Discovery and verification of panels of T-lymphocyte proteins as biomarkers of Parkinson’s disease

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The diagnosis of Parkinson’s disease (PD) is currently based on the clinical evaluation of extrapyramidal signs with a considerable error rate. The identification of specific markers might allow PD diagnosis before the onset of classical motor symptoms. By two-dimensional electrophoresis we identified proteome alterations in T-lymphocytes of 17 control subjects and 15 PD patients. The observed changes were used to build predictive models that were verified by the leave-one-out cross-validation. We further built two functions able to stage the subjects. We chose to verify by Western blotting the identity of spots corresponding to β-fibrinogen and transaldolase, two recurrent proteins in six out of 20 spots. β-Fibrinogen levels are lowered in PD patients, whereas a heavy transaldolase set of isoforms was more abundant. Eventually, we identified a list of seven proteins showing different levels in early-onset with respect to late-onset PD patients.
Two-dimensional electrophoresis profiling of T-cell proteins. We obtained T-cell protein expression profiles from the enrolled groups by 2-DE. First, we screened the profiles to identify proteins or protein modifications whose changes were linked to confounding factors such as therapy and age. Spots showing linear Pearson correlation with age (evaluated in control subjects only) or daily L-DOPA dose, or showing significant differences between patients treated or not with dopamine agonists (Wilcoxon test, p<0.05) were excluded from the further analysis. By comparing 2-DE maps from 15 PD patients to 17 control subjects, we selected 20 protein spots showing different levels in the two groups (Wilcoxon test, p<0.05) (Fig. 1 and Supplemental Fig. 1). Proteins corresponding to selected spots were identified by LC-MS/MS as reported in Table 1.

Figure 1 | Identification of 20 spots that discriminate PD patients from control subjects. Their position in the 2-DE map of T-cells from a control subject (a), the fold of change in Log2 scale (b) and the Wilcoxon test p value (c) are reported.
Table 1 | Identity of protein spots

| Spot No. | Protein Description | Uniprot Id | Correlation | p |
|---|---|---|---|---|
| A. PD patients vs. control subjects (Wilcoxon) |
| 86 | Vinculin | P18206 | PD | 0.003 |
| 87 | Vinculin | P18206 | PD | 0.018 |
| 329 | Vimentin | P08670 | PD | 0.006 |
| 335 | Talin-1 | Q9Y490 | PD | 0.016 |
| 362 | Beta-fibrinogen | P02675 | PD | 0.005 |
| 365 | Beta-fibrinogen | P02675 | PD | 0.003 |
| 368 | Beta-fibrinogen | P02675 | PD | 0.014 |
| 369 | Beta-fibrinogen | P02675 | PD | <0.001 |
| 382 | Filamin-A | P21333 | PD | <0.001 |
| 405 | Lymphocyte-specific Protein 1 | P33241 | PD | 0.025 |
| 414 | Septin-6 | Q14141 | PD | 0.047 |
| 591 | Vimentin | P08670 | PD | 0.003 |
| 598 | Moesin | P26038 | PD | 0.12 |
| 657 | Gelsolin | P06396 | PD | 0.023 |
| 676 | Transaldolase | P37837 | PD | 0.007 |
| 679 | Transaldolase | P37837 | PD | 0.014 |
| 842 | Twinfilin-2 | Q6BSO | PD | 0.034 |
| 871 | Rho GDP dissociation inhibitor isoform 2 | P52566 | PD | 0.031 |
| 1639 | beta actin fragment | P60709 | PD | 0.030 |
| 1641 | 14-3-3 epsilon | P62258 | PD | 0.002 |
| B. LOPD patients vs. EOPD patients (Wilcoxon) |
| 351 | Beta-tubulin | P07437 | LOPD | 0.047 |
| 363 | Protein disulfide isomerase A3 | P30101 | LOPD | 0.013 |
| 392 | Vimentin | P08670 | LOPD | 0.046 |
| 505 | Plastin-2 | P13796 | LOPD | 0.013 |
| 792 | Purine nucleoside phosphorylase | P00491 | LOPD | 0.016 |
| 940 | Glutathione S-transferase P | P09211 | LOPD | 0.007 |
| 1787 | PDCD6 interacting protein | Q8WUM4 | LOPD | 0.047 |

(see Supplemental Table 1 for details on protein identification by mass spectrometry). Multiple identifications were refined by matching with reference 2-DE maps available in the SWISS-2DPAGE database (http://world-2dpage.expasy.org/swiss-2dpage/). In particular, the LYMPHOCYTE_HUMAN map was used for disambiguation. In the case ambiguous assignment was not resolved by matching (see Supplemental Results), the correct assignment was verified by Western blotting (Supplemental Fig. 2 and Supplemental Fig. 3). All peptides attributable to keratins or trypsin (common contaminants) were eliminated from the mascot search.

