ORIGINAL ARTICLE

Inflammatory profile discriminates clinical subtypes in *LRRK2*-associated Parkinson's disease

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Keywords:
inflammation, *LRRK2*, Parkinson, phenotype

Received 13 September 2016
Accepted 7 November 2016

European Journal of Neurology 2017, **24:** 427–436

doi:10.1111/ene.13223

Background and purpose: The presentation of Parkinson's disease patients with mutations in the *LRRK2* gene (PD_{LRRK2}) is highly variable, suggesting a strong influence of modifying factors. In this context, inflammation is a potential candidate inducing clinical subtypes.

Methods: An extensive battery of peripheral inflammatory markers was measured in human serum in a multicentre cohort of 142 PD_{LRRK2} patients from the MJFF *LRRK2* Consortium, stratified by three different subtypes as recently proposed for idiopathic Parkinson's disease: diffuse/malignant, intermediate and mainly pure motor.

Results: Patients classified as diffuse/malignant presented with the highest levels of the pro-inflammatory proteins interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1-β (MIP-1-β) paralleled by high levels of the neurotrophic protein brain-derived neurotrophic factor (BDNF). It was also possible to distinguish the clinical subtypes based on their inflammatory profile by using discriminant and area under the receiver operating characteristic curve analysis.

Conclusions: Inflammation seems to be associated with the presence of a specific clinical subtype in PD_{LRRK2} that is characterized by a broad and more severely affected spectrum of motor and non-motor symptoms. The pro-inflammatory metabolites IL-8, MCP-1 and MIP-1-β as well as BDNF are interesting candidates to be included in biomarker panels that aim to differentiate subtypes in PD_{LRRK2} and predict progression.

Introduction

Parkinson's disease (PD) is a complex disorder with heterogeneity in phenotypes and variability in progression of motor and non-motor symptoms. Such a phenomenon also holds true for LRRK2-associated PD (PD_{LRRK2}) where even amongst patients with the

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same mutation phenotypic characteristics are highly variable. A possible explanation might be inter-individual heterogeneity/modification in the underlying pathological processes [1–4]. These findings suggest that different phenotypes exist which might help to detect subtype-specific pathways that could serve as a basis for individualized treatment strategies.

From a clinical point of view, PD_{LRRK2} resembles idiopathic Parkinson's disease (IPD) possibly indicating that subtypes described in IPD may also exist in PD_{LRRK2} [5]. Fereshtehnejad and colleagues recently reported new clinical subtypes of IPD that predicted longitudinal progression. The combination of mild cognitive impairment, orthostatic hypotension and rapid eye movement (REM) sleep behaviour disorder (RBD) indicated a diffuse/malignant subtype that was associated with the most rapid progression rate as opposed to a mainly pure motor subtype [6]. However, the underlying biological causes of such different phenotypes remain to be elucidated and one could imagine more widespread pathways in the diffuse/malignant subtype.

Postmortem and biomarker analyses as well as genetic studies provide evidence for a relevant role of inflammation in the pathogenesis of PD (reviewed in references7–14). Although levels of cytokines are highly variable but generally in the normal range in many PD patients, a substantial proportion show elevated levels of these proteins in serum and cerebrospinal fluid (CSF). This increase indicates an activation of the innate immune system with involvement of astrocytes and activation of microglia [7,9,15]. In this context, astrocytes endocytose α-synuclein species secreted from neurons and induce glial inclusions and inflammatory processes [16]. Neuropathological and neuroimaging studies find a hugely varying extent of neuroinflammation in IPD [7]. However, at this point it is unclear whether such processes are primarily disease-causing or rather disease-maintaining and whether inflammatory profiles relate only to a specific subgroup of patients or are associated with disease progression. It is also unclear why anti-inflammatory drugs fail to have an effect on clinical symptoms despite substantial evidence for a role for inflammation in PD. Two scenarios might explain these paradoxes: (i) only a proportion of PD patients suffer from concomitant inflammation, and (ii) inflammatory cascades differ between subgroups of PD patients.

There is increasing evidence for the involvement of LRRK2 in inflammatory pathways, linking PD_{LRRK2} to the immune system [9,10,17]. This study evaluates whether peripheral inflammatory markers differ between predefined clinical phenotypes as proposed by Fereshtehnejad and colleagues and thereby help to explain the clinical variability.

Participants and methods

Centres and participants

In 2008, the Michael J. Fox Foundation established an international consortium to investigate the role of *LRRK2* in Parkinson's disease (www.michaeljfox.org/page.html?lrrk2-cohort-consortium). The consortium brought together leading groups and experts focusing on genetic forms of PD from nine countries across four continents (Canada, China, France, Germany, Israel, Norway, Spain, Tunisia and the USA). At this point, all centres have already been following cohorts with genetically proven PD_{LRRK2} as well as asymptomatic mutation carriers.

In total, clinical data and serum samples of 149 PD_{LRRK2} patients recruited in Canada, France, Germany, Norway, Spain and the USA were available for the present study. Of these, 142 PD_{LRRK2} patients with proven pathogenic mutations (113 p.G2019S, 23 p.R1441G, 3 p.I2020T, 2 p.N1437H, 1 p.R1441C) had complete sets of clinical data and serum samples and were included in the present analyses.

Ethical approval and consent to participate

The study was approved by the respective local ethics committees of the participating centres (Tübingen: 391/2011BO2). All participants gave written informed consent.

