

Cytokines, miRNAs, and Antioxidants as Combined Non-Invasive Biomarkers for Parkinson's Disease

Amr Ghit (✉ amr.saadshehatariz01@universitadipavia.it)

Universita degli Studi di Pavia Dipartimento di Biologia e Biotechnologie Lazzaro Spallanzani

<https://orcid.org/0000-0002-7153-2413>

Hany El Deeb

Alexandria University Faculty of Medicine

Research Article

Keywords: Parkinson's disease, blood-based biomarker, cytokines, α -synuclein, oxidative stress, miRNA

Posted Date: November 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1028650/v1>

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Version of Record: A version of this preprint was published at Journal of Molecular Neuroscience on February 23rd, 2022. See the published version at <https://doi.org/10.1007/s12031-022-01984-1>.

Abstract

Background:

Parkinson's disease (PD) is one of the most common long-term degenerative disorders of the CNS that primarily affects the human locomotor system. Owing to the heterogeneity of PD etiology and the lack of appropriate diagnostic tests, blood-based biomarkers became the most promising method for diagnosing PD. Even though various biomarkers for PD have been found, their specificity and sensitivity are not optimum when used alone. Therefore, the aim of this study was directed to evaluate changes in a group of sensitive blood-based biomarkers in the same PD patients compared to healthy individuals. Serum samples were collected from 20 PD patients and 15 age-matched healthy controls. We analyzed serum levels of cytokines (IL10, IL12, and TNF- α), α -synuclein proteins, miRNAs (miR-214, miR-221, and miR-141) and antioxidants (UA, PON1, ARE).

Results:

Our results showed an increase in sera levels of cytokines in PD patients as well as a positive correlation among them. Also, we found a significant increase in sera levels of α -synuclein protein associated with a decrease in miR-214 which regulates its gene expression. Lastly, we observed a decrease in sera levels of miR-221, miR-141, UA, PON1, and ARE, which have a prominent role against oxidative stress.

Conclusion:

Because of the many etiologies of PD, a single measure is unlikely to become a useful biomarker. Therefore, to correctly predict disease state and progression, a mix of noninvasive biomarkers is required. Although considerable work has to be done, this study sheds light on the role of certain biomarkers in the diagnosis of PD.

Background

Parkinson's disease (PD) is the second most common idiopathic neurodegenerative disorder after Alzheimer's disease (AD). It affects approximately 1% of individuals above age 60 [1]. PD characterized by two main pathological hallmarks: 1) death of dopaminergic neurons in the substantia nigra pars compacta (SNpc), followed by a depletion of dopamine (DA) levels in the striatum, 2) abnormal deposition of α -synuclein protein in the cytoplasm of certain neurons in several different brain regions [2, 3]. It is not yet clear what causes degeneration of dopaminergic neurons in SNpc. However, mitochondrial dysfunction, oxidative stress, and accumulation of misfolded proteins may be involved in PD pathogenesis [3–5].

Elevated levels of inflammatory proteins in the brain and CSF have been reported [6]. Autopsy studies and animal models of PD showed the presence of activated microglia in the SN [7]. Furthermore, using nonaspirin nonsteroidal anti-inflammatory drugs decrease the risk of PD [8]. MicroRNA (miRNA) is a

small non-coding RNA (about 22 nucleotides) that regulates the expression of a wide range of target genes. MiRNAs dysregulation is associated with the pathogenesis of PD [9]. Increasing reactive oxygen species (ROS) and/or impairment of the anti-oxidant mechanisms lead to oxidative stress [10] which initiate α -synuclein aggregation and dopaminergic neuron death in PD [11]. Enzymatic (ex. Paraoxonase, PON1/ Arylesterase, ARE) and non-enzymatic (ex. Uric acid, UA) systems both defend against oxidative stress and reduce the incidence of neurodegeneration [12, 13].

The use of blood-based biomarkers is suggested to be the most promising method for diagnosing PD because it is fast, easy, and inexpensive [14]. Despite the fact that several biomarkers of PD have been identified, their specificity and sensitivity when used alone are not ideal.

