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## **Disturbed expression of autophagy genes in blood of Parkinson's disease patients**

Saïd El Haddad<sup>a</sup>, Amandine Serrano<sup>a</sup>, Frédéric Moal<sup>b</sup>, Thierry Normand<sup>a</sup>, Chloé Robin<sup>a</sup>, Stéphane Charpentier<sup>a</sup>, Antoine Valery<sup>c</sup>, Fabienne Brulé-Morabito<sup>a</sup>, Pascal Auzou<sup>d</sup>, Lucile Mollet<sup>a</sup>, Canan Ozsancak<sup>d</sup>, Alain Legrand<sup>a,\*</sup>

<sup>a</sup> *Centre de Biophysique Moléculaire, CNRS UPR 4301, affiliated with the Université d'Orléans – Pôle Universitaire Centre Val de Loire, Rue Charles Sadron, 45071 Orléans Cedex 2, France*

<sup>b</sup> *LIFO Bat. 3IA, Université d'Orléans, Rue Léonard de Vinci, B.P. 6759F-45067 ORLEANS Cedex 2, France*

<sup>c</sup> *Département d'Information Médicale, Centre Hospitalier Régional d'Orléans-La Source CHRO, Orléans, France*

<sup>d</sup> *Service de Neurologie, Centre Hospitalier Régional d'Orléans-La Source CHRO, Orléans, France*

**\* Corresponding author at:** Centre de Biophysique Moléculaire, CNRS UPR4301, Rue Charles Sadron, CS80054, 45071 Orléans Cedex 2, France. Tel.: +33 238255536; E-mail: [alain.legrand@cnrs.fr](mailto:alain.legrand@cnrs.fr)

## **Abstract**

Parkinson's disease (PD) is a common neurodegenerative disorder which affects dopaminergic neurons leading to alteration of numerous cellular pathways. Several reports highlight that PD disturbs also other cells than CNS neurons including PBMCs, which could lead, among other things, to dysfunctions of immune functions. Because autophagy could be altered in PD, a monocentric pilot study was performed to quantify the transcripts levels of several autophagy genes in blood cells. *MAP1LC3B*, *GABARAP*, *GABARAPL1*, *GABARAPL2* and *P62/SQSTM1* were found to be overexpressed in patients. On the contrary, transcripts for *HSPA8* and *GAPDH* were both decreased. Expression of *MAP1LC3B* and *GABARAP* was able to successfully segregate PD patients from healthy controls. The accuracy of this segregation was substantially increased when combined expressions of *MAP1LC3B* and *GAPDH* or *GABARAP* and *GAPDH* were used as categorical variables. This pilot study suggests that autophagy genes expression is dysregulated in PD patients and may open new perspectives for the characterisation of prediction markers.

**Keywords:** Parkinson's disease; Autophagy genes; Differential gene expression; Blood cells; Droplet Digital PCR.

## **1. Introduction**

Parkinson's disease (PD) is a common neurodegenerative disorder triggered by a loss of dopaminergic neurons in the substantia nigra (Poewe et al., 2017). PD usually begins in the sixth or seventh decade of life and is manifested by motor and non-motor symptoms (Fahn, 2003). The molecular processes leading to neuropathological alterations and cell death are far from being deciphered and the main drugs available nowadays are designed to compensate for the lack of dopamine and cannot cure the disease (Fahn, 2003). A hallmark of the affected cells is the presence of abnormal deposits, called Lewy bodies, which contain a high level of alpha-synuclein (Spillantini et al., 1997). However, various pathways could lead to neuronal death (Michel et al., 2016) and the functional consequence of Alpha-synuclein inclusions and the link with cytotoxicity is still under debate (Shulman et al., 2011). Decoding the mechanisms underlying the development and progression of the disease is a major research challenge that could lead to key therapeutic progresses. From a diagnosis point of view, identification of PD is still challenging, especially in the early stages in the absence of motor symptoms. Currently, PD diagnosis is based on clinical criteria and typical symptoms which appear when the majority of the dopaminergic neurons have already died (Cookson et al., 2008).