Clinical investigations

The consortium followed standardized data acquisition protocols to ensure that tests conducted at multiple sites can be pooled. Next to demographics such as sex, age, age at onset and disease duration, the following clinical parameters were analysed in the present study. Diagnosis of PD was defined according to the UK Brain Bank criteria with the exception that a positive family history for PD was not considered an exclusion criterion [18]. Severity of motor symptoms was assessed using part III of the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS-III) [19]. Disease stage was classified according to the modified Hoehn and Yahr (H&Y) scale [20]. Cognitive function was tested with the Montreal Cognitive Assessment (MoCA). A cut-off of ≤26 out of 30 points indicated cognitive impairment [21]. A cut-off of ≥5 points in the REM Sleep Behaviour Disorder Screening Questionnaire (RBD Questionnaire) was interpreted as the presence of RBD [22]. The Epworth Sleepiness Scale (ESS) was used to assess excessive daytime sleepiness [23]. Mood

disturbances were evaluated using the Geriatric Depression Scale (GDS) [24]. The Scales for Outcomes in Parkinson's Disease – Autonomic (SCOPA-AUT) was used to assess autonomic dysfunction [25]. Orthostatic dysfunction was present if a subject met at least 1 point in items 14 (light-headedness when standing up), 15 (light-headedness after long standing) or 16 (fainted) of the SCOPA-AUT. Olfactory dysfunction was tested using the University of Pennsylvania Smell Identification Test (UPSIT) [26].

Clinical subtypes

Based on the clinical subtypes reported by Fereshtehnejad and colleagues, all patients were categorized into one of the following subgroups: (i) diffuse/malignant – presence of cognitive impairment + RBD + orthostatic dysfunction ($n = 10$); (ii) intermediate – presence of one or two of cognitive impairment, RBD or orthostatic dysfunction ($n = 103$); (iii) mainly pure motor – absence of cognitive impairment, RBD and orthostatic dysfunction ($n = 29$).

Biomaterial and analyses of inflammatory markers in serum

Standard operating procedures were defined for the collection, preparation and storage of biomaterial. Serum samples were collected between 8.00 and 11.00 a.m. after overnight fasting, prepared and stored according to standardized operating procedures as defined by the MJFF Consortium. Serum was centrifuged at 2000 *g*, 4°C for 10 min and stored at −80°C within 60 min after collection. Levels of 29 immune-associated markers were measured as follows: samples were thawed at room temperature, vortexed, spun at 18 000 *g* for 1 min and pipetted into a master microtitre plate for multiplexed immunoassay. The kit components of the multiplexed immunoassay were kindly provided by Myriad RBM, Austin, TX, USA (http://rbm.myriad.com). After dilution with assay diluents in a ratio of 1:5, an aliquot of 10 µl diluted serum was introduced into one of the capture microsphere multiplexes followed by incubation at room temperature for 1 h. Reporter antibodies were added followed by incubation for an additional hour at room temperature. Streptavidin-phycoerythrin solution was added for development and incubated for 1 h at room temperature. For control purposes, calibrators and controls were included on each microtitre plate. Standard curve, control and sample quality control were performed to ensure proper assay performance (see Table S2 for details on limit of detection, lower limit of quantitation, average concentrations as well as intra- and inter-assay coefficients of variability). Samples were tested in singles. Analysis was performed using the Luminex 100/200 instrument and data were interpreted using the software developed and provided by Myriad RBM.

The following four serum markers were excluded from analysis due to missing values in >5% of study participants: interleukin 1α (IL-1α), IL-7, IL-13, IL-15. Of the remaining, 1% of the overall data were missing values, which were replaced by the overall group mean of the respective parameter. A total of 24 inflammatory serum markers as well as the neurotrophin brain-derived neurotrophic factor (BDNF) were included in the analyses. For a list of all assessed markers see Table 2.

Statistics

Statistical analysis was performed using SPSS 22.0 software for Windows (SPSS Inc., Chicago, IL, USA). An association between age and disease duration with levels of inflammatory markers was tested using Spearman's correlation; manual correction for multiple testing according to Bonferroni was applied by defining $P \leq 0.002$ as statistically significant. Dichotomous data were analysed using the chi-squared test.

To evaluate differences in clinical characteristics as well as in inflammatory profiles based on the clinical subtypes, non-parametric analysis using the Kruskal–Wallis test was performed for group comparisons. This test was preferred to account for the small sample size of the diffuse/malignant and mainly pure motor subgroups. In the case of significant differences in continuous variables, the pair-wise *post hoc* Dunn test, which is the equivalent test to Bonferroni for non-parametric analysis including automatic correction for multiple testing, was applied.

Discriminant analysis including all assessed serum markers was performed to unbiasedly test whether specific inflammatory profiles could discriminate the clinical phenotypes and thereby correctly classify patients to the respective clinical subtype based on the serum marker profile. Discriminant analysis undertakes the same task as linear regression by predicting an outcome. The difference between the two methods is the classification of the dependent variable which should be categorical when using discriminant analysis (in our case the clinical subgroups diffuse/malignant, intermediate, mainly pure motor) as opposed to linear regression where the dependent variable is an interval variable, thereby impeding this method for our analysis. The variable 'clinical subgroup' was introduced as dependent variable whereas all inflammatory markers were entered at once without prior selection (unbiased

analysis) as predictors (independent variables). The discriminant analysis weights the effect of all inflammatory markers in order to identify and combine the most important ones which are referred to as discriminant score.

