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Upregulation of APAF1 and CSF1R in Peripheral Blood Mononuclear Cells of Parkinson's Disease

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Abstract: Increased oxidative stress and neuroinflammation play a crucial role in the pathogenesis of Parkinson's disease (PD). In this study, the expression levels of 52 genes related to oxidative stress and inflammation were measured in peripheral blood mononuclear cells of the discovery cohort including 48 PD patients and 25 healthy controls. Four genes, including *ALDH1A*, *APAF1*, *CR1*, and *CSF1R*, were found to be upregulated in PD patients. The expression patterns of these genes were validated in a second cohort of 101 PD patients and 61 healthy controls. The results confirmed the upregulation of *APAF1* (PD: 0.34 ± 0.18, control: 0.26 ± 0.11, $p < 0.001$) and *CSF1R* (PD: 0.38 ± 0.12, control: 0.33 ± 0.10, $p = 0.005$) in PD patients. The expression level of *APAF1* was correlated with the scores of the Unified Parkinson's Disease Rating Scale (UPDRS, r = 0.235, $p = 0.018$) and 39-item PD questionnaire (PDQ-39, r = 0.250, $p = 0.012$). The expression level of *CSF1R* was negatively correlated with the scores of the mini-mental status examination (MMSE, r = −0.200, $p = 0.047$) and Montréal Cognitive Assessment (MoCA, r = −0.226, $p = 0.023$). These results highly suggest that oxidative stress biomarkers in peripheral blood may be useful in monitoring the progression of motor disabilities and cognitive decline in PD patients.

Keywords: oxidative stress; *APAF1*; *CSF1R*

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1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by tremors, slowness of movement, and freezing of gait [1]. The loss of dopaminergic neurons in the substantia nigra of the ventral midbrain accompanied by the presence of eosinophilic cytoplasmic inclusion bodies (Lewy bodies) enriched with α-synuclein is a pathological hallmark of PD [2]. This neurodegeneration is associated with a deficiency of dopamine in the striatum. The exact pathogenesis of PD is unknown, but oxidative stress is thought to play a role in the neurodegeneration associated with the disease [3]. Neurons are particularly vulnerable to oxidative stress due to their high levels of unsaturated lipids, and dopamine metabolism also generates hydrogen peroxide and other reactive oxygen species (ROS) [4]. Iron deposition [5], mitochondrial dysfunction [6], inflammation mediated by microglial activation [7], and reduced levels of antioxidants and antioxidant enzymes [8] also contribute to increased ROS levels, which expose dopaminergic neurons to chronic oxidative stress. Genes linked to inherited PD, such as α-synuclein (*SNCA*), parkin (*PARK2*), DJ-1 (*PARK7*), PTEN induced kinase 1 (*PINK1*), and leucine-rich repeat kinase 2 (*LRRK2*), are also associated with mechanisms that increase oxidative stress [9]. Identifying oxidative stress or inflammation biomarkers in patients with PD could provide insights into the roles of oxidative stress and inflammation involved in pathogenesis and potentially aid in the detection and monitoring of disease progression.

Cerebrospinal fluid (CSF) is considered to be a good source of biomarkers of PD because it comes into direct contact with the diseased brain tissue. However, CSF is

not as easily accessible as other tissues such as blood or peripheral blood mononuclear cells (PBMCs), which can be collected through minimally invasive methods. Several molecules involved in oxidative stress have been identified in PD patients, including elevated plasma levels of homocysteine [10], which are known to cause dopaminergic neuronal loss by inhibiting mitochondrial activity and increasing oxidative stress [11]. High levels of malondialdehyde (MDA), a product of lipid peroxidation, have also been reported in the plasma of PD patients [12–15]. Increased levels of 8-hydroxydeoxyguanosine (8-OHdG), an oxidized DNA damage marker have been shown in the peripheral blood of PD patients [12,16,17]. A meta-analysis showing elevated concentrations of 8-OHdG, MDA, nitrite, and ferritin, and reduced levels of catalase, uric acid, and glutathione in the peripheral blood of PD patients, further supports increased oxidative stress in PD [18]. The expression levels of nuclear factor erythroid 2-related factor 2 (*NRF2*), an anti-oxidative factor involved in the pathogenic processes of PD [19,20], are also elevated in the PBMCs of PD patients [21]. Activation of the transcription factor nuclear factor kappa B (*NF-κB*) that controls target genes encoding proinflammatory cytokines and chemokines has been shown in brain regions of PD at post-mortem [22]. Microarray analysis of substantia nigra from PD patients also showed that reactive astrocytes appear to be responsible for the activation of microglia which in turn releases proinflammatory cytokines contributing further to neurodegeneration [23]. Serum interleukins (IL-2, IL-10, IL-6, IL-4), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and soluble TNF-α receptor-1 concentrations were elevated in PD patients [24,25]. Plasma cytokine levels were significantly correlated with the disease severity in PD patients [26]. These studies suggest elevated inflammatory factors in the peripheral blood of PD patients.