Different reports show that PD could affect other cells than CNS neurons including PBMCs of the immune system (Chen et al., 2018; Caggiu et al., 2019). Among them, numerous studies performed on blood from PD patients showed alteration of production of non-coding RNA and mRNA related to pathways also dysregulated in the pathological brain, such as dopamine metabolism, protein degradation, mitochondrial function, apoptosis or inflammation (Nagai et al., 1996; Barbanti et al., 1999; Scherzer et al., 2007; Buttarelli et al., 2009; Martins et al., 2011; Mutez et al., 2011; Calligaris et al., 2015; Locascio et al., 2015; Santiago and Potashkin, 2015; Santiago et al., 2016; Shamir et al., 2017), for reviews see (Gwinn et al., 2017; Borraigeiro et al., 2018). Moreover, dysregulation of macroautophagy and chaperone-mediated autophagy (CMA) pathways has been observed in the brains of patients and in animal models of PD, indicating the emerging role of autophagy in this disease (Alvarez-Erviti et al., 2010; Gonzalez-Polo et al., 2013). As already observed in the affected brain, significant activation of the autophagy response was also identified within PBMCs of patients with PD (Prigione et al., 2010).

Hence, we undertook a pilot study to test whether the transcript levels of genes related to autophagy were altered in PBMCs of PD patients. Subsequently several machine learning algorithms

were used to test whether the highlighted differential expressions could be a basis of discrimination between PD patients and controls. These classification algorithms are commonly used to differentiate groups of populations and represent auxiliary tools to help with diagnostic and prognostic problems in medical domains, such as significance of specific biomarkers, importance of clinical parameters, therapy planning or patient management (Tarca et al., 2007). In this regard, the expression of the *MAP1LC3B* and *GABARAP* genes could constitute predictive data to segregate PBMCs according to PD status.

## **2. Materials and Methods**

### *2.1. Ethical review*

The study was approved by the Person's Protection Committee of Tours Hospital, France, (CPP#2014-S20) and written consent was obtained from every participant. Blood samples for the control group were obtained with informed consent and did not require additional ethical review. All informed consent followed the ethical guidelines set forth by the Declaration of Helsinki (1964). This article does not contain any studies with animals performed by any of the authors.

### *2.2. Participants*

For this pilot study, 36 patients diagnosed with PD (female = 14, male = 22) were recruited from the Neurological Unit of the Orléans Hospital (CHRO, Centre Hospitalier Régional d'Orléans, France). Motor symptoms were rated according to the international Unified Parkinson's Disease Rating Scale (UPDRS), including Hoehn and Yahr score (Supplementary Table S1). The control group consisted of 40 healthy controls (female = 7, male = 33) recruited among volunteer blood donors in the same hospital (French Blood Establishment). They satisfied the criteria for blood donation according to the rules of the French Blood Establishment (weight: more than 50 Kg, age: between 18 and 70 years old, no transfusion, no graft, good health, travel restriction, negative for many viruses, including HIV and Hepatitis B and C, ...). Blood samples were collected in the same conditions for both groups in non-fasting conditions. Four sets of RT-PCR were performed, each one containing both samples from patients and controls. The mean ages of PD patients and control groups were respectively  $67.5 \pm 10$  years and  $57 \pm 9$  years.

### *2.3. Genes tested in the study*

The mRNA levels of several macroautophagy genes related to the regulation and formation of autophagosomes were analysed. Among these genes were: *BECN1* (encoding ATG6/Beclin-1), essential to autophagosome initiation, *MAP1LC3B* (Microtubule-associated protein 1A/1B light chain 3B), *GABARAP* (GABA<sub>A</sub>-receptor-associated protein), *GABARAPL1* and *GABARAPL2* (GABARAP-like proteins 1 and 2, respectively), crucial to autophagosome formation, elongation and maturation, *ATG9a*, involved in phagophore expansion and *p62/SQSTM1* (Sequestosome-1), a non *ATG gene*, an adapter that can drive selected substrates to the autophagosomes (Johansen and Lamark, 2014). *HSPA8*, a gene encoding Hsc70 linked to CMA (Chiang et al., 1989; Sala et al., 2014), was analysed since its expression has already been described as reduced in PBMCs of PD patients (Sala et al., 2014; Papagiannakis et al., 2015). *GAPDH*, a multifunctional gene, was also included in this analysis. This gene is often used as a calibration reference for quantification of gene expression (Kozera and Rapacz, 2013; Borrageiro et al., 2018) but is also related to autophagy and neurodegenerative diseases, for reviews, see (Gerszon and Rodacka, 2018; Butera et al., 2019). Finally, two other genes have been tested: *SNCA*, the gene encoding Alpha-synuclein, for its relevance in PD development, and *GALIG*, a gene encoding Cytogaligin, a protein that has been shown to interact with Alpha-synuclein (El Haddad et al., 2018). The list of these genes is reported in Supplementary Table S2.