As a result, combining diverse blood-based biomarkers will considerably increase diagnostic accuracy and promote personalized medication [15]. Accordingly, the current study is an attempt to evaluate three different sets of blood-based biomarkers in the same PD patients. The selection of biomarkers that included cytokines (IL10, IL12, and TNF- α), miRNAs (miR-214, miR-221, miR-141) and antioxidants (UA, PON1 and ARE) was based on previous studies of great importance for understanding the pathogenesis of PD.

Methods

Participants

A total of 20 patients with PD were recruited between 2017 and 2018 from outpatient clinics of Nariman Hospital at Faculty of Medicine, Alexandria University. Baseline assessments included disease duration, family history, medication history, and comorbid conditions. In addition, 15 healthy individuals were selected from the same community as a control group. They had no signs or symptoms suggesting cognitive impairment or neurological disease, and they had a negative family history of movement disorders. The study was approved by the Ethics Committee Faculty of Medicine, Alexandria University. Written informed consent was obtained from all participants.

Blood sample collection

Venous blood samples (5 mL) were collected by vein puncture using coagulation-promoting tubes. The blood samples were left to stand for about 30 min at 4°C before centrifugation at 2000 rpm for 15 min. Serum was removed and stored at -80°C in the centrifuge tubes until assays were performed.

Measurements

All assays were performed according to the manufacturer's instructions mentioned below by experts at Department of Biochemistry, Faculty of Science, Alexandria University.

Immune Markers and α -Synuclein proteins

Serum levels of circulating IL10, IL12, TNF- α , and α -Synuclein proteins were measured with immunoassay using commercially available ELISA kits (BIOMATIK; EKU05178, EKU05193, EKU07946, EKU07560).

MiRNAs

RNA extraction

All sera samples were placed on ice before being centrifuged at 20,000 g for 15 minutes at 4°C to eliminate any leftover cell debris before RNA extraction. After that, 200 μ L of serum were carefully transferred to RNase-free tubes for total RNA isolation using miRNeasy Serum/Plasma Kit (Qiagen, Germany) according to the manufacturer's protocol as previously described [16]. Briefly, serum was mixed with denaturing buffer, placed on a vortex mixer at 3,000 rpm for 15 seconds, and left at room temperature for 5 minutes. Total RNAs were eluted by 50 μ L of nuclease-free water (Ambion TX). The RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, DE).

Reverse transcription and quantitative real-time PCR

MicroRNAs were reverse transcribed and the resulting cDNA was amplified using miScript SYBR Green PCR kit (Qiagen, Germany) and microRNA specific primers. Subsequently, 2 μ L of the product was used to detect miRNAs expression by quantitative real-time PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Germany). The PCR primer sequences were as follows: mir-141 (5'-TTCCATGGGTTGTGTCAGTG-3'); mir-214 (5'-ACAGCAGGCACAGGC-3'); mir-221 (5'-AGCTACATTGTCTGCTGGGTT-3'). Expression levels of Targets miRNAs in each sample were normalized using the miRNeasy Serum/Plasma SpikeIn Control (cat. no. 219610) and corresponding Ce_miR-39_1 miScript Primer Assay that is recommended to monitor miRNA purification and amplification. The fold change of miRNAs expression was calculated based on the threshold cycle (CT) value using the following formula: $2^{-\Delta\Delta CT}$ [17].

Antioxidants

Serum urate concentration was determined by the colourimetric method (at $\lambda = 570$ nm) of the Uric Acid Assay Kit (ab65344) according to the manufacturer's protocol as previously described [18]. The activity of paraoxonase (PON1) was measured spectrophotometrically using paraoxon (O, O-diethyl-o-p-nitrophenyl phosphate; Sigma Chemical Co) as a substrate. The rate of production of 4-nitrophenol was measured at 412 nm with a spectrophotometer (Techcomp 8500 II UV/VIS, China) as already described [19]. Arylesterase activity was measured spectrophotometrically using phenylacetate (Sigma Co, London, UK) as a substrate and the rate of formation of phenol was measured by monitoring the increase in absorbance at 217 nm at 25°C following an established procedure [20].