Previous gene expression studies have shown that the expression of several oxidative stress- or inflammation-related genes such as heat-shock protein 70-interacting protein (*ST13*), proteasome 20S subunit alpha 2 (*PSMA2*), aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*), BCL11 transcription factor B (*BCL11B*), nuclear-encoded mitochondrial gene (LRPPRC), interleukin 1 beta (*IL-1β*) and complement receptor 1 (*CR1*) was altered in the peripheral blood of PD patients compared to the healthy controls [27–33]. In this study, we measured the gene expression levels of a panel of genes involved in oxidative stress or inflammation including previously reported differentially expressed genes as described above in PBMCs of PD patients. We further examined if the expression levels of identified genes were significantly correlated with clinical scores of motor or cognitive impairments of PD patients.

2. Results

2.1. Expression Profiles of Peripheral Blood Mononuclear Cells in the Discovery Cohort for PD

To identify candidate peripheral gene expression markers for oxidative stress for PD, we examined the expression profile of PBMCs using an in-house q-PCR array that included 52 candidate genes involved in oxidative, chaperon, and inflammation pathways in a discovery cohort of 48 PD patients and 25 controls (Table 1). *ALDH1A1* (PD vs. control: 0.063 ± 0.022 vs. 0.053 ± 0.016, $p = 0.047$), apoptotic protease activating factor 1 (*APAF1*, PD vs. control: 0.459 ± 0.188 vs. 0.354 ± 0.082, $p = 0.006$), *CR1* (PD vs. control: 0.073 ± 0.031 μM vs. 0.054 ± 0.032, $p = 0.026$), and colony stimulating factor 1 receptor (*CSF1R*, PD vs. control: 0.375 ± 0.082 μM vs. 0.325 ± 0.094, $p = 0.032$) were significantly upregulated in PBMCs of PD patients (Table 2).

2.2. Validation of Candidate Gene Expression Markers in a Validation Cohort

The identified candidate gene expression markers were further validated in an independent validation cohort including 101 PD patients and 61 controls. Scores for Clinical Dementia Rating (CDR), Beck Depression Inventory-II (BDI-II), Hamilton Depression Scale (HAM-D), 39-item PD questionnaire (PDQ-39), Neuropsychiatric Inventory (NPI), UPDRS, and UPDRS part III were significantly higher in PD patients compared to controls (all $p < 0.001$). Conversely, scores for MMSE and MoCA were significantly lower in PD patients

(all $p < 0.001$). The plasma level of α-synuclein was significantly higher in PD patients compared to controls (PD: 190.07 ± 159.53 fM, control: 110.70 ± 65.78 fM, $p < 0.001$). The levels of pre-prandial glucose, glycohemoglobin, albumin, triglycerides, and cholesterol were similar between the two groups. When stratifying PD patients by disease severity, we found that those in the advanced stage had a significantly higher mean age than those in the early stage ($p = 0.003$). Scores for CDR, BDI-II, HAM-D, PDQ-39, NPI, UPDRS, and UPDRS part III were also significantly higher in advanced-stage PD patients compared to early-stage PD patients (all $p < 0.001$). Advanced-stage PD patients had lower scores for MMSE, MoCA, and Activities of Daily Living score (ADL) compared to early-stage PD patients (all $p < 0.001$). The detailed results are displayed in Table 3.

Table 1. Clinical characteristics of the discovery cohort.

	Control (n = 25)	PD (n = 48)
Age (years)	66.33 ± 8.56	68.58 ± 10.79
Male (%)	155 (60.00)	30 (62.50)
Hoehn and Yahr stage		2.14 ± 0.95
LEDD (mg)		849.28 ± 616.80
UPDRS		
Total	1.12 ± 1.88	53.38 ± 31.25 *
Part III	0.16 ± 0.78	32.56 ± 19.60 *

LEDD: levodopa equivalent daily dosage; PD: Parkinson's disease; UPDRS: Unified Parkinson's Disease Rating Scale. *: Statistically significant in comparison with control, $p < 0.05$, Two-tailed Student's t test.

Table 2. Expression levels of 52 genes in the discovery cohort of Parkinson's disease patients (PD) and controls.