#### 2.4. Isolation of PBMC, RNA extraction and reverse transcription (RT)

Peripheral blood samples (7 ml) were collected from patients and healthy controls into EDTA vacutainer tubes. Between  $7 \times 10^6$  and  $22 \times 10^6$  PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Premium (GE Healthcare, Little Chalfont, UK). Total RNA was isolated by using the NucleoSpin® RNA II Kit (Macherey-Nagel, Hoerd, France). cDNA was synthesised from 500 ng of total RNA with the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and stored at -20 °C.

#### 2.5. Droplet Digital PCR (ddPCR)

Absolute transcript quantification was performed using the ddPCR technology following manufacturer's instructions (Bio-Rad). For each tested gene, the linear range of the ddPCR was estimated using tenfold serial dilutions of cloned cDNA as substrate. In all cases, 20 ng of input reverse-transcribed RNA (cDNA) showed no saturation of positive droplets resulting in a suitable dynamic

range of ddPCR and making possible the use the Poisson's algorithm (Hindson et al., 2011; Zhao et al., 2016). The complete list of primers is reported in Supplementary Table S2. The PCR reaction was set up in a volume of 22  $\mu$ l containing 20 ng of reverse-transcribed RNA (cDNA), 100 nM of each primer and 11  $\mu$ l of ddPCR EvaGreen® Supermix (2X) (Thermo Fisher Scientific). The mix reaction was placed into an 8-channel cartridge (Bio-Rad Laboratories) and 70  $\mu$ l of droplet generating oil were added. Droplets were produced using the QX200™ droplet generator (Bio-Rad Laboratories). PCR was performed on the sealed plate with the following program: 95 °C heat activation for 5 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a terminal extension cycle 72 °C for 5 min. All steps had a ramp rate of 2 °C/s. The droplets were stabilised by an ultimate step: 4 °C for 5 min and 90 °C for 5 min. Droplet fluorescence was subsequently analysed using the QX200™ reader (Bio-Rad Laboratories). Absolute quantification was performed with QuantaSoft (Bio-Rad). Values were expressed as number of cDNA per ng of input reverse-transcribed RNA (cDNA). The specificity of the amplified fragments was confirmed by DNA sequencing (Eurofins, Germany).

## 2.6. Statistical analysis

Statistical analyses were performed with the Mann-Whitney test using StatEL software ([www.adsciences.edu](http://www.adsciences.edu)). p values were adjusted for multiple comparisons, using the Holm-Sidak method, and the statistical significance was set as p value less than 0.01. Sample size calculation was performed to guarantee a desired power of 0.8 ([www.stat.ubc.ca/~rollin/stats/ssize/n2.html](http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html)). Influences of sex and severity of the disease (Hoehn and Yahr score) were evaluated with the Mann-Whitney test and the Kruskal-Wallis test, respectively. After checking of the normal distribution of *GAPDH* expression in samples using a Shapiro-Wilks test, a Student's t test was applied to analyse the statistical differences between samples.

## 2.7. Predictions with machine learning algorithms (classifiers)

Predictions were performed with the software Scikit-learn, a Python module integrating state-of-the-art machine learning engines (Pedregosa et al., 2011). All appropriate features were processed with three standard classification methods: support vector machine (SVM with linear Kernel), logistic regression (LR) and random forests (RF). Stratified five-fold cross-validations were performed to assess classification performance. Briefly, datasets were randomly divided into five equal parts. Four of them

were used as training sets and the last one was used in turn as the test set. The accuracy is computed on this remaining fifth part. This process is repeated 5 times on each part to calculate the mean accuracy and the 95% confidence interval. All samples in each part were selected to test the classifier trained by samples from the other parts. Consequently, final evaluation is carried out on tested sets that were not used to define the model coefficients. Determination of the predictive accuracy enabled us to evaluate the global performance of the model. Accuracy was calculated as the ratio of all correct predictions (true positive plus true negative) to the total number of cases evaluated.