Correlation of selected spot volumes with Hoehn and Yahr score and disease duration. We evaluated the occurrence of linear correlation of selected spot volumes with the Hoehn and Yahr score and the duration of the disease, to test if some of the proposed biomarkers changed with time or disease severity. Correlation with the Hoehn and Yahr score was observed for spots 86, 362, 365, 369, 368, 369, 405, 598 and 1641 (Supplemental Fig. 4). These spot volumes were linearly combined to afford a function that significantly tracks the Hoehn and Yahr score. Multiple regression on PD patients allowed us to determine a set of coefficients for the eight spots listed above (Supplemental Table 2). In this way, the spot volume combination linearly correlates with the Hoehn and Yahr score ($r = 0.674$, p = 0.006) (Fig. 2a). The spots 369, 598 and 1641 correlate with both parameters.

Linear discriminant analysis of selected spot levels. We analyzed all spots (n = 20) showing different levels in PD patients by LDA so to obtain a likelihood score (PD Score) expressed as a linear combination of relative spot volumes (Supplemental Table 3). The spots were selected on the basis of uncorrected $p < 0.05$ so to avoid the possible exclusion of factors that could contribute to the model even if their change should not be considered as significant in multiple testing. Spot combinations were significantly different in PD patients with respect to control subjects (Wilcoxon test, $p < 10^{-4}$), with a cutoff value of 0.67. To effectively test the performance of the model, each subject was iteratively excluded from the training set and predicted on the basis of the other subjects. According to the “leave-one-out” procedure we obtained 87% sensitivity and 81% specificity. Predictions so obtained were used to build a ROC curve with an area under curve of 0.906 (Fig. 3).

A simplification of the model was achieved by ranking the 20 spots in terms of their ability to discriminate PD patients from control subjects. Thus, the six spots showing the worst contribution (weight $< 0.3$) were discarded and the LDA was performed on the remaining 14 spots (Supplemental Table 3). Likelihood scores (PD Score) were significantly different in PD patients with respect to control subjects (Wilcoxon test, $p < 10^{-4}$), with a cutoff value of $-0.31$. In this case, the “leave-one-out” cross-validation procedure of the model allowed us to obtain 100% sensitivity and 94% specificity. Predictions obtained so far were used to build a ROC curve with an area under curve of 0.996 (Fig. 3).

We further simplified the model by removing spots with weight $< 1$, thus obtaining a 9-spot model (Supplemental Table 3). Again, LDA yielded likelihood scores (PD Score) significantly different in
PD patients with respect to control subjects (Wilcoxon test, p < 10^{-8}), with a cutoff value of -2.90. The “leave-one-out” cross-validation procedure of the model allowed us to obtain 100% sensitivity and 88% specificity, and the ROC curve built with these predictions had an area under curve of 0.992 (Fig. 3). For comparison, results of the three proposed predictive models are summarized in Table 2.

To test the power of our analysis we evaluated the intra-group variance and the difference of mean values for each spot included in the three models (equation (4)). The minimum number of subjects required for a significant verification is in the range from 10 to 30 for most spots (Supplemental Table 4).

**Total beta-fibrinogen levels are reduced in PD patients.** We clustered spot groups by aggregative nesting according to Pearson correlation (Supplemental Fig. 6). Spots 362, 365, 368 and 369, all corresponding to β-fibrinogen, were clustered in a single outgroup, showing the most stringent similarity among spots considered in each model. To verify their proper identification and confirm the differences observed by 2-DE with another technique, we measured total β-fibrinogen levels by Western blotting in three control subjects and three PD patients, taken from the training set, and correlated them to the sum of relative volumes for spots 362, 365, 368 and 369 (r = 0.889; p = 1.7×10^{-4}). Downregulation of β-fibrinogen involves all 2-DE isoforms, without an appreciable qualitative change of the pattern (Fig. 4).