Gene	Control (N = 25)	PD (N = 48)	p Value
ALDH1A1	0.053 ± 0.017	0.063 ± 0.023	0.047
CR1	0.054 ± 0.033	0.073 ± 0.031	0.026
CSF1R	0.325 ± 0.096	0.375 ± 0.084	0.032
APAF1	0.354 ± 0.084	0.459 ± 0.190	0.002
HIP2	0.043 ± 0.014	0.046 ± 0.012	0.408
EGF	0.009 ± 0.010	0.006 ± 0.003	0.153
HSPB8	0.003 ± 0.005	0.001 ± 0.001	0.128
PSMA7	0.580 ± 0.126	0.564 ± 0.149	0.639
BCL11B	0.148 ± 0.067	0.144 ± 0.057	0.818
ST13	1.075 ± 0.319	1.053 ± 0.519	0.821
C3	0.010 ± 0.003	0.011 ± 0.004	0.117
CSF1	0.080 ± 0.038	0.092 ± 0.070	0.349
APOA1	0.001 ± 0.000	0.001 ± 0.000	0.808
FAS	0.018 ± 0.010	0.021 ± 0.013	0.330
CR2	0.007 ± 0.003	0.006 ± 0.004	0.309
C3AR1	0.152 ± 0.087	0.144 ± 0.075	0.680
NLRP1	0.527 ± 0.137	0.548 ± 0.200	0.601
NLRP3	0.074 ± 0.026	0.087 ± 0.038	0.116
Nrf2	0.596 ± 0.136	0.650 ± 0.222	0.200
HMOX1	0.248 ± 0.082	0.264 ± 0.084	0.435
HMOX2	0.112 ± 0.040	0.094 ± 0.031	0.058
GCLM	0.016 ± 0.009	0.018 ± 0.013	0.326
BAD	0.020 ± 0.007	0.018 ± 0.007	0.323
NQO1	0.014 ± 0.016	0.014 ± 0.025	0.888
LRPPRC	0.007 ± 0.010	0.006 ± 0.010	0.684
PSMA2	0.006 ± 0.004	0.007 ± 0.004	0.414
PPARGC1A	0.005 ± 0.010	0.004 ± 0.013	0.771
C4A	0.007 ± 0.003	0.007 ± 0.004	0.680
C4B	0.103 ± 0.168	0.103 ± 0.181	0.998
C1qA	0.026 ± 0.014	0.026 ± 0.016	0.986
C5AR1	3.929 ± 1.122	4.022 ± 2.105	0.807

Table 2. *Cont.*

Gene	Control (N = 25)	PD (N = 48)	*p* Value
CASP1	0.475 ± 0.179	0.570 ± 0.253	0.067
IL-1beta	0.347 ± 0.208	0.399 ± 0.258	0.352
CASP3	0.092 ± 0.043	0.096 ± 0.038	0.723
CASP6	1.178 ± 5.757	0.024 ± 0.013	0.326
HSF1	0.167 ± 0.041	0.162 ± 0.055	0.683
HSP90AA1	0.274 ± 0.129	0.270 ± 0.118	0.914
OPA1	0.022 ± 0.012	0.022 ± 0.010	0.820
MFN1	0.037 ± 0.024	0.040 ± 0.018	0.557
MFN2	0.069 ± 0.028	0.081 ± 0.033	0.115
HSPA5	0.047 ± 0.032	0.057 ± 0.060	0.334
HSPB1	0.227 ± 0.098	0.211 ± 0.119	0.542
HSPD1	0.229 ± 0.085	0.200 ± 0.085	0.181
DNM1L	0.019 ± 0.010	0.020 ± 0.008	0.588
BCL2	0.071 ± 0.034	0.069 ± 0.034	0.796
BID	0.490 ± 0.303	0.478 ± 0.156	0.849
GSS	0.049 ± 0.009	0.047 ± 0.012	0.688
GSR	0.180 ± 0.055	0.188 ± 0.053	0.550
IKBKB	0.100 ± 0.030	0.088 ± 0.028	0.122
CASP9	0.046 ± 0.023	0.045 ± 0.022	0.857
DNAJB1	0.250 ± 0.078	0.257 ± 0.087	0.749
NRF1	0.011 ± 0.005	0.012 ± 0.004	0.825

Table 3. Demographic characteristics and blood biochemical parameters of the validation cohort.