Statistical Analysis

All data were evaluated using Excel software and presented as the means \pm standard deviations (SDs). Statistical analysis was performed using SPSS (version 23). Differences between PD patients and

healthy individuals were evaluated using unpaired two-tailed student t-test. Results with *P* values of 0.05 or less were considered significant. Correlations between certain indices in the different groups were detected by Spearman's tests and linear regression analysis.

Results

Subjects

The demographic and clinical characteristics of subjects are summarized in Table 1. No statistically significant differences were observed among patients with PD and healthy controls concerning age or sex.

Table 1.			
Demographic and clinical characteristic of patients with PD and healthy controls			
	PD patients (n=20)	Healthy controls (n=15)	p-value*
Male/female	11/9	10/5	0.334
Age (years)	61.7 ± 14.6	61.8 ± 4.9	0.923
Disease duration (years)	4.3 ± 4.0	-	-
Treatment duration (years)	3.9 ± 3.4	-	-
Data are presented as mean ± standard deviation unless otherwise stated.			
* Unpaired two-tailed student t-test.			

Serum levels of Immune and non-immune markers

Laboratory findings of the PD patients and controls are summarized in Table 2. The mean serum levels of circulating cytokines (IL10, IL12, and TNF- α) and α -Synuclein proteins in patients with PD were significantly higher than those in healthy individuals ($p < 0.0001$) (Fig. 1A; 1B; 1C; 1D). On the other hand, serum levels of microRNAs (miR-214, miR-221, miR-141) as well as antioxidants (UA, PON1, and ARE) in patients with PD were significantly lower than those in healthy individuals ($p < 0.0001$) (Fig. 1E; 1F; 1G; 1H).

Table 2.			
Serum levels of immune and non-immune markers in PD patients and healthy controls.			
	PD (n=20) (mean ± SD)	HC (n=15) (mean ± SD)	p-value*
IL10 (pg / ml)	10.7 ± 2.2	2.5 ± 0.3	<0.0001
IL12 (pg / ml)	9.3 ± 1.7	2.1 ± 0.3	<0.0001
TNF-α (pg / ml)	47.0 ± 10.0	14.2 ± 6.1	<0.0001
α-Synuclein (pg/ml)	84.2 ± 2.6	69.4 ± 2.7	<0.0001
miR-214	0.2 ± 0.1	0.9 ± 0.2	<0.0001
miR-221	0.5 ± 0.1	0.9 ± 0.2	<0.0001
miR-141	0.4 ± 0.1	0.9 ± 0.2	<0.0001
UA (mg/dl)	3.32 ± 0.3	5.4 ± 0.5	<0.0001
PON1 (U/mL)	181.4 ± 7.9	425.2 ± 28.1	<0.0001
ARE (U/L)	120.7 ± 16.8	196.9 ± 11.7	<0.0001
* Unpaired two-tailed student t-test. Values are mean ± SD.			
<i>PD</i> Parkinson's disease, <i>HC</i> healthy control, <i>IL</i> interleukin, <i>TNF-α</i> tumor necrotic factor-alpha, <i>miR</i> microRNA, <i>UA</i> uric acid, <i>PON1</i> paraoxonase1, <i>ARE</i> arylesterase, <i>pg/ml</i> Picogram per millilitre, <i>mg/dl</i> milligrams per deciliter, <i>U/mL</i> Units Per Millilitre, <i>U/L</i> units per litre.			