**Total transaldolase levels are increased in PD patients.** Spots 676 and 679, both corresponding to transaldolase, showed increased levels in PD patients by 2-DE quantification and close correlation by aggregative nesting (Supplemental Fig. 6). To confirm this finding we evaluated transaldolase expression by two-dimensional Western blotting. Fig. 5 shows the characteristic 2-DE pattern of transaldolase, with two trains of spots at different apparent molecular weight. In particular, its pattern was altered in three PD patients (two LO PD and one EO PD) with respect to three control subjects (two healthy subjects and one with atypical parkinsonism), patients showing a higher abundance of the higher molecular weight isoforms.

**Linear discriminant analysis of spots showing differences between late-onset and early-onset patients.** To test the possibility to discriminate PD subtypes, we compared LO PD patients to EO PD patients (Fig. 6). We selected 7 protein spots (Wilcoxon test, p < 0.05) and identified them by LC-MS/MS (Table 1; see Supplemental Table 4 | Predictors of PD severity and duration. Panels a and b: a linear combination of spots 86 (vinculin), 362 (vinculin), 365 (β-fibrinogen), 368 (β-fibrinogen), 369 (β-fibrinogen), 405 (lymphocyte-specific protein 1), 598 (moesin) and 1641 (14-3-3 epsilon) correlates with the Hoehn and Yahr score. Panels c and d: a linear combination of spots 369 (β-fibrinogen), 591 (vimentin), 598 (moesin) and 1641 (14-3-3 epsilon) correlates with the disease duration. Linear correlations are significant both excluding (a, c) and including (b, d) control subjects. P values refer to slope different from zero.
We performed a linear discriminant analysis on selected spots in LO patients with respect to EO patients so to obtain a likelihood score (LO vs. EO Score) expressed as a linear combination of relative spot volumes (Supplemental Table 5). Spot combinations were significantly different in LO PD patients with respect to EO PD patients (Wilcoxon test, \( p = 0.006 \)), with a cutoff value of 11.0. To effectively test the performance of the model, we performed the "leave-one-out" procedure described above and obtained 71% sensitivity and 100% specificity. Predictions obtained so far were used to build a ROC curve with an area under curve of 0.911 (Fig. 7).

In this study we showed that T-lymphocyte proteome changes may be a valid tool to classify PD patients. Furthermore, we demonstrated that linear combinations of selected spots of the 2-DE gel display linear correlation with parameters such as disease duration (expressed as years from onset) and Hoehn and Yahr score. Eventually, we found a set of seven spots that can differentiate LO from EO PD patients.

The main advantage of this investigation is to merge the powerful ability of 2-DE to identify quantitative changes in single protein isoforms or post-translational modifications with a multiparametric approach where distinct 2-DE spots are combined in a predictive function. A similar result might not be reached by antibody-based techniques such as ELISA, where total protein levels are measured4. The combination of several spot volumes increased the specificity of the proposed biomarkers, while maintaining high sensitivity. Indeed, we were able to obtain ROC AUC values higher than 0.99 both in the 14-spot and in the 9-spot model. The 20-spot dataset was selected on the basis of the non-parametric Wilcoxon test, since the distribution of spot volumes deviates from normality and intra-group variance values are dissimilar. Therefore, LDA does not grant the best classification results when all 20 spots are included in the discriminant model. In an original way, we calculated the weight of each spot in the model (equation (3)), so to exclude those with a limited contribution. In this way, performance parameters of the models were markedly improved (Table 2).
Performance parameters obtained in this investigation need to be compared to similar biomarker discovery and validation studies appeared in the literature so far\textsuperscript{3,4,5,14}. Targeted studies usually evaluate single biomarker alterations at the peripheral level that were first observed in the affected tissue\textsuperscript{15}. A marked improvement is achieved when two or more biomarkers are measured in order to predict a disease probability based on multiple observations. Recently, a panel of four gene transcripts, selected on the basis of their altered levels in the substantia nigra, was measured in total blood cells of a large group of sporadic PD patients with respect to a smaller group of control subjects, but verified on the training set itself\textsuperscript{16}. Measurement of $\alpha$-synuclein, tau and total protein concentrations in CSF of patients with PD, Alzheimer disease, Dementia with Lewy Body, multiple system atrophy and non-neurodegenerative neurological disorders allowed Mollenhauer and coworkers to define a very accurate predictive model (ROC AUC $= 0.909$ for the training set); in a subsequent validation phase this model displayed a AUC $= 0.706$ for the classification of PD patients and control subjects\textsuperscript{17}. Unbiased discovery studies that rely on proteomics\textsuperscript{4} or metabolomics\textsuperscript{18} have the great advantage to identify a larger panel of targets to be included in the predictive model and to explore a greater universe of candidates without limiting to those that have been related to pathogenesis. We intentionally avoided to focus on the identity of selected proteins. Indeed, every single protein is far from being a significant element for PD early diagnosis\textsuperscript{4,15}. What we wanted to point out was a protein fingerprinting of the disease state, more than a functional correlation between proteins altered at the central level and those that mirror neurodegeneration at the peripheral level. Nevertheless, we chose to verify the identity of spots corresponding to $\beta$-fibrinogen and transaldolase, not only in order to validate the results with an independent technique, but also for the predominant role of these spots in the models. Although little is known on the role of $\beta$-fibrinogen in T-lymphocytes, its downregulation complements