	Control (n = 61)	PD		
		Early Stage (n = 81)	Advanced Stage (n = 20)	Total (n = 101)
Age, year	67.18 ± 8.06	65.31 ± 10.72	73.00 ± 9.18 [b,c]	66.83 ± 10.87
Male, No (%)	30 (49.18 %)	42 (51.86%)	11 (55.00%)	53 (52.48%)
Age at onset		61.78 ±15.83	61.05 ± 13.78	61.63 ±15.45
Duration of PD		5.47 ± 7.94	12.31 ± 6.23	6.83 ± 8.10
BMI	24.14 ± 3.04	24.51 ± 3.45	23.67 ± 3.39	24.35 ± 3.45
Pre-prandial Glucose, mg/dL	99.32 ± 11.16	105.40 ± 22.67	99.35 ± 19.59	104.20 ± 22.22
Glycohemoglobin, %	5.87 ± 0.42	6.00 ± 0.66	5.81 ± 0.37	5.96 ± 0.62
Albumin, mg/dL	4.43 ± 0.20	4.46 ± 0.22	4.24 ± 0.25	4.42 ± 0.24
Total cholesterol, mg/dL	184.14 ± 28.94	177.05 ± 35.36	166.70 ± 29.19	175.00 ± 34.47
LDL-C, mg/dL	107.84 ± 27.71	104.35 ± 31.09	95.15 ± 30.32	102.52 ± 31.75
HDL-C, mg/dL	55.13 ± 13.99	53.19 ± 15.53	53.50 ± 12.73	53.25 ± 15.02
Triglyceride, mg/dL	103.87 ± 63.02	112.74 ± 60.98	89.35 ± 37.45	108.11 ± 57.86
Creatinine, mg/dL	0.78 ± 0.22	0.89 ± 0.38	0.79 ± 0.24	0.87 ± 0.36
MMSE	29.51 ± 0.88	27.47 ± 3.69 [b]	21.75 ± 6.50 [b,c]	26.34 ± 4.95 [a]
MoCA	27.56 ± 4.26	24.59 ± 5.54 [b]	17.70 ± 8.33 [b,c]	23.23 ± 6.77 [a]
CDR	0.20 ± 0.24	0.31 ± 0.24 [b]	0.68 ± 0.36 [b,c]	0.39 ± 0.31 [a]
BDI-II	1.64 ± 2.86	6.26 ± 1.89 [b]	15.40 ± 7.64 [b,c]	8.07 ± 6.63 [a]
HAM-D	1.61 ± 2.68	5.15 ± 3.68 [b]	10.35 ± 6.52 [b,c]	6.18 ± 4.86 [a]
ADL	98.11 ± 10.79	99.69 ± 1.44	71.00 ± 27.18 [b,c]	94.01 ± 16.69
PDQ-39	5.72 ± 8.15	23.09 ± 18.22 [b]	66.95 ± 32.36 [b,c]	31.77 ± 27.92 [a]
NPI	0.52 ± 1.68	1.98 ± 2.83	7.20 ± 7.28 [b,c]	3.01 ± 4.61 [a]
UPDRS				
Total	1.54 ± 2.17	28.46 ± 13.26	70.45 ± 25.15 [b,c]	36.68 ± 23.94 [a]
Part III	0.38 ± 1.20	17.63 ± 9.26	42.42 ± 15.19 [b,c]	22.44 ± 14.49 [a]
LEDD		445.91 ± 429.28	1306.55 ± 65.57 [d]	616.34 ± 592.05
α-Synuclein (Femtomolar)	110.70 ± 65.78	186.22 ± 157.41 [b]	205.64 ± 166.69 [b]	190.07 ± 159.53 [a]

Table 3. *Cont.*

	Control (n = 61)	PD		
		Early Stage (n = 81)	Advanced Stage (n = 20)	Total (n = 101)
Relative gene expression				
APAF1	0.26 ± 0.11	0.32 ± 0.18 [b]	0.43 ± 0.16 [b,c]	0.34 ± 0.18 [a]
CR1	0.10 ± 0.06	0.12 ± 0.08	0.10 ± 0.06	0.12 ± 0.07
CSF1R	0.33 ± 0.10	0.38 ± 0.13 [b]	0.37 ± 0.08 [b]	0.38 ± 0.12 [a]
ALDH1A1	0.08 ± 0.03	0.10 ± 0.09	0.8 ± 0.04	0.10 ± 0.08

ADL: Activities of Daily Living; BDI-II: Beck Depression Inventory II; BMI: body mass index; CDR: Clinical Dementia Rating; HAM-D: Hamilton Depression Rating Scale; HDL-C: high-density lipoprotein-cholesterol; LEDD: Levodopa Equivalent Daily Dose; LDL-C: low-density lipoprotein-cholesterol; MMSE: Mini-Mental State Examination; MoCA: Montreal Cognitive Assessment; NPI: Neuropsychiatric Inventory Questionnaire; PD: Parkinson's disease; PDQ-39: 39-item PD questionnaire; UPDRS: Unified Parkinson's Disease Rating Scale. [a]: Statistically significant in comparison with controls, $p < 0.05$, Two-tailed Student's t test. [b]: Statistically significant in comparison with control, $p < 0.05$, one-way analysis of variance (ANOVA) with Tukey's post hoc test. [c]: Statistically significant in comparison with PD at early stage, $p < 0.05$, one-way analysis of variance (ANOVA) with Tukey's post hoc test. [d]: Statistically significant in comparison with PD at early stage, $p < 0.05$, Two-tailed Student's *t* test.