To estimate the accuracy of those inflammatory serum markers that came up as the most promising ones in the discriminant analyses, the area under the receiver operating characteristic curve (AUC) was calculated with clinical subgroup as conditional variable.

Results

Clinical and demographic features of the subgroups are shown in Table 1.

After correction for multiple testing, Spearman's correlation revealed no significant association between age or disease duration with levels of any of the inflammatory markers.

The three different clinical subtypes did not differ significantly with regard to sex, age, age at onset or disease duration.

The following group-specific data within parentheses are given in the order diffuse/malignant versus intermediate versus mainly pure motor. Patients with the diffuse/malignant and intermediate subtype had higher MDS-UPDRS-III ratings (31 vs. 20 vs. 11; $P = 0.001$) as well as H&Y stages (2.8 vs. 2.0 vs. 2.0; $P = 0.018$) compared to participants classified as mainly pure motor. Levodopa-equivalent daily dosages were no different between the three subtypes (770 vs. 700 vs. 560; $P = 0.502$). By definition, cognitive performance assessed by MoCA scores was worse in patients with the diffuse/malignant and intermediate subtype compared to patients classified as mainly pure motor (24 vs. 24 vs. 27; $P = 0.00002$). Likewise, patients of the diffuse/malignant subgroup had higher RBD ratings (6.5 vs. 3.0 vs. 2.0; $P = 0.00007$) whereas ESS was not different between the three subtypes (10 vs. 7 vs. 6; $P = 0.389$). Overall autonomic dysfunction (total SCOPA-AUT score) was more prominent in patients with the diffuse/malignant and intermediate subtypes compared to patients classified as mainly pure motor (21 vs. 16 vs. 9; $P = 0.00002$). The same was true for mood disturbances (GDS) (8 vs. 4 vs. 1; $P = 0.00007$). UPSIT scores indicated worst olfactory dysfunction in the intermediate subtype (24.0 vs. 19.5 vs. 28.0; $P = 0.001$).

Clinical and demographic data stratified by mutation (p.G2019S vs. p.R1441G) were not significantly different and are given in Table S1.

Patients with the diffuse/malignant subtype had higher levels of pro-inflammatory proteins, namely IL-8 (22.95 vs. 12.60 vs. 10.30 pg/ml; $P = 0.0003$), monocyte chemotactic protein 1 (MCP-1) (596.50 vs. 358.00 vs. 310.00 pg/ml; $P = 0.026$) and macrophage inflammatory protein 1-β (MIP-1-β) (277.00 vs. 207.00 vs. 207.00 pg/ml; $P = 0.013$) as well as the neurotrophin BDNF (26.05 vs. 14.70 vs. 19.40 pg/ml; $P = 0.010$) compared to patients classified as intermediate and mainly pure motor. For details see Table 2 and Fig. 1.

Discriminant analysis including all three subtypes (diffuse/malignant versus intermediate versus mainly pure motor) revealed an overall discrimination of 76.1%. The most important predictors for discrimination of these subgroups were MCP-1 (structure matrix coefficient 0.380; $P = 0.025$), BDNF (structure matrix coefficient 0.376; $P = 0.021$) and tumour necrosis factor α (TNF-α) (structure matrix coefficient 0.300; $P = 0.070$) in discriminant function 1 as well as fatty-acid-binding protein (structure matrix coefficient 0.344; $P = 0.074$) in discriminant function 2. For details see Fig. 2.

Discriminant analysis of the two extreme clinical subtypes (diffuse/malignant versus mainly pure motor) including all 25 assessed serum markers revealed IL-8 as the most important predictor for discrimination of these subgroups (structure matrix coefficient 0.372; $P = 0.0005$). Overall, 94.9% of the cases could be correctly classified to the respective clinical subtype based on the inflammatory profile.

Area under the curve analysis of the two extreme clinical subtypes (diffuse/malignant versus mainly pure motor) revealed an excellent area for IL-8 (0.895; standard error 0.058) and good areas for MCP-1 (area 0.736; standard error 0.091) and MIP-1-β (0.767; standard error 0.075). For an overview see Fig. 3.

Discussion

By assessing inflammatory profiles in three different clinical subtypes of PD_{LRRK2} patients (diffuse/malignant versus intermediate versus mainly pure motor) it is shown that patients classified as diffuse/malignant present with the highest levels of the pro-inflammatory proteins IL-8, MCP-1 and MIP-1-β as well as the neurotrophin BDNF. It seems also possible to discriminate the two extreme clinical subtypes (diffuse/malignant versus mainly pure motor) based on their inflammatory marker profile. In this context, higher levels of pro-inflammatory proteins might be indicative of a more severe and broader motor as well as non-motor phenotype of PD involving not only the central nervous system (CNS) (cognitive impairment, RBD and mood disturbances besides the 'usual' motor dysfunction) but possibly also the peripheral nervous system (PNS) (autonomic dysfunction as

Table 1 Overview of demographic and clinical data for the three clinical subtypes