**Figure 4** | Western blotting validation of $\beta$-fibrinogen. Panel a: Western blotting of total $\beta$-fibrinogen from six subjects selected among those enrolled in the biomarker discovery study. Panel b: Linear correlation of total $\beta$-fibrinogen levels as measured by 2-DE spot volume and by Western blotting. The correlation coefficient is calculated according to equation (1). Panel c: Two representative 2-DE Western blotting experiments.

**Figure 5** | Two-dimensional Western blotting of transaldolase, showing two distinct pI distributions at different MW. Samples have been selected in the training set among those showing larger discrepancies in the 2-DE quantification to validate the correct assignment of protein spots.
Figure 6 | Identification of 7 spots that discriminate late-onset (LO) from early-onset (EO) patients. For each of the 7 spots, the position in the 2-DE map of T-cells from a control subject (a), the fold of change in Log2 scale (b) and the Wilcoxon test p value (c) are reported.
the preliminary evidence that two distinct isoforms of α-fibrinogen either correlate with the disease state or with disease duration in PBL of PD patients<sup>12</sup>. Noticeably, the 2-DE β-fibrinogen pattern is not significantly modified in PD patients with respect to control subjects and a lower total β-fibrinogen level was observed in lymphocytes of PD patients. On the other hand, transaldolase, a key enzyme of the pentose phosphate pathway (PPP), has a characteristic pattern in 2-DE<sup>19</sup> and has been involved in apoptotic cell death both in cancer and in neurodegeneration<sup>20</sup>. Pattern modifications have been linked to PPP regulation<sup>19</sup>. When comparing PD patients with control subjects, it appeared that PD patients have a remarkably higher abundance of the heavy isoforms. Although the experiment was limited to a small number of subjects to confirm proteomic results, the ratio between the two forms could contribute to the diagnostic panel.

A limitation that might be envisaged in the present investigation is the limited number of cases and control subjects included in the study. It should be noticed, however, that we have enrolled a sufficient number of subjects (about 15 per each group) based on established guidelines for biomarker discovery studies<sup>15</sup>. Being aware that, insufficient number of subjects (about 15 per each group) based on established guidelines for biomarker discovery studies<sup>15</sup>. Being aware that, a lower total β-fibrinogen level was observed in lymphocytes of PD patients. On the other hand, transaldolase, a key enzyme of the pentose phosphate pathway (PPP), has a characteristic pattern in 2-DE<sup>19</sup> and has been involved in apoptotic cell death both in cancer and in neurodegeneration<sup>20</sup>. Pattern modifications have been linked to PPP regulation<sup>19</sup>. When comparing PD patients with control subjects, it appeared that PD patients have a remarkably higher abundance of the heavy isoforms. Although the experiment was limited to a small number of subjects to confirm proteomic results, the ratio between the two forms could contribute to the diagnostic panel.

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phosphate-buffered saline (PBS) and stratified in two 50 ml tubes on top of 15 ml of Lypholyte®-H (Cedarlane) each. After centrifugation (800 x g, 20 min, 20 °C), peripheral blood mononuclear cells (PBMC) were removed and pelleted through centrifugation (400 x g, 15 min, 20 °C). Pellets were washed twice with 10 ml of magnetic-activated cell sorting (MACS) buffer (Miltenyi Biotec). The isolation of T lymphocytes was achieved by MACS with the Pan T-cell isolation kit (Miltenyi Biotec) using the manufacturer's protocol.