In PBMCs, higher expression levels of *APAF1* (PD: 0.34 ± 0.18, control: 0.26 ± 0.11, $p < 0.001$) and *CSF1R* (PD: 0.38 ± 0.12, control: 0.33 ± 0.10, $p = 0.005$) were demonstrated in PD patients compared with controls (Table 2). Furthermore, PD patients at the advanced stage demonstrated higher expression levels of *APAF1* compared with the early stage (advanced stage: 0.43 ± 0.16, control: 0.32 ± 0.18, $p = 0.011$) (Table 2 and Figure 1A). The expression level of *APAF1* was correlated with scores of UPDRS (r = 0.235, $p = 0.018$, Figure 1B) and PDQ-39 (r = 0.250, $p = 0.012$, Figure 1C). The expression level of *CSF1R* was negatively correlated with scores of MMSE (r = −0.200, $p = 0.047$, Figure 1D) and MoCA (r = −0.226, $p = 0.023$, Figure 1E).

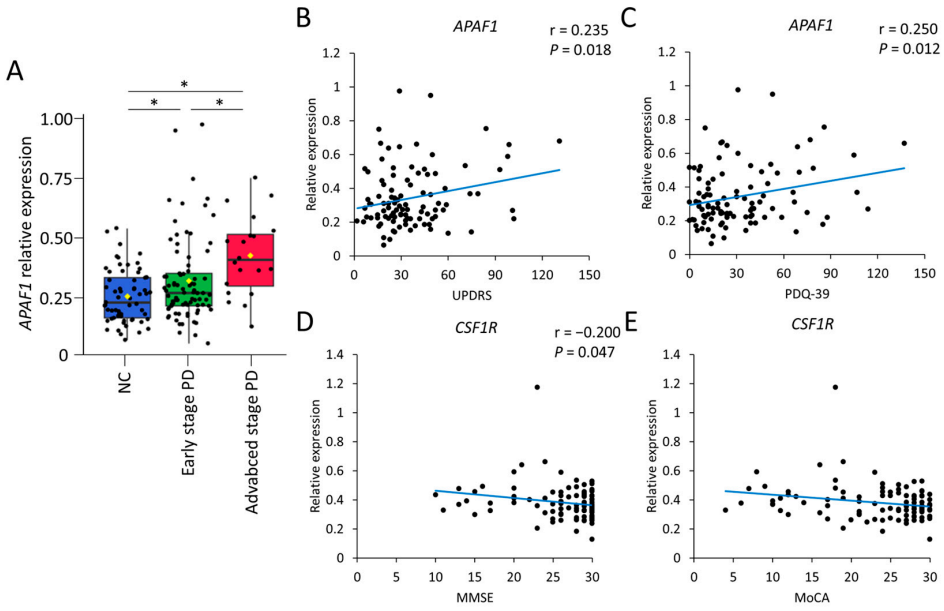

Figure 1. (**A**) Difference in expression level of *APAF1* between Parkinson's disease (PD) patients at early (N = 81) and advanced stages (N = 20) compared to controls (N = 61). (**B**,**C**) The correlation between expression level of *APAF1* and scores of Unified Parkinson's Disease Rating Scale (UPDRS) or 39-item PD questionnaire (PDQ-39). (**D**,**E**) The correlation between expression level of *CSF1R* and the scores of mini-mental state examination (MMSE) or Montreal Cognitive Assessment (MoCA). *: Statistically significant between two groups, $p < 0.05$, one-way analysis of variance (ANOVA) with Tukey's post hoc test.

3. Discussion

This study analyzed gene expression related to oxidative stress or inflammation in patients with PD. Using a two-stage validation process, we found that the expression of *APAF1* and *CSF1R* was increased in PBMCs of PD patients, particularly in advanced-stage patients. Although we have found increased levels of *ALDH1A1* and *CR1* in PD patients compared to the controls from the discovery cohort, these results were not confirmed in the validation cohort. Interestingly, these two genes have been shown to be differentially expressed in peripheral blood in PD by previous gene expression studies [28,29,31]. The inconsistent results may arise from different approaches used. The expression levels of *APAF1* were correlated with scores for parkinsonism, as measured by the UPDRS and PDQ-39, while the expression levels of *CSF1R* were negatively correlated with scores for cognitive function, as measured by the MMSE and MoCA. These findings suggest that peripheral oxidative stress biomarkers may be useful for detecting and monitoring neurodegeneration in PD.