	Malignant/ diffuse $N = 10$	Intermediate $N = 103$	Mainly pure motor $N = 29$	*P* value malignant/ diffuse, intermediate, mainly pure motor	*P* value *post hoc* Dunn test, malignant/ diffuse, intermediate	*P* value *post hoc* Dunn test, malignant/ diffuse, mainly pure motor	*P* value *post hoc* Dunn test, intermediate, mainly pure motor
p.G2019S mutation (%)	**100**	**77.7**	**79.3**	0.247			
Male sex (%)	**30**	**50.5**	**48.3**	0.465			
Regular anti-inflammatory medication (% of individuals)	**0**	**7.2**	**8**	0.667			
Age (years)	**69**	**67**	64	0.152			
	53.0–80.0	38.0–89.0	35.0–86.0				
	8.6	11.9	13.3				
Age at onset (years)	**60**	**56**	**55**	0.225			
	53.0–70.0	23.0–82.0	29.0–78.0				
	5.6	11.6	12.8				
Disease duration (years)	**10**	**10**	**10**	0.206			
	1.0–18.0	1.0–34.0	1.0–19.0				
	5.1	6.3	5.2				
UPDRS-III	**31**	**20**	11	**0.001**	0.222	**0.003**	**0.008**
	3.0–45.0	2.0–82.0	2.0–48.0				
	11.8	14.7	9.6				
H&Y	**2.8**	**2**	**2**	**0.018**	0.91	0.058	**0.041**
	1.0–4.0	1.0–5.0	1.0–3.0				
	0.9	0.9	0.6				
LEDD	**770**	**700**	**555**	0.502			
	0.0–2600.0	100.0–3400.0	0.0–1920.0				
	835.6	665.7	503.6				
MoCA	**24**	**24**	**27**	**0.00002**	0.999	**0.00002**	**0.00003**
	14.0–26.0	5.0–30.0	27.0–30.0				
	4.7	5.3	1.5				
RBD Questionnaire	**6.5**	**3**	**2**	**0.00007**	**0.001**	**0.00003**	0.142
	5.0–11.0	0.0–12.0	0.0–4.0				
	1.8	2.9	1.3				
ESS	**10**	**7**	**6**	0.389			
	1.0–24.0	0.0–21.0	0.0–24.0				
	8.3	5.6	5.1				
SCOPA-AUT	**21**	**16**	**9**	**0.00002**	0.084	**0.00005**	**0.005**
	13.0–51.0	0.0–45.0	0.0–26.0				
	14.7	10.7	7.2				
GDS	**8**	**4**	**1**	**0.00007**	0.235	**0.001**	**0.001**
	1.0–14.0	0.0–15.0	0.0–11.0				
	5.4	3.8	2.3				
UPSIT	**24**	**19.5**	**28**	**0.001**	0.999	0.555	**0.0005**
	10.0–33.0	0.0–38.0	0.0–38.0				
	7.6	9.4	7.9				

ESS, Epworth Sleepiness Scale; GDS, Geriatric Depression Scale; H&Y, Hoehn and Yahr Scale; LEDD, levodopa-equivalent daily dosage; MoCA, Montreal Cognitive Assessment; RBD, rapid eye movement sleep behaviour disorder; UPDRS-III, Unified Parkinson's Disease Rating Scale; UPSIT, University of Pennsylvania Smell Identification Test. Data are presented as median with range and standard deviation. *P* values reflect results from the Kruskal–Wallis test and, in the case of significant differences between the three clinical subtypes, from the pair-wise *post hoc* Dunn test including correction for multiple comparisons.

indicated by higher total scores of SCOPA AUT). Interestingly, it has already been shown in IPD patients that higher peripheral levels of BDNF, IL-8, MCP-1 and MIP-1 are associated with more severe motor impairment assessed with UPDRS-III, timed up and go and H&Y staging [27,28]. Moreover, higher IL-8 plasma levels were associated with dementia in PD patients carrying a homozygous or heterozygous mutation in the glucocerebrosidase (*GBA*) gene (PD_{GBA}) [29]. Based on these findings in IPD, PD_{GBA} and now also in PD_{LRRK2}, it is intriguing to hypothesize that inflammatory processes act as driving forces

Table 2 Overview of levels of inflammatory-related and neurotrophic markers for the three clinical subtypes