Correlations among serum levels of immune and non-immune markers in PD patients and healthy controls are shown in Table 3. In PD patients, correlations among cytokines were significant at p -values <0.01. Serum levels of IL10 and IL12 were positively correlated ($R=0.913$, $p<0.0001$) (Fig. 2A) with a related variance of 83%, serum levels of TNF-α and IL10 were positively correlated ($R= 0.714$, $p=0.002$) (Fig. 2B) with a related variance of 51%, and serum levels of TNF-α and IL12 were positively correlated ($R=0.75$, $p<0.0001$) (Fig. 2C) with a related variance of 56%. Besides, the positive correlation between antioxidant enzymes, PON1 and ARE was significant ($R=0.651$, $p=0.002$) with 42% of a related variance (Fig. 2D). On the other hand, there was a negative correlation between serum levels of miR-221 and TNF-α ($R= - 0.564$, $p=0.01$) (Fig. 2E) with a related variance of 32%. Interestingly, there were no correlations among serum levels of the previously mentioned values in healthy controls (see right sides of Fig. 2).

Table 3.

Correlation between serum levels of immune and non-immune markers in PD patients and healthy controls evaluated by the Spearman rank correlation.

	R*		p-value**		R ²		Related variance	
	PD	HC	PD	HC	PD	HC	PD	HC
IL10; IL12	0.913	0.364	<0.0001	0.182	0.83	0.13	83%	13%
TNF- α ; IL10	0.714	0.191	0.002	0.494	0.51	0.04	51%	4%
TNF- α ; IL12	0.75	0.087	<0.0001	0.759	0.56	0.01	56%	1%
PON1;ARE	0.651	-0.327	0.002	0.234	0.42	0.11	42%	11%
miR-221;TNF- α	-0.564	-0.323	0.01	0.240	0.32	0.10	32%	10%

*R correlation coefficient.

**Correlation is significant at the 0.01 level (2-tailed).

Discussion

Changes in the levels of cytokines may enhance apoptosis and subsequent phagocytosis of DA neurons [21]. Previous observations found that concentrations of IL-1b, IL-2, IL-6, IL-10, and TNF- α , and C - reactive protein increased in PD patients [22]. Recently, a study found a positive correlation between serum levels of IL-10 and gastrointestinal symptoms in PD [23]. In the current study, the mean serum levels of circulating cytokines (IL10, IL12, and TNF- α) in PD patients were higher than those in healthy controls (Fig. 1A; 1B; 1C), and this is consistent with results observed in previous studies [24, 25]. Immune markers are thus potential predictive biomarkers in the diagnosis of PD. Although IL-10 is an anti-inflammatory cytokine that negatively regulates TNF- α and IL-12 [26], the positive correlation between IL10 and IL12 (R=0.913, p<0.0001), and TNF- α and IL10 (R=0.714, p=0.002) indicate immunological disturbances in PD patients. On the other hand, the positive correlation between TNF- α and IL12 (R=0.75, p<0.0001), and its absence among healthy controls (Fig. 2A; 2B; 2C) suggest that neuroprotective mechanisms are involved in PD. These findings agree with a previous study showed increased levels of IL-10, as well as a positive correlation between IL-10 and IL-12 [27].

MiRNAs are quite stable in serum and plasma among individuals in the same species [28]. The stability of miRNAs in the blood may be due to the protection of binding proteins or microvesicles which encapsulate miRNAs [29]. Pathological conditions may influence the secretion of microvesicles which in turn can alter the blood miRNA profile. Since the miRNA can be directly detected in the blood, it can be used as a diagnostic biomarker for various diseases [30, 31].

The significance of miRNAs in the pathogenesis of PD as well as the challenges of using them as biomarkers were discussed in detail [9, 32, 33].

Aggregation of α -synuclein proteins forming Lewy Bodies represents the pathological hallmark of PD [2, 3]. In our study, serum levels of α -synuclein protein (Fig. 1D) were increased in parallel with the significant decline in miR-214 (Fig. 1E) which regulate α -synuclein gene expression [34]. Therefore, circulating α -synuclein protein and miR-214 may play a role in diagnosing PD. However, we can't say that they are useful for monitoring the degree of progression and severity of PD because, in some studies, α -syn levels were correlated to PD severity, but not in others [35].