### 3. Results

#### 3.1. Quantification of mRNA transcripts in PBMCs of patients with PD

mRNA levels from genes related to macroautophagy (*BECN1*, *MAP1LC3B*, *GABARAP*, *GABARAPL1*, *GABARAPL2*, *p62/SQSTM1* and *ATG9a*), to CMA (*HSPA8*) and to mitophagy (*GAPDH*) were analysed by RT-ddPCR for absolute quantification in PBMCs from a population of 36 patients diagnosed with PD and compared to 40 healthy blood donors (Table 1). Two other genes were also included in the study: *SNCA*, (the Alpha-synuclein encoding gene) and *GALIG* (an Alpha-synuclein-interacting protein encoding gene). Transcript quantifications and statistical analyses are reported in Table 1 and Fig. 1.

The four genes from the *ATG8* family (*MAP1LC3B*, *GABARAP*, *GABARAPL1* and *GABARAPL2*) and the autophagolysosome adapter *p62/SQSTM1* showed a statistically significant increase in transcript levels in PD patients. On the contrary, *HSPA8* exhibited lower levels of mRNA in PD patients. A power analysis was performed (power = 0.8) and established that the samples had an appropriate size.

No statistical significant difference was detected between PD patients and control subjects regarding the expression of the two remaining genes associated with autophagy (*BECN1* and *ATG9a*) as well as for the *SNCA* and *GALIG* genes. *GAPDH* displayed a mild reduction of expression in patients and the Mann Whitney test indicated a p-value at the limit of significance (p-value <0.01) as calculated after application of the Holm-Sidak method (Table 1). The statistical significance between the two groups was confirmed by application of a Student's t-test after checking of the normal distribution of the samples expressing *GAPDH* by the Shapiro-Wilks test (p-value < 6.8.10<sup>-3</sup>).

Genes whose number of transcripts were found statistically different in patients were further tested to highlight a possible influence of disease severity (based on the Hoehn and Yahr score). No statistical links were evidenced when Hoehn and Yahr scores were taken into account (Supplementary Table S3).



In addition, due to the significant difference in gender distribution in the two groups, we have compared the gene expression levels between females and males from the Parkinson group. No significant difference was evidenced whatever the gene analyzed. The same observation was made when comparing females and males from the control group (Supplementary Table S4). Finally, we have compared the expression levels of males from the patients and controls groups. These data confirmed the statistical analysis reported in Table 1. Another point to consider is related to age distribution which was not evenly matched in control and patients groups ( $67.5 \pm 10$  years vs  $57 \pm 9$  years). This disproportion resulted from the natural age difference between the patient population and that one of volunteer blood donors. To take into account this age discrepancy, we have restrained the statistical analyses to subgroups containing patients and controls within the same age range (42 to 67 years). These subgroups represent 42 % (15 individuals) and 85 % (35 individuals) of the total PD population and controls respectively. The results, reported in Supplemental Table S5, confirmed the data that were obtained with the complete groups and described in Table 1. Consequently, even if the two population displayed imperfect matching for age, it remains that it is not a discriminatory factor between the two studied populations. Moreover, when considering *GAPDH* expression in these subgroups of the same age range, a net significant difference was evidenced in patients and controls groups confirming that *GAPDH* expression was significantly lower in PD patients.

It should be noted that a positive correlation was evidenced between age and transcript levels for the *MAP1LC3B*, *GABARAP* and *GAPDH* genes but only in the PD group and not in the control group (Supplementary Fig. S1 and Supplementary Table S6). For these genes, the increase of transcripts in the PD group was not a direct consequence of age but instead can be attributed to the disease.

### 3.2. Classification prediction with machine learning algorithms

These discrepancies in transcript levels of the autophagy genes between PD patients and controls prompted us to consider a prognostic study using mRNA quantification. Is it possible to predict with good accuracy whether a blood sample arises from a sick or a non-sick individual? To answer this question, we have used different standard classifiers (i.e., SVM, LR and RF) with the datasets from each individual gene. The three classifiers produced similar data. Consequently, only the results issued from the SVM classifier are reported in Table 2. The complete data issued from the three classifiers are reported in Supplementary Table S7. These results confirmed the box plot data (Fig. 1) and the most

discriminant characters of the *MAP1LC3B* and *GABARAP* genes with a predictive performance of  $0.842 \pm 0.218$  and  $0.832 \pm 0.213$ , respectively (Table 2). However, taking into account the individual data for these two genes was not sufficient enough to allow accurate clinical prediction.