Serum marker	Malignant/ diffuse $N = 10$	Intermediate $N = 103$	Mainly pure motor $N = 29$	*P* value Kruskal–Wallis test, malignant/ diffuse, intermediate, mainly pure motor	*P* value *post hoc* Dunn test, malignant/ diffuse, intermediate	*P* value *post hoc* Dunn test, malignant/ diffuse, mainly pure motor	*P* value *post hoc* Dunn t est, intermediate, mainly pure motor
Alpha fetoprotein (ng/ml)	**0.92** 0.34–2.28 0.64	**0.87** 0.12–14.10 1.42	**0.82** 0.12–4.59 1.06	0.666			
BDNF (ng/ml)	**26.05** 15.40–31.30 5.18	**14.07** 1.52–44.60 9.41	**19.04** 4.81–55.40 11.79	**0.01**	**0.009**	0.102	0.981
ENA-78 (ng/ml)	**2.27** 0.99–5.32 1.7	**1.49** 0.36–8.36 1.36	**1.78** 0.43–5.42 1.1	0.225			
FABP (ng/ml)	**4.18** 2.46–18.90 5.27	**4.33** 0.67–26.70 3.69	**3.03** 0.40–7.87 1.83	0.128			
GH (ng/ml)	**2.53** 0.08–6.76 2.62	**1.11** 0.08–30.80 5.06	**1.92** 0.15–18.40 4.18	0.317			
ICAM-1 (ng/ml)	**142** 62.00–240.00 54.86	**157** 51.60–247.00 38.54	**152** 113.00–262.00 30.13	0.865			
IgE (U/ml)	**17.28** 1.03–905.00 278.44	**18.06** 1.03–633.00 110.76	**15.01** 1.59–94.30 22.51	0.52			
IL-1-β (pg/ml)	**2** 1.19–4.53 1.16	**2.07** 0.29–5.22 0.9	**2.01** 0.56–3.07 0.59	0.765			
IL-4 (pg/ml)	**18.1** 10.80–23.20 3.72	**14.77** 5.57–27.10 4.67	**14.77** 4.43–20.90 4.55	0.101			
IL-6 (pg/ml)	**5.56** 2.14–14.70 3.89	**4.74** 1.27–16.30 2.37	**4.44** 2.63–6.76 1.3	0.314			
IL-8 (pg/ml)	**22.95** 9.18–60.70 14.16	**12.6** 2.93–20.90 20.33	**10.3** 4.30–24.60 5.82	**0.0003**	**0.002**	**0.0006**	0.31
IL-10 (pg/ml)	**3.39** 1.80–7.59 1.68	**3.57** 1.78–19.20 2.8	**3.26** 1.78–7.95 1.5	0.147			
IL-12 p40 (ng/ml)	**0.24** 0.10–0.49 0.12	**0.23** 0.07–0.62 0.09	**0.19** 0.13–0.44 0.09	0.556			
IL-16 (pg/ml)	**545** 253.00–1310.00 307.94	**506** 232.00–982.00 165.11	**564** 287.00–1080.00 188.93	0.387			
IL-18 (pg/ml)	**269.5** 193.00–348.00 50.77	**293** 101.00–771.00 149.55	**258** 141.00–651.00 137.8	0.337			
Leptin (ng/ml)	**10.18** 0.71–23.40 7.84	**11.9** 0.81–113.00 15.66	**7.88** 0.63–69.80 18.3	0.174			
MCP-1 (pg/ml)	**596.5** 215.00–742.00 178.27	**358** 99.90–802.00 142.4	**310** 160.00–1189.00 241.03	**0.026**	**0.048**	**0.022**	0.999
MDC (pg/ml)	**416** 159.00–927.00 215.59	**462** 125.00–940.00 161.26	**506** 268.00–3860.00 647.48	0.725			

(continued)

Table 2 *(Continued)*

Serum marker	Malignant/ diffuse $N = 10$	Intermediate $N = 103$	Mainly pure motor $N = 29$	*P* value Kruskal–Wallis test, malignant/ diffuse, intermediate, mainly pure motor	*P* value *post hoc* Dunn test, malignant/ diffuse, intermediate	*P* value *post hoc* Dunn test, malignant/ diffuse, mainly pure motor	*P* value *post hoc* Dunn t est, intermediate, mainly pure motor
MIP-1-β (pg/ml)	**277** 226.00–481.00 94.77	**207** 18.40–1530.00 173.52	**207** 81.20–498.00 120.52	**0.013**	**0.014**	**0.015**	**0.999**
MMP-3 (ng/ml)	**19.65** 7.43–120.00 33.29	**18.1** 6.31–54.40 10.73	**18.3** 7.57–46.40 10.49	0.92			
MMP-9 (ng/ml)	**22.2** 15.40–46.20 10.6	**23.6** 9.28–121.00 12.16	**23.6** 15.60–35.50 5.59	0.997			
SCF (pg/ml)	**329** 161.00–527.00 98.8	**254** 78.30–635.00 104.81	**252** 183.00–401.00 67.13	0.197			
TF (ng/ml)	**0.28** 0.15–0.63 0.18	**0.3** 0.13–0.74 0.11	**0.27** 0.12–0.51 0.1	0.442			
TNF-α (pg/ml)	**64.8** 57.30–143.00 32.2	**65.5** 21.20–125.00 20.07	**65.5** 35.70–100.00 13.35	0.498			
TPO (pg/ml)	**2.62** 1.17–3.76 0.7	**2.06** 0.19–3.70 0.82	**2.07** 0.74–4.08 0.87	0.086			

BDNF, brain-derived neurotrophic factor; ENA-78, epithelial-derived neutrophil-activating peptide 78; FABP, fatty-acid-binding protein; GH, growth hormone; ICAM-1, intercellular adhesion molecule 1; IgE, immunoglobulin G; IL, interleukin; MCP-1, monocyte chemotactic protein 1; MDC, macrophage-derived chemokine; MIP-1-β, macrophage inflammatory protein 1-β; MMP, matrix-metalloproteinase; SCF, stem cell factor; TF, transfer factor; TNF-α, tumour necrosis factor α; TPO, thrombopoetin. Data are presented as median with range and standard deviation. *P* values reflect results from the Kruskal–Wallis test and, in the case of significant differences between the three clinical subtypes, results from the pair-wise *post hoc* Dunn test including automatic correction for multiple comparisons.

promoting a more severe and broader phenotype in PD patients. Interestingly, these processes seem similar across diverse populations of PD patients and irrespective of the presence/absence of underlying disease-causing mutations. However, the mechanisms that promote inflammation in the different PD cohorts might vary and remain to be elucidated.