The damages induced by ROS accumulate and trigger the release of cytochrome c from mitochondria during the induction of apoptosis. Cytosolic cytochrome c binds to APAF1, which adopts a heptameric quaternary structure and recruits procaspase-9 to form apoptosome [34]. The overexpression of *APAF1* promotes apoptosis in various cell models [35,36], highlighting its central role in the activation of the intrinsic apoptotic pathway. *APAF1* is highly expressed in the substantia nigra of patients with PD [37]. In accordance with the previous report, our findings showed upregulation of *APAF1* in PBMCs of PD patients. The overexpression of **a** dominant negative variant of *APAF1* suppressed both apoptosis and nigrostriatal degeneration in MPTP-treated mice [38]. The use of **a** pramipexole transdermal patch has been associated with the downregulation of *APAF1* in the 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated PD mouse model [39]. Apoptosis induced by the overexpression of *LRRK2* variants for familial PD can be prevented by genetic ablation of *APAF1* [40]. Therefore, APAF1 appears to be a potential target for PD treatment through apoptosis inhibition. We further found a correlation between the expression level of *APAF1* in PBMCs and the scores of UPDRS or PDQ-39, suggesting the potential of APAF1 as a biomarker to monitor motor disabilities during PD progression.

Belonging to the class III transmembrane tyrosine kinase receptor family, CSF1R is a major regulator of microglial development and maintenance in the brain [41]. The binding of the ligand, CSF1 or IL-34, induces homodimerization and autophosphorylation of CSF1R to activate phosphoinositide 3-kinase (PI3K)/Akt, protein kinase C (PKC), and extracellular signal-regulated kinase 1/2 (ERK1/2)pathways [42]. The deficiency of CSF1R leads to a significant reduction in microglial density in rodents [43,44]. On the other hand, the overexpression or activation of CSF1R in microglia upregulate the expression of pro-inflammatory cytokines IL-1β, macrophage inflammatory proteins-1α (MIP-1α), inducible nitric oxide synthase (iNOS), and IL-6 [45], which increase the production of ROS [46]. In substantia nigra, *CSF1* and *CSF1R* expression was increased in PD patients compared with controls [47]. CSF1R expression is also upregulated in the striatum of an MPTP-treated PD mouse model [47]. *CSF1* rs1058885 T variant, which is proposed to reduce the CSF1 activity, is less common in PD patients [48]. Treatment with the CSF1R inhibitor GW2580 significantly attenuates microglial activation, dopaminergic neuron loss, and motor behavioral deficits in an MPTP-induced mouse model [47]. Furthermore, our results consistently found the upregulation of *CSF1R* in PBMCs of PD patients. The levels of *CSF1R* were correlated with cognitive impairment measured by MMSE and MoCA. Similar to our results, the upregulation of CSF1R has been reported in the microglia of post-mortem brain samples from patients with Alzheimer's disease (AD) [49]. Deletion of CSF1R in *APPSwe/PS1*AD mice delayed cognitive decline [50]. These studies indicate the involvement of *CSF1R* expression in cognitive decline during neurodegeneration, while targeting CSF1R signaling may be a viable neuroprotective strategy for PD and other neurodegenerative diseases.

This study has several limitations that may affect the results. The sample size may not be large enough to detect small changes in gene expression in PD. The low proportion of patients with advanced PD may also make it difficult to detect differences in gene expression between the two different PD stages. Additionally, the potential interactions of medications taken by the patients may contribute to differences between the groups. However, our study still provides valuable information on gene expression in PBMCs of PD patients and suggests **a** potential therapeutic benefit of inhibiting *APAF1* and *CSF1R* in these patients. Further research on larger and independent patient groups is needed to confirm these findings.

4. Materials and Methods

4.1. Patient Recruitment

Patients with PD were recruited from the neurology clinics of Chang Gung Memorial Hospital. The diagnosis of PD was based on the United Kingdom PD Society Brain Bank clinical diagnostic criteria by two neurologists specialized in movement disorders (KH Chang and CM Chen) [51]. Controls were recruited from neurology outpatient clinics by a convenience sample of individuals seen at the time of recruitment, and were frequency matched for the sex and age of patients. All subjects received clinical assessment including UPDRS [52], Hoehn and Yahr stage [53], ADL [54], PDQ-39 [55], CDR [56], MMSE [57], MoCA [58], NPI [59], BDI-II [60], and HAM-D [61].

The blood was collected in a PaxgeneTM blood RNA tube (Pre-AnalytiX, Qiagen, Hilden, Germany). Total RNA from leukocytes was extracted using the PaxgeneTM blood RNA Extraction Kit (Pre-AnalytiX, Qiagen) and purified and concentrated using the RNeasy MinElute spin column (RNeasyH MinEluteHCleanup Kit, Qiagen). RNA quality was determined using the A260/A280 absorption ratio and capillary electrophoresis on an Agilent 2100 Bioanalyzer automated analysis system (Agilent, Santa Clara, CA, USA).