In order to improve these predictive performances, we challenged, using the same classifiers, models combining data from *MAP1LC3B* and *GABARAP* gene expression with those of each one of the other genes. This led to substantially enhanced predictive accuracy and confidence (Table 2). The most informative prediction occurred while taking into account the dataset from the pair of genes *MAP1LC3B* and *GAPDH* and the pair of genes *GABARAP* and *GAPDH* (Table 2). For instance, using the SVM classifier, accuracy was increased to 94.8% ( $\pm 0.099$ ) for the pair *MAP1LC3B-GAPDH* and to 94.7% ( $\pm 0.100$ ) for pair *GABARAP-GAPDH* (Table 2). When the three genes *MAP1LC3B*, *GABARAP* and *GAPDH* were used as variable input, only a negligible additional gain was observed (data not shown). Fig. 2A shows the distribution of PD vs. control subjects for the expression of the *MAP1LC3B* and *GAPDH* genes (Fig. 2A, upper panel) and *GABARAP* and *GAPDH* genes (Fig. 2A, lower panel) and illustrates the discriminative capacity of these models.

Figure 2A displays the spread of *MAP1LC3B* data in patients and controls. It shows that *GAPDH* helps to discriminate patients whose *MAP1LC3B* data are close to those of control subjects. The divergence of the expression of *MAP1LC3B* and *GABARAP* in PD patients and controls can also be further visualised by plotting the distributions of *MAP1LC3B/GAPDH* vs. *GABARAP/GAPDH* transcript ratios for each individual (Fig. 2B). This distribution is clearly more homogeneous in the control population than in the PD population, which is very disperse. Indeed, a significant correlation is evidenced in the control population (Spearman correlation index = 0.595,  $p > 0.0002$ ) but not in the PD patient population. These results are in agreement with the conclusion of the machine learning algorithms and reflect a dysregulation process for the expression of these genes in the blood cells of the patients.

#### 4. Discussion

Several reports point out a relationship between PD and dysregulation of macroautophagy (Janda et al., 2012) and/or CMA (Alvarez-Erviti et al., 2010). In order to determine whether the transcription of genes related to autophagy could also be disturbed, we have compared their mRNA levels in PBMCs

from patients with sporadic PD to those from healthy subjects. We have used the ddPCR technology as a quantitative method because it allows absolute cDNA quantification and avoids the use of multiple reference genes. The potential reference genes do not always have a true steady expression but frequently exhibit unavoidable individual variations (Kozera and Rapacz, 2013). An increased number of transcripts was evidenced in blood from PD patients for genes of the *ATG8* family, namely *MAP1LC3B*, *GABARAP*, *GABARAPL1* and *GABARAPL2*, as well as for that of the selective autophagy receptor *p62/SQSTM1*. The most notable difference was for *MAP1LC3B* mRNA for which a 2.5 times fold increase was observed in PD patients over the control group. *MAP1LC3B* mRNA distribution showed very little variation in healthy subjects, and the median is the minimum rate observed in PD patients. By itself, this gene displayed a very discriminating value of 84% accuracy between patients and controls. There was a similar pattern with *GABARAP*, *GABARAPL1*, *GABARAPL2* and also *p62/SQSTM1* but distributions intersected more. An increased level of *MAP1LC3B* mRNA in the blood of PD patients has already been reported. However, the experiment was performed using a non-quantitative procedure (Wu et al., 2011). By contrast, another publication, based on whole-transcriptome assay by microarray, reported the differential expression of *ATG* genes but not *MAP1LC3B* in PBMCs of a cohort of Japanese patients with PD (Miki et al., 2018). It is frequent that there is little consensus at the level of transcript amounts across independent studies (Borrageiro et al., 2018). One explanation probably arises from the heterogeneous genetic backgrounds of the PD populations. Conversely, a statistically significant decrease of *HSPA8* (Hsc70) transcripts was detected in PD patients. These observations are consistent with the fact that CMA is reduced in the brain of PD patients (Alvarez-Erviti et al., 2010; Murphy et al., 2015). Two studies also showed decreased expression of the *HSPA8* gene at the transcript level in the blood of patients (Sala et al., 2014; Papagiannakis et al., 2015). It may seem surprising that *GAPDH* transcripts displayed a mild but significant decrease in patients insofar as *GAPDH* was commonly used as a calibration reference in gene expression experiments (de Jonge et al., 2007). However, *GAPDH* is now described as a multifunctional protein and its role extends well beyond its function in glucose metabolism. Its use as a reference gene should be done with caution (Kozera and Rapacz, 2013). Interestingly, several observations indicated it could be involved in the pathogenesis of PD. Genetic variants of the *GAPDH* gene are associated to a high risk of PD (Liu et al., 2015; Ping et al., 2018). Furthermore, *GAPDH* has been identified as a major protein reduced in abundance in lenses from eyes of PD patients (Klettner et

