-γ level in study subjects

	INF-γ	**TNF- α**
Mean	**2.880**	**1.9240**
SD	3.786	1.8894
Median	1.44100	1.365506
IQR	0.44-3.93	0.38-2.85
Minimum	.005	.0145
Maximum	20.496	7.3855

The mean gene expression for INF-γ is 2.880 and 1.9240 for TNF-α. The median for INF-γ is 1.44100 with standard deviation of 3.786, while median for TNF-α is 1.365506 with standard deviation of 1.8894. The maximum gene expression of INF-γ is 20.496 while that of TNF- α is 7.3855. The minimum gene expression for INF-γ is 0.005 with interquartile range of 0.44-3.93. The minimum gene expression of INF-γ is 0.0145 with interquartile range of 0.38-2.85.

Table 3: Different parameters in study subjects

	Hb	MCV	MCH	MCHC	PLT
Mean	11.51	84.775	28.876	33.664	265.70
Std. Deviation	1.89	5.4648	2.3114	1.6893	106.984
Median	11.6	84.250	28.750	33.650	238.50
IQR	9.82-12.9	81.27-88.95	26.82-30.55	32.92-34.3	191.75-324.5

The mean haemoglobin is 11.51g/dl (11-15) with standard deviation of 1.89. The median for 44 participants is 11.6 with interquartile range of 9.82-12.9. The mean MCV for the 44 participants is 84.775fL (80-100) with standard deviation of 5.4648. The median MCV for 44 participants is 84.250 with interquartile range of 81.27-88.95. The mean MCH for the 44 participants is 28.876pg (27-34) with standard deviation of 2.3114. The median MCH for 44 participants is 28.750 with interquartile range of 26.8230.55. The mean MCHC for the 44 participants is 33.664g/dL (32-36) with standard deviation of 1.6893. The median MCV for 44 participants is 33.650 with interquartile range of 32.92-34.3. The mean Platelet count for the 44 participants is 265.70 10*3/Ul (100-400 10*3/uL) with standard deviation of 106.984. The median Platelet count for 44 participants is 238.50 with interquartile range of 191.75-324.5.

Table 4: Correlation of severity of parkinsonism with TNF- α and INF-γ level in study subjects

		INF-γ	TNF- α
WRS	r value	-0.01	-0.06
	p value	0.91	0.70
H & Y scale	r value	-0.04	0.01
	p value	0.77	0.91

The R- value of INF- γ in correlation with webster rating scale is 0.01, while P- value is 0.91. The R- value of TNF- α in correlation with webster rating scale is -0.06, while P- value is 0.70. Similarly, the R- value of INF- γ in correlation with Hoehn and Yahr scale of PD is -0.04, while P-value is 0.77. The R- value of TNF- α in correlation with Hoehn and Yahr scale is 0.01, while P- value is 0.91.

Table 5: Association of severity of parkinsonism using WRS with TNF- α and INF-γ level in study subjects

	Early illness	Moderate	Severe or advanced	p value
INF-γ	2.02 (0.72-7.80)	1.05 (0.32-3.92)	2.70 (1.64-4.47)	0.31
TNF- α	1.96 (0.44-3.46)	1.03 (0.27-2.48)	1.70 (1.28-2.73)	0.57

The gene expression of INF-γ in early illness is 2.02(0.72-7.80), while in moderate disability is 1.05(0.323.92) and in severe or advanced disability is 2.70(1.64-4.47). Maximum gene expression of INF-γ was seen severe or advanced disability stage. The gene expression of TNF- α in early illness was 1.96(0.44-3.46), while in moderate disability is 1.03(0.27-2.48) and in

severe or advanced disability is 1.70(1.28-2.73). Maximum TNF- α gene expression was seen in early illness. The P-value of INF-γ in correlation with WRS is 0.31. The P-value of TNF- α in correlation with WRS is 0.57.

Table 6: Association of severity of parkinsonism using H & Y scale with TNF- α and INF-γ level in study subjects

	Grade II	Grade III	Grade IV	Grade V	p value
INF-γ	2.02 (1.02-6.83)	1.01 (0.36-3.24)	1.44 (0.18-3.80)	2.70	0.48
TNF- α	1.48 (0.59-2.71)	1.46 (0.25-3.04)	1.12 (0.39-1.80)	1.95	0.65

The gene expression of INF-γ in Maximum in Grade V 2.70 followed by Grade II 2.02 while the value of INF-γ in Grade III and Grade IV is 1.01(0.36-3.24) and 1.44(0.18-3.80) respectively. The gene expression of TNF- α in Maximum in Grade V 1.95 followed by Grade II 1.48 while the value of TNF- α in Grade III and Grade IV is 1.46(0.25-3.04) and 1.12(0.39-1.80) respectively.