4.2. Measurement of α-Synuclein in Plasma

We used the immunomagnetic reduction assay to measure the plasma levels of α-synuclein [62]. Frozen human plasma samples were brought to room temperature for 20 min and then mixed with reagents (MF-ASC–0060, MagQu, New Taipei City, Taiwan) for α-synuclein assay. Calibrators (CA-DEX-0060 and CA-DEX-0080, MagQu) and control solutions (CL-ASC-000T, MagQu) were also used in each batch of measurements. The immunomagnetic reduction analyzer (XacPro-S361, MagQu) was utilized to perform duplicate measurements of α-synuclein for each sample. The average concentration of the duplicated measurements was reported, and plasma α-synuclein levels were determined by technicians who were blinded to the clinical diagnosis.

4.3. Profiling of Relevant Gene Expression Related to Reactive Oxygen Species (ROS) and Inflammation Using a Quantitative Polymerase Chain Reaction (q-PCR) Array

Reverse transcription (RT) was performed using Superscript III (Invitrogen, Waltham, MA, USA) with an initial concentration of 5 μg total RNA. We established an in-house human panel for ROS profiling analysis using real-time qPCR with SYBR green reagents (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 1 min for 40 cycles, on the ABI 7900 HT RT-PCR system (Applied Biosystems). Each sample was analyzed in duplicate. Relative expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method, ΔCt = Ct (target gene) − Ct (*GAPDH*), where Ct indicates the cycle threshold (the fractional cycle number at which the fluorescent signal reaches the detection threshold). The primer sequences for the selected 52 genes are listed in Table 4.

Table 4. Genes and primers of q-PCR array related to oxidative stress and inflammation.

Gene Symbol		Gene Primer (5′→3′)
ALDH1A1	aldehyde dehydrogenase 1 family member A1	F-gttgtcaaaccagcagagca R-caagtcggcatcagctaaca
CR1	complement C3b/C4b receptor 1	F-cccgaactctgcaaacaaat R-gtttagcacgaggcagaagg
CSF1R	colony-stimulating factor 1 receptor	F-aaggtggctgtgaagatgct R-ccttccttcgcagaaagttg
APAF1	apoptotic peptidase activating factor 1	F-tggccttcagcagttctttt R-gggagcaggaatagtgtcca
HIP2	ubiquitin-conjugating enzyme E2 K(UBE2K)	F-ggtgtggcacagtttgtcag R-caccacaacaaagcaccatc
EGF	epidermal growth factor	F-tagctcagtgcagcctcaaa R-gcaccatggctaatgcctat
HSPB8	heat shock protein family B (small) member 8	F-acagccaggaagtcacctgt R-ggcctaacacaaccaagcat
PSMA7	proteasome 20S subunit alpha 7	F-tgatggcactcctaggctct R-gcctcatgacagcaagttca
BCL11B	BCL11 transcription factor B	F-ggagggaatgggagagaaag R-ggatcatctgcttccgtgtt
ST13	ST13 Hsp70 interacting protein	F-ctggaatgcctggactcaat R-aggttgcttttccttcagca
C3	complement C3	F-ggaaaaggaggatggaaagc R-caatggccatgatgtactcg
CSF1	colony-stimulating factor 1	F-ggagacctcgtgccaaatta R-ggccttgtcatgctcttcat
APOA1	apolipoprotein A1	F-tggatgtgctcaaagacagc R-tcacctcctccagatccttg
FAS	Fas cell surface death receptor	F-gccacctttctttctgcaa R-actggagagcagacagcaca
CR2	complement C3d receptor 2	F-caaggcacaattccttggtt R-ctccaggtgcctctttcttg
C3AR1	complement C3a receptor 1	F-tcccttcctttatgccctct R-gtttttgaagtccgctgctc
NLRP1	NLR family pyrin domain containing 1	F-ccagaaacctgaaggagctg R-tgagcacattgaagctcagg
NLRP3	NLR family pyrin domain containing 3	F-aaaggaagtggactgcgaga R-ctggtttaccaggccaaaga
Nrf2	NFE2 like bZIP transcription factor 2(NFE2L2)	F-catgccctcacctgctactt R-tgttctggtgatgccacact
HMOX1	heme oxygenase 1	F-tccgatgggtccttacactc R-taaggaagccagccaagaga
HMOX2	heme oxygenase 2	F-tccggtagtccctgtttttg R-ttctgggtgagagggatgag
GCLM	glutamate-cysteine ligase modifier subunit	F-tcctacctgcaccctcaact R-tgtgaacatcagcctggaaa
BAD	BCL2 associated agonist of cell death	F-caggcctatgcaaaaagagg R-taaacctggctcgcgactta
NQO1	NAD(P)H quinone dehydrogenase 1	F-ttactatgggatggggtcca R-tttcaatgcaccacaagagg
LRPPRC	leucine-rich pentatricopeptide repeat containing	F-cttggcccagtggacatagt R-gaggctgaggcacaagaatc
PSMA2	proteasome 20S subunit alpha 2	F-gccctcttcgctatcagatg R-accaacaggaaccagcaaac
PPARGC1A	PPARG coactivator 1 alpha	F-tttccttttgccatggaatc R-gaaagaaccgctgaacaagc
C4A	complement C4A (Rodgers blood group)	F-cccaatatgatccctgatgg R-ccactgctctgtcttgtcca
C4B	complement C4B (Chido blood group)	F-acggcttccaggttaaggtt R-ctcctcgatccagctattcg