al., 2017). Accumulating evidence has demonstrated that GAPDH co-localises with alpha-synuclein and promotes formation of Lewy bodies (Mazzola and Sirover, 2002; Tsuchiya et al., 2005; Olah et al., 2006). Binding of alpha-synuclein to partially oxidized GAPDH induces subsequent inactivation of the enzyme (Barinova et al., 2018). DOPAL (3,4-dihydroxyphenylacetaldehyde) an aldehyde metabolite of dopamine, acts as an endogenous neurotoxin in PD, causes extensive GAPDH aggregation and irreversibly inhibited enzyme activity thus leading to cell cytotoxicity (Vanle et al., 2017). Whether GAPDH expression alteration in PD patients is related to autophagy dysregulation was not addressed in this paper. However the link between GAPDH impairment and autophagy dysfunction has been reported. At first, it has been shown that GAPDH associates with damaged mitochondria and initiates their uptake into lysosomal-like structures to promote their elimination by mitophagy in an independent pathway of macroautophagy (Yogalingam et al., 2013). Secondly, expression of GAPDH preserve cells from caspase-independent cell death through elevation in glycolysis. This protection reflected an increase in and a dependence upon autophagy (Colell et al., 2007). Finally, GAPDH has been shown to be a pivotal and central regulator of autophagy under glucose deficiency (Chang et al., 2015). The remaining genes for macroautophagy tested in this study, *BECN1* and *ATG9a*, did not appear discriminatory at all.

Criteria related to sex, Hoehn and Yahr score and age were verified as being non-discriminating and having no impact on gene expression. For the *MAP1LC3B*, *GABARAP* and *GAPDH* genes, their expression increased with age in PD patients but not in the controls. The remaining genes for macroautophagy tested in this study *BECN1* and *ATG9a* did not appear discriminatory at all. Conversely, a statistically significant decrease of *HSPA8* (Hsc70) transcripts was detected in PD patients and controls. These observations are consistent with the fact that CMA is reduced in the brain of PD patients (Alvarez-Erviti et al., 2010; Murphy et al., 2015). Two studies also showed decreased expression of the *HSPA8* gene at the transcript level in the blood of patients (Sala et al., 2014; Papagiannakis et al., 2015).

An increased level of *MAP1LC3B* mRNA in the blood of PD patients has already been reported. However, the experiment was performed using a non-quantitative procedure (Wu et al., 2011). By contrast, another publication, based on whole-transcriptome assay by microarray, reported the differential expression of *ATG* genes but not *MAP1LC3B* in PBMCs of a cohort of Japanese patients with PD (Miki et al., 2018). It is frequent that there is little consensus at the level of transcript amounts

across independent studies (Borragero et al., 2018). One explanation probably comes from the heterogeneous genetic backgrounds of the PD populations.

These results led us to use machine learning algorithms to test prediction models based on the data of the differentially expressed genes in PD patients. The most discriminant predictions were obtained with data from the *MAP1LC3B* and *GABARAP* genes with an accuracy of 84% and 83%, respectively (Table 2). However, taking into account data from additional genes helped to greatly increase accuracy and confidence. The most significant increase was obtained when combining data from the pairs of genes *MAP1LC3B* + *GAPDH* and *GABARAP* + *GAPDH* (Table 2). *GAPDH* data increased prediction accuracy when *MAP1LC3B* or *GABARAP* data were close between PD patients and controls (Table 2). Thus, considering these predictive data, accuracy for prediction of an individual having PD or not was increased to almost 95%. Surprisingly, although both *MAP1LC3B* and *GABARAP* transcripts increased in patients, the combination of their datasets did not significantly increase prediction accuracy. This is probably linked to a significant individual variability generated by the dysregulation of the expression of each of these two genes. From a functional point of view, it is noteworthy that the increase of *MAP1LC3B* and *GABARAP* gene expression is paralleled by a reduction of *GAPDH* gene expression. Are these observations linked or, on the contrary, do they represent independent pathways? The decrease of *GAPDH* expression in patients could impede the elimination of damaged mitochondria via mitophagy thus increasing cellular cytotoxicity (Klettner et al., 2017). Then, activation of *MAP1LC3B* or *GABARAP* gene expression could be a pathway to promote autophagy and favour cellular protection to counteract the deleterious effect of impaired mitophagy. Such questions will be important specific points to investigate.