**Two-dimensional electrophoresis and image analysis.** T-lymphocytes were resuspended in 120 μl UTC (7 μM urea, 2 M thiourea and 4% 3-(cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) with a protease inhibitors cocktail (Sigma-Aldrich). Cells were left in this solution for 30 min to allow a complete cell lysis, sonicated (3 x 5 sec) and centrifuged (12000 x g, 20 min, 10 °C) to precipitate the cellular debris. Protein concentration in the extracts was determined according to Bradford. Total proteins (200 μg) were separated through two-dimensional electrophoresis (2-DE) using 18 cm IPG DryStrips with a nonlinear 3–10 pH gradient (GE Healthcare) followed by 14%T SDS-PAGE. The gel images were acquired (12 bit grayscale) with the GelDoc-It Imaging System (UVP) and analyzed with ImageMaster 2D Platinum (GE Healthcare). A common set of about 300 spots was selected for statistical analysis. Spots that were missing in more than 25% of gels were not taken into account.

**Statistical analysis.** Spot volumes were normalized by the total volume of a subset of spots common to all gels. Missing spot values were replaced by the mean value of the spot volume of the group or, if the mean was lower than the 98th percentile, by the minimum value observed in the group. Relative volumes were analyzed by the non-parametric Wilcoxon test to find significant differences (p < 0.05) in patients with PD with respect to control subjects, in early-onset patients with respect to late-onset patients and in PD patients treated or not with dopamine agonists.

The Pearson linear correlation coefficient r was evaluated according to equation (1), where x is the independent variable (i.e., Hoehen and Yahr score, age, years from onset, daily L-DOPA dose), y is the relative spot volume, t is the mean x value, t is the mean y value, x̄ is the x x̄ y product, sxy is the product of t and t mean values.

\[ r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}} \]

Predictive models for the classification of PD patients with respect to control subjects and of early-onset patients with respect to late-onset patients were built by linear discriminant analysis (LDA) of the spots identified as described above. In this case, missing values were set to the lower 0.05% confidence interval according to Fisher's test. At least 2 different peptides had to be assigned. Peptide and protein identifications corresponding to keratins or tropins were not taken into account.

**Quantitative Western blotting analysis.** For one-dimensional Western blotting, cell lyses (20 μg) were denatured in Laemmli sample buffer for 5 min at 98 °C and electrophoresed on 10% SDS-PAGE gel. 2-DE was performed as described above. Gels were transferred to polyvinylidene difluoride (PVDF) membranes at 1 mA/cm², 1.5 h (TE77pwr, Hoefer). Membranes were saturated in 5% non-fat milk in TBS-T (0.1 M Tris-HCl pH 7.4, 1.5 M NaCl and 0.5% Tween-20) and incubated in the same buffer at 4 °C overnight with goat anti-fibrinogen polyclonal antibody (Abnova PAB11318), 1:10000 dilution, with mouse anti-transaldolase polyclonal antibody (Abcam ab64767), 1:500 dilution, or with mouse anti-β-actin monoclonal antibody (GenTex GTX23280), 1:3000 dilution. Membranes were then washed with TBS-T and incubated with peroxidase-conjugated anti-goat-IgG antibody (Millipore AP106P), 1:8000 in 5% milk-TBS-T, or anti-mouse-IgG antibody (Millipore 12-349), 1:3000 in 5% milk-TBS-T, respectively, for chemiluminescence detection (Millipore). Photographic films were scanned with a Tropomyosin V759. Proteomic characterization of Jurkat T leukemic cells after dopamine stimulation: A model of circulating dopamine-sensitive cells. *Biosciences*. 93, 892–898 (2011).

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Author contributions
TA, ACP and SO performed the 2-DE and Western blot experiments. CC and MZ enrolled the patients and collected clinical/demographic data. DC identified the proteins by mass spectrometry. MF wrote the software routines in R and analyzed the data. TA and MF designed the experiments. TA, MF and LL wrote the manuscript. All authors critically revised the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: TA, MF and LL are listed as inventors in the patent application IT/TO2011A001241 entitled “Method for the in vitro diagnosis of Parkinson’s disease”.

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