Interestingly, miR-221 decreased the expression of pro-apoptotic genes (Bim, BNIP3L, Foxo3a, and BMF) which in turn promote neuronal survival against oxidative stress [36]. Ding et al. (2016) suggested that increased serum levels of miR-195 and decreased serum levels of miR-221 may be used as non-invasive biomarkers for the diagnosis of PD [37]. Likewise, miR-141 down-regulates Keap1 gene expression which has a direct effect on the activation of the transcription factor Nrf2 which in turn regulates the expression of antioxidant enzymes [38]. In addition, miR-141 and miR-214 were decreased significantly in serum of PD patients compared with healthy individuals [39]. In agreement with the previous studies, we found a significant decline in serum levels of miR-221 and miR-141 in PD patients than those in healthy controls (Fig. 1E) reflecting their role in the protection of dopaminergic neurons against oxidative stress and apoptosis. Therefore, a combination of miR-214, miR-221, and miR-141 may help in distinguishing PD patients from healthy individuals. Of note, increasing the severity of neuroinflammation is suggested to be related to the level of oxidative stress in PD [40], which can be supported by the negative correlations between miR-221 and TNF- α which have been absent from healthy controls (Fig. 2E).

Post-mortem studies on brains from PD patients found decreased amounts of antioxidants in the SN suggested that oxidative stress in the CNS damages the SN, which in turn promote dopaminergic neuronal loss [41]. A recent study found that serum levels of uric acid are a potential biomarker for motor and non-motor features and disease progression [42]. Low levels of serum uric acid have been reported in PD patients with memory impairment, depression, anxiety, and cardiovascular symptoms [43]. In agreement with the previous observations, we found a decrease in serum levels of UA in PD patients.

PD is marked by an imbalance between Acetylcholine (ACh) and DA in CNS which mainly results from the degeneration of dopaminergic neurons in the SN [44]. Free radicals such as peroxide and organophosphate oxons, attack and inhibit acetylcholinesterase, thus increasing acetylcholine, which in turn exacerbates the imbalance between ACh and DA. These free radicals are blocked by Paraoxonase 1 (PON1) enzyme (is a glycoprotein enzyme synthesized in the liver) preventing the imbalance between ACh and DA [45, 46]. In the current study, the decrease in the level of PON1 and ARE enzymes (Fig. 1G; 1H) as well as the positive correlation between them in PD patients (Fig. 2D; $R=0.651$, $p=0.002$) without any notable correlation among healthy controls supporting their role against oxidative stress in PD. Therefore, circulating UA, PON1 and ARE can be used in diagnosing of PD.

Conclusions

A single measure is unlikely to become a useful biomarker due to the multiple etiologies of PD. Therefore, evaluation of different types of noninvasive biomarkers is needed to accurately predict disease status and progression. Although much remains to be done, this study highlights the involvement of specific cytokines, miRNAs, and antioxidants in understanding the pathogenesis of PD more clearly, and will aid in the clinical diagnosis of the disease.

Abbreviations

ACh	Acetylcholine
AD	Alzheimer's disease
ARE	Arylesterase
DA	Dopamine
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
miRNAs	MicroRNAs
PD	Parkinson's disease
PON1	Paraoxonase
ROS	Reactive oxygen species
SNpc	Substantia nigra pars compacta
TNF- α	Tumor necrotic factor-alpha
UA	Uric acid

Declarations

Data availability statement (DAS)

The datasets generated during and/or analysed during the current study are available on request from the corresponding author.

ETHICAL STATEMENT:

Ethics approval and consent to participate:

The study was approved by the Ethics Committee Faculty of Medicine, Alexandria University. Written informed consent was obtained from all participants.

Consent for publication:

Not Applicable

Availability of data and materials:

All data generated or analyzed during this study are included in this manuscript.