Table 4. *Cont.*

Gene Symbol		Gene Primer (5′→3′)
C1qA	complement C1q A chain	F-gaaatctgcctgtccatcgt R-gcagatgggaagatgaggaa
C5AR1	complement C5a receptor 1	F-atgccatctggttcctcaac R-caggaaggagggtatggtca
CASP1	caspase 1	F-tgttcctgtgatgtggagga R-tgcccacagacattcatacag
IL-1beta	interleukin 1 beta	F-ttcgacacatgggataacga R-tctttcaacacgcaggacag
CASP3	caspase 3	F-tttttcagaggggatcgttg R-cggcctccactggtatttta
CASP6	caspase 6	F-gaagcaggttccctgttttg R-ctcccaaagtgctgggatta
HSF1	heat shock transcription factor 1	F-gacataaagatccgccagga R-ctgcaccagtgagatcagga
HSP90AA1	heat shock protein 90kDa alpha family class A member 1	F-ggcagaggctgataagaacg R-ttcttccatgcgtgatgtgt
OPA1	OPA1, mitochondrial dynamin-like GTPase	F-ccacagatttctcccaagga R-attactgtggggcatggaga
MFN1	mitofusin 1	F-tggggctgtgagctcttaat R-acactccttggtggttccag
MFN2	mitofusin 2	F-tgttggctcagtgcttcatc R-aagtccctccttgtcccagt
HSPA5	heat shock protein family A (Hsp70) member 5	F-tttcacagtgcccaagagtg R-tgatcactcactccccatca
HSPB1	heat shock protein family B (small) member 1	F-acgagatcaccatcccagtc R-tttgacaggtggttgctttg
HSPD1	heat shock protein family D (Hsp60) member 1	F-ttcaggttgtggcagtcaag R-tggtcacaatgacctctcca
DNM1L	dynamin 1-like	F-cagtgtgccaaaggcagtaa R-gatgagtctcccggatttca
BCL2	B-cell CLL/lymphoma 2	F-aagattgatgggatcgttgc R-gcggaacacttgattctggt
BID	BH3 interacting domain death agonist	F-gcaggcctaccctagagaca R-tccatcccatttctggctaa
GSS	glutathione synthetase	F-accgctcgtctctttgacat R-ttgccagcttctttggtctt
GSR	glutathione reductase	F-agtgggactcacggaagatg R-ttcactgcaacagcaaaacc
IKBKB	inhibitor of nuclear factor kappa B kinase subunit beta	F-agcatgaatgcctctcgact R-gccgtgaaactctggtcttg
CASP9	caspase 9	F-ttccctcattttgctccaac R-tggtgcacgcctgtagtaag
DNAJB1	DnaJ heat shock protein family (Hsp40) member B1	F-acagtgaacgtccccactct R-agtccttggggagctcagat
NRF1	nuclear respiratory factor 1	F-gtggcaggacttctttctgc R-taattccatgcgggtttcat
GAPDH	gyceraldehyde-3-phosphate dehydrogenase	F-cgagatccctccaaaatcaa R-ttcacacccatgacgaacat

F: forward primers, R: reversed primers.

4.4. Statistical Analysis

All statistical analyses were conducted using SPSS version 19.0 (SPSS, Chicago, IL, USA). Baseline characteristics and metabolite concentrations are presented as mean ± standard deviation for continuous variables and counts (percentages) for categorical variables. Comparisons between the control group and PD groups, including early PD and advanced PD, were performed using an independent Student's *t* test or one-way analysis of variance (ANOVA) with Tukey's post hoc test. The Pearson correlation coefficient (r) was used to

analyze correlations between levels of gene expression and age or clinical assessment such as UPDRS, H&Y, ADL, PDQ-39, CDR, MMSE, MoCA, NPI, BDI-II, and HAM-D. A p value of <0.05 was considered statistically significant.

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