Of note, age at onset was similar across subtypes, possibly suggesting that the immune system does not contribute in an important way to the development of PD_{LRRK2} in very early (prodromal) phases, but rather leads to an acceleration of the disease process and to an increased probability of occurrence of additional symptoms in the clinically manifest disease, at least in a subset of patients. However, this interpretation needs validation in a longitudinal cohort of asymptomatic mutation carriers in whom inflammatory profiles are repeatedly assessed in relation to conversion to PD over time.

All pro-inflammatory mediators found to be important in our study (IL-8, MCP-1 and MIP-1-β) represent monocyte-lineage-associated pro-inflammatory chemokines. This is of special interest since LRRK2 is highly expressed in monocytes [30] and exposure to bacterial lipopolysaccharides resulted in an upregulation of LRRK2 protein as well as impaired autophagy in macrophages [10,31]. IL-8 is produced by macrophages and promotes chemotaxis causing granulocytes to migrate toward sites of infectious/injured tissue where, as a second function of IL-8, phagocytosis is induced. Interestingly, secretion of IL-8 is increased by oxidative stress which in turn promotes inflammation and thereby further increases oxidative stress, a phenomenon that is widely discussed as a key event in the pathogenesis of PD (reviewed in reference 32). Of note, PD_{LRRK2} patients carrying the p.G2019S mutation show higher CSF levels of IL-8 compared to IPD [33]. MIP-1 is also produced by macrophages and promotes chemotaxis as well as the synthesis of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α [34] whereas MCP-1 has a chemotactic function on monocytes. BDNF is a widely expressed neurotrophin which plays a crucial role in neuronal survival. Decreased levels are associated with the

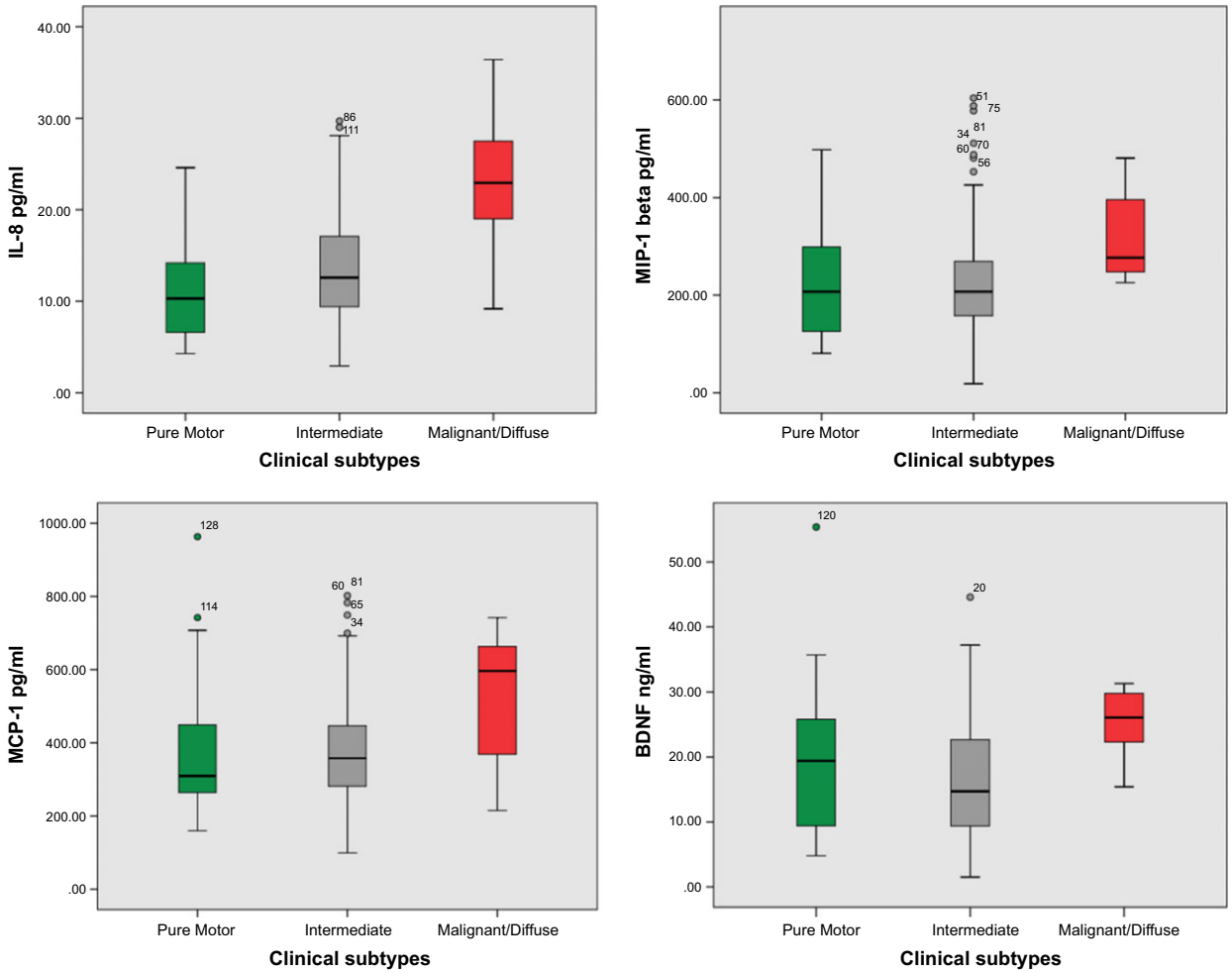

Figure 1 Box-plots of levels of the most significantly different inflammatory-related and neurotrophic markers for the three clinical subtypes. Although overlaps of levels between the groups are detectable, the group of diffuse/malignant shows almost no outliers suggesting that group differences are not due to single outliers in this subgroup. [Colour figure can be viewed at wileyonlinelibrary.com].