Competing interests:

Not applicable

Funding:

Not applicable

Authors' contributions:

AG conceived and designed the study. HED examined patients clinically and selected the appropriate cases. AG and HED collected samples and patient data. AG performed statistical data analysis and interpretation, and wrote the manuscript. HED revised statistical data analysis and interpretation. The authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

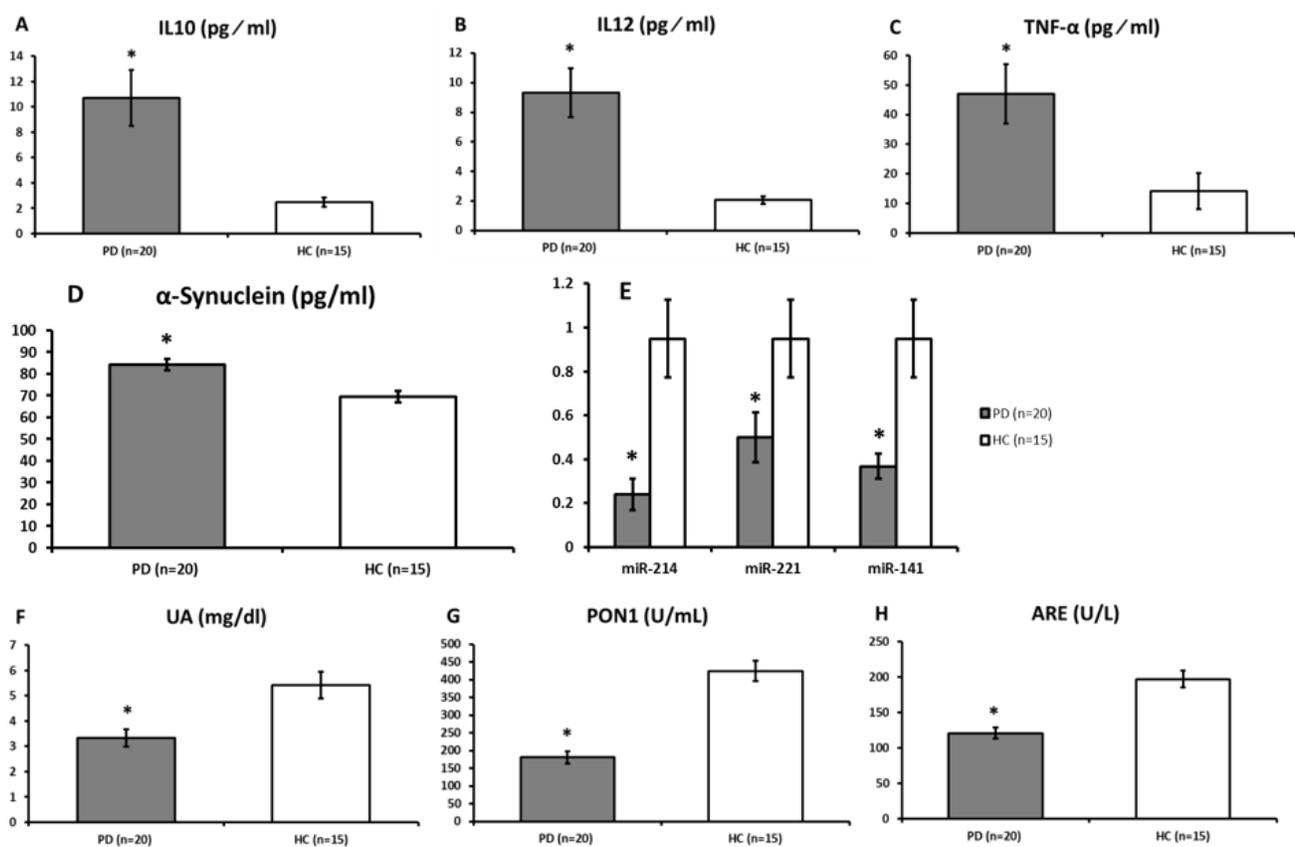


Figure 1

Comparison of serum levels of immune and non-immune markers in PD patients and healthy controls. Bars represent mean values; error bars represent the standard deviation; *P < 0.0001. (A) IL10; (B) IL12; (C) TNF- α ; (D) α -Synuclein protein; (E) MicroRNAs (miR-214, miR-221, and miR-141); (F) UA; (G) PON1; (H) ARE.