The R-value of Haemoglobin in correlation with WRS is -0.01, while with H&Y scale is -0.02. The P-value of haemoglobin in correlation with WRS is 0.95, while with H&Y scale is 0.85 which is statistically not significant.

4. DISCUSSION

The individuals living with Parkinson's disease in the world's largest population is estimated at 5,000,000, and this count is expected to double within next 20 years due to the increasing age of the population. The average age of onset is approximately sixth decade and the lifetime risk is approximately about 3% in men and 2% in women.The prevalence of PD differs throughout the world. The prevalence is much lower in continent of Asia and Africa when compared to Europe and America.[11] The annual incidence of PD in the United Kingdom is 18/100 000 and prevalence is 180/100 000.[12] The generalized incidence of PD in India is 70/100 000. However, 328/100 000 cases were identified among a Parsi community in Mumbai.[13]

A total of 44 participants took part in the study. The current study revealed that of the total 44 cases, 15 cases are female which is 34.1 % and 29 participants were male which is 65.9 % of the case. Majority of the participants belonged to age group of 71-80. Minimum number of participants belonged to age group up to 60 years. The age distribution range was 57-88, while the mean age of participants was 73.16±8.80 years. In the study by El-Kattan MM et al[14] included 30 patients in which 25(83.3%) were male and 5(16.7%) females. The age distribution ranged from 49 to 73 years with mean age of 55±7 years. A study by Kouchaki E et al[15], included 83 cases in which 52 male and 31 female. The mean age of cases was 65.73 with standard deviation of ±11.20. In our study of 44 cases, we found that 34 cases, which is 77.3% of the cases had hypertension. Thus, we can conclude that co-morbidities like Hypertension and T2DM are important risk factors for PD and early treatment and efficient/ aggressive treatment of co-morbidities could slow the progression of PD.

In the study by El-Kattan MM et al[14], they reported that using Hoehn and Yahr scores ranged from 1 to 3. The median value was 2 and mean was 2.4 ± 0.8[166]. In our study we found that maximum patients 17/44, which is 38.6% of the participants belonged to Grade III. The least number of cases belonged to Grade IV, which is 6.8% of the cases while 12 cases belong to grade 2 and 4 each which is 27.3 % of the total cases. Using the wester rating scale in our study we concluded that majority of participants 29/44 belonged to moderate disability, which is 65.9 %of total participants. Only 5 participants belonged to severe or advanced disability, which is 11.4 % of the total participants. In another study by Kouchaki E et al[15] they concluded that the serum level of tnf- α in patients were significantly higher than in healthy subjects. they also concluded that TNF- α levels were significantly higher in severe forms of PD than those in mild and moderate forms and it correlates with severity of PD using the H&Y scale.

In our study we found that the Mean gene expression of INF-γ was 2.880 and median of 1.44100. The level of INF-γ in correlation to H&Y scale was maximum in Grade V and least in Grade III. This may suggest that as severity of PD progresses the higher the value of inflammatory cytokines. While in correlation with WRS we found that INF-γ levels were maximum in severe or advanced disability PD. The P-value in correlation with WPS is 0.31and H&Y scale is 0.48, both of which are statistically not significant. But the gene expression of both inflammatory cytokines was higher in severe form of PD then in mild or moderate form. Furthermore, INF-γ is abundant in microglia while TNF-α is prevalent in astroglia within the SNc in cases of long-term Parkinsonism. The research indicated that the continuous secretion of INF-γ and TNF-α, along with other inflammatory cytokines, plays a key role in sustaining glial activation in Parkinsonism and could potentially lead to neuronal deterioration. Oxidative stress is an important pathogenesis of cell degeneration in PD. When correlated with WRS the P-

Value is <0.001 and in correlation with H&Y Scale is <0.001, which is statistically significant. . Our theory is that advanced PD patients often experience difficulties with swallowing, known as dysphagia, which can impact their ability to consume essential nutrients and consequently lead to poor nutritional health.

5. CONCLUSION

We concluded in our study that gene expression of INF-γ and TNF-α were higher in patient with Severe form of PD. But the P-Value of these inflammatory markers were not statistically significant. A multi-centric study cross- sectional study should be done for further study.

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