occurrence of PD and Alzheimer's disease (AD) [35]. However, increased serum levels of BDNF have been found in PD patients with longer disease duration and more severe motor impairment [28] as well as in patients with mild cognitive impairment and manifest AD [36]. Our results indicate that inflammatory processes are paralleled by increased production of BDNF, possibly as an attempt to counteract tissue damage. This hypothesis is supported by previous studies. In inflammatory brain lesions, activated monocytes directly secrete bioactive BDNF which promotes neuronal survival *in vitro* [37]. Moreover it was demonstrated that BDNF is positively associated with inflammatory markers in AD [38]. Whether a direct mechanistic link exists between LRRK2, monocyte-associated chemokines as well as BDNF and to what degree such interactions are modified by mutations in the *LRRK2* gene need to be further established. Taking all evidence into account so far, inflammatory processes seem not specific to distinct genes but rather are associated with PD or neurodegeneration in general.

As a consequence of the limitations of this study, future studies should address the following aspects. (i) Since our analyses are underpowered, findings from this exploratory study need to be replicated in larger cohorts, preferably also in other PD cohorts with and without different mutations. Such an approach would further allow differentiating whether findings are related to *LRRK2* or represent a more general aspect of PD-associated endophenotypes. (ii) Peripheral serum markers should be analysed in relation to CNS-derived material such as CSF to evaluate whether both systems (PNS and CNS) act in parallel and/or are involved to a different degree. (iii) Information on concomitant immune-related diseases along

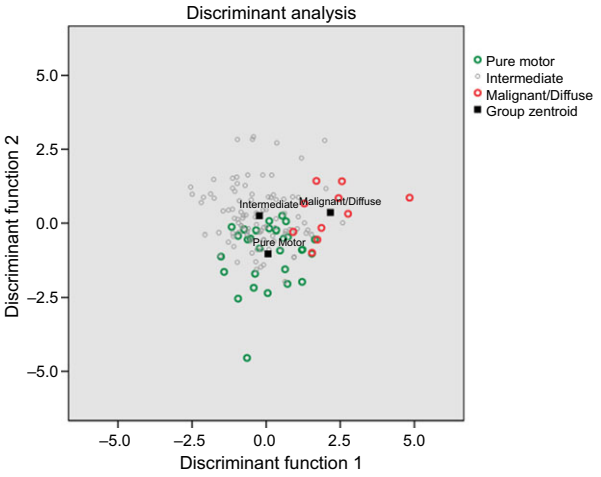

Figure 2 Illustration of the distribution of the discriminant function scores for the three clinical subtypes (diffuse/malignant versus intermediate versus mainly pure motor). The minimal overlap between the two extreme phenotypes diffuse/malignant versus mainly pure motor indicates a relevant discrimination of 94.9% of these clinical subtypes based on activated pro-inflammatory serum marker profiles. Twenty-eight out of 29 mainly pure and 9 out of 10 diffuse/malignant patients have been classified correctly to the respective subgroup. The wide overlap of individuals classified as intermediate with the other two subtypes suggests a poorer discrimination (76.1%) due to largely variable levels of activated pro-inflammatory markers in this subgroup. [Colour figure can be viewed at wileyonlinelibrary.com].

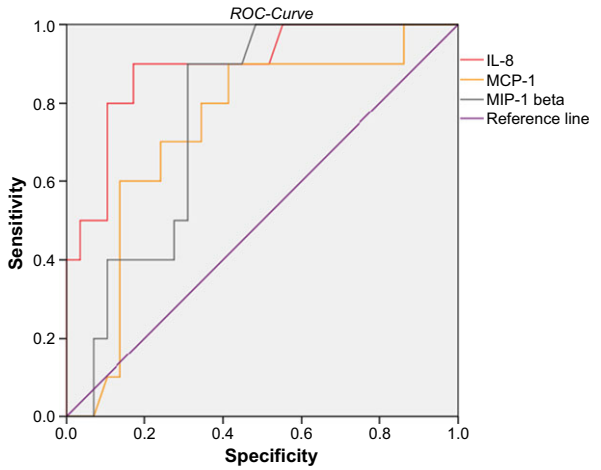

Figure 3 AUC of IL-8, MCP-1 and MIP-1-β in order to distinguish the extreme subtypes diffuse/malignant versus mainly pure motor. Of the three pro-inflammatory markers IL-8, MCP-1 and MIP-1-β that have been identified as the most important ones in group comparison and discriminant analyses, IL-8 had the best AUC with an excellent area of 0.895. ROC, receiver operating characteristic. [Colour figure can be viewed at wileyonlinelibrary.com].

with anti-inflammatory therapies should be assessed. (iv) Studies should be performed in patients with early disease to see if the same distinctions are present at that stage. Pre-manifest *LRRK2* mutation carriers were not investigated since it was previously shown that these individuals do not present elevated levels of inflammatory markers compared to healthy control individuals, even in subgroup analyses in those participants who presented prodromal symptoms of PD [17]. (v) Longitudinal evaluations are needed to directly demonstrate the rate of progression of PD across the subtypes and to assess changes in inflammatory markers over the course of the disease. (vi) If possible in terms of sample size, mutation-specific subgroup analyses should be made to investigate potential differences in the inflammatory profile based on different *LRRK2* mutations within different functional domains. Identifying the reason for different degrees of activation of the immune system might lead to specific disease modifying treatment options. Several scenarios could be envisioned: the increase in inflammatory activity is due to an ongoing disease process such as infection or neurodegeneration itself. Following this line, immune activation would constitute a PD subtype for which immune modulation would be a targeted treatment candidate, which could potentially convert a rapidly progressing 'malignant' case into a more benign form of PD.