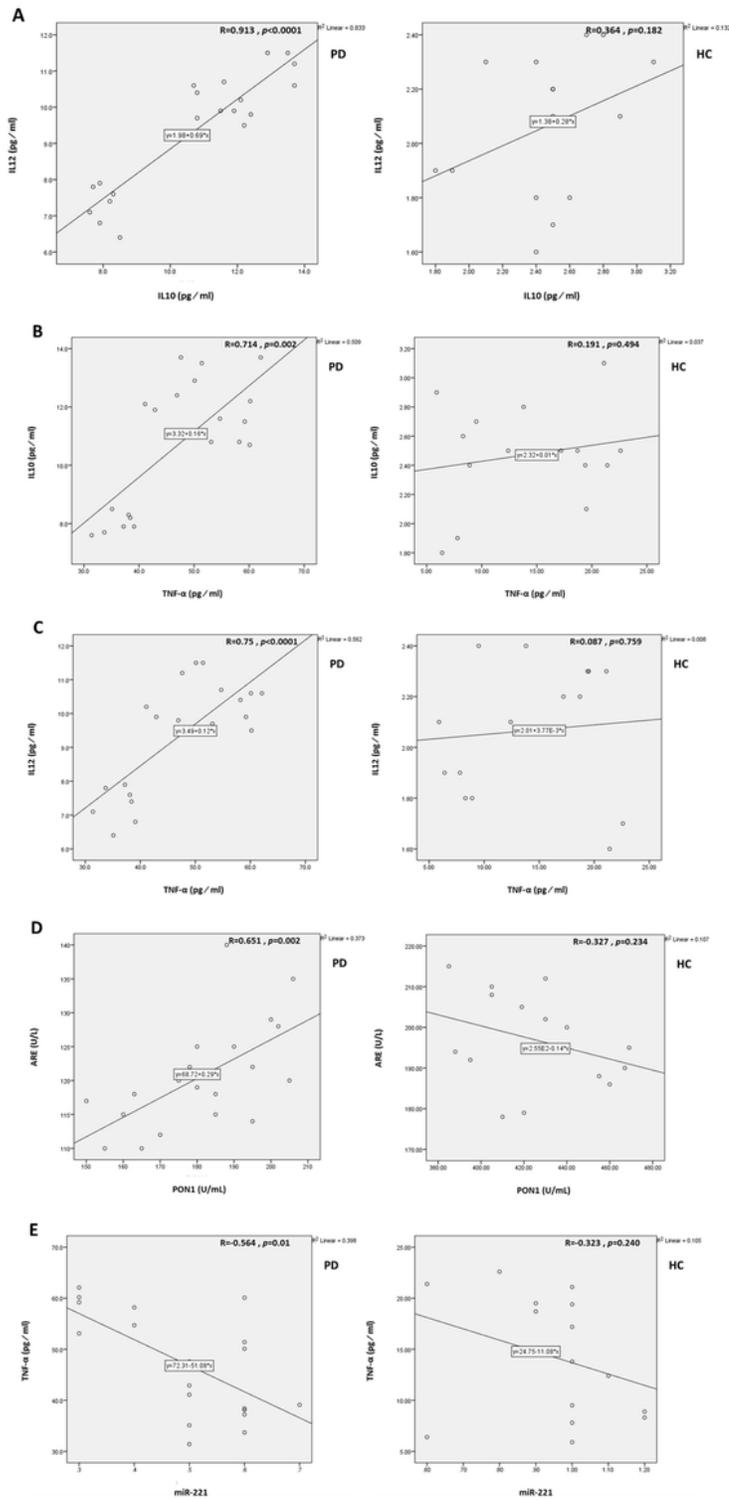


Figure 2

Correlations between serum levels of immune and non-immune markers in PD patients and healthy controls. (A) IL10 and IL12; (B) TNF- α and IL10; (C) TNF- α and IL12; (D) PON1 and ARE; (E) miR-221 and TNF- α .