In conclusion, it has been shown that elevation of specific inflammatory markers as well as BDNF in serum seems to be associated with the malignant/diffuse subtype [6] in PD_{LRRK2} that is characterized by a broad and more severe spectrum of motor and non-motor symptoms, compared to the mainly pure motor subtype. The pro-inflammatory mediators IL-8, MCP-1 and MIP-1 may serve as interesting parameters to be included in biomarker panels that aim to differentiate subtypes in PD_{LRRK2}. However, the predictive value of these markers for future progression remains to be investigated.

Eventually, our results may be of relevance for PD in general; e.g. the proportion of patients classified as diffuse/malignant appears to be higher in IPD and PD_{GBA} since they more frequently present with RBD [39] and cognitive impairment [2,40] compared to PD_{LRRK2} patients. Stressing this point, it was recently shown longitudinally that higher levels of inflammatory markers predispose to more severe impairment of motor symptoms and cognitive decline in 230 IPD patients [41]. If the prognostic value of inflammatory profiles proves true, one would have a marker for patient stratification in clinical trials and could envision that targeted modifying treatment strategies such as immune modulating therapeutics could be of benefit for the subgroup of PD patients who present with this inflammatory-driven phenotype.

Acknowledgement

We thank all patients and families for supporting this research.

Data and biospecimens used in the analyses were obtained from the MJFF LRRK2 Cohort Consortium. The MJFF LRRK2 Cohort Consortium is coordinated and funded by the Michael J. Fox Foundation for Parkinson's Research. The analysis was generously supported by the MJFF Grant 'Influence of Immune Subtypes on the LRRK2 Phenotype'.

Disclosure of conflicts of interest

KB has received a research grant from the University of Tuebingen (TUEFF) and the German Society of Parkinson's Disease (dPV), funding from the Michael J. Fox Foundation for Parkinson's Research (MJFF), travel grants from the Movement Disorders Society and speaker honoraria from Lundbeck. CP-S has received funding from the MJFF. TOJ serves on the editorial board of *Proteomics*, *Drug Discovery Today*. He has received funding for travel or consultancy from Luminex and Myriad RBM. TG serves on the editorial boards of *Parkinsonism and Related Disorders*, *Movement Disorders* and *Journal of Neurology*; holds a patent re: KASPP (LRRK2) Gene, its Production and Use for the Detection and Treatment of Neurodegenerative Diseases; serves as a consultant for Cephalon Inc. and Merck Serono; serves on speaker's bureaus of Novartis, Merck Serono, Schwarz Pharma, Boehringer Ingelheim and Valeant Pharmaceuticals International; and receives research support from Novartis, the European Union, BMBF (the Federal Ministry of Education and Research) and Helmholtz Association. CM has received funding from the MJFF, the Canadian Institutes of Health Research, the National Parkinson Foundation, the Parkinson Society Canada, the International Parkinson and Movement Disorders Society, the Parkinson Study Group, the Parkinson Disease Foundation and honoraria from Allon Therapeutics, Horizon Pharma and EMD Serono. JOA has received funding from the MJFF and from Norwegian University of Science and Technology/St Olavs Hospital. TF received funding from the MJFF. AB received funding from the MJFF. ET received honoraria for consultancy from Novartis, TEVA, Boehringer Ingelheim, UCB, Solvay and Lundbeck, and he received funding for research from the Spanish Network for Research on Neurodegenerative Disorders (CIBERNED) – Instituto Carlos III (ISCIII), MJFF and Fondo de Investigaciones Sanitarias de la Seguridad Social (FISS). DB has served on scientific advisory boards for Novartis, UCB/Schwarz Pharma, Lundbeck and Teva Pharmaceutical Industries Ltd; has received funding for travel or speaker honoraria from Boehringer Ingelheim, Lundbeck Inc., Novartis, GlaxoSmithKline, UCB/Schwarz Pharma, Merck Serono, Johnson & Johnson and Teva Pharmaceutical Industries Ltd; and has received research support from Janssen, Teva Pharmaceutical Industries Ltd, Solvay Pharmaceuticals Inc./Abbott, Boehringer, UCB, MJFF, BMBF, dPV (German Parkinson's Disease Association), Neuroallianz and Center of Integrative Neurosciences. WM serves on the editorial board of *PLoS One*, received funding from the European Union, the MJFF, Robert Bosch Foundation, Neuroalliance and Janssen. He received speaker honoraria from GlaxoSmithKline, UCB, Licher MT and Rölke Pharma. CS, NSM, AA, DV, JRM, ML, JCC, FC, TK, AB, BS and JFMM have nothing to disclose. There are no conflicts of interest of any of the authors.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Overview of demographic and clinical data stratified by mutation.
Table S2. Overview of quality control of all assessed inflammatory markers (analytes).

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