We are conscious that this report represents an initial study and it will benefit from a larger cohort size. Nevertheless, considering the preliminary results, this pilot study is promising and opens new perspectives. Characterisation of a specific autophagy gene expression pattern in the peripheral blood of PD patients would represent a simple and non-invasive method to implement, since it is exclusively based on the use of quantitative PCR, a standard technology, without the need for sophisticated and burdensome methods based on high throughput sequencing methods. Also, this study was limited to PD and could not be extended at the present time to other neurodegenerative diseases including atypical parkinsonisms. Such investigations will be performed and should help to establish whether the alterations of *ATG* gene expression are specific to PD.

We are aware that the significant changes in *ATG genes* expression do not necessarily mean a change in protein production rate or even a change in the process of autophagy which were not addressed in this work. Nevertheless, beyond their predictive aspect, our results suggest that some cellular dysfunction related to autophagy and occurring in the brain of PD patients could also be evidenced in peripheral blood cells. Therefore, it is possible that other autophagy genes than those tested in this report are also dysregulated. This would reinforce the predictive data generated by our study.

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### **Credit Author Statement**

SEH, AS, TN and CR, methodology, software, AL, SC, TN, FB and LM, conceptualization, designed experiments; AV, statistical analysis, writing - editing; FM predictions with classifiers; writing - editing, AL, PA, CO and LM, Supervision ; SHE, SC and AL, writing, original draft, review and editing wrote the manuscript with additional contributions by the other authors.

### **Conflict of interest**

The authors declare that they have no conflict of interest.

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## Table and Figures Captions

**Table 1**

Quantification of gene expression

GENE	Mean cDNA copies number per ng of reverse transcribed RNA (cDNA)		p-value
	PD: mean +/-SD	CONTROLS: mean +/-SD	
<i>BECN1</i>	711.1 ± 105.8	686.6 ± 116.6	0.21
<i>MAP1LC3B</i>	3,908.5 ± 1,734.2	1,359.0 ± 244.3	<b>&lt; 1.0 x 10<sup>-5</sup></b>
<i>GABARAP</i>	9,890.6 ± 2,218.8	6,218.4 ± 1341.8	<b>&lt; 1.0 x 10<sup>-5</sup></b>
<i>GABARAPL1</i>	531.5 ± 158.4	436.8 ± 103.5	<b>5.4 x 10<sup>-4</sup></b>
<i>GABARAPL2</i>	2,193.0 ± 972.5	1,320.1 ± 225.1	<b>&lt; 1.0 x 10<sup>-5</sup></b>
<i>p62/SQSTM1</i>	2,131.2 ± 704.6	1,558.2 ± 326.0	<b>1.5 x 10<sup>-5</sup></b>
<i>ATG9a</i>	437.4 ± 86.9	434.3 ± 75.4	0.79
<i>HSPA8</i>	3,965.1 ± 1,443.	5,427.8 ± 1,202.4	<b>&lt;1.0 x 10<sup>-5</sup></b>
<i>GAPDH</i>	12,122.8 ± 2,475.6	13,738.5 ± 2,730.5	<b>0.01</b>
<i>SNCA</i>	236.2 ± 79.4	261.5 ± 95.1	0.23
<i>GALIG</i>	67.3 ± 24.7	72.0 ± 21.5	0.29

Mean and standard deviation ( $\pm$ SD) of cDNA copies number per ng of reverse transcribed RNA (cDNA) in blood cells from the Parkinson's disease patients group (PD) and the healthy blood donors group (CONTROLS). Statistical analyses (p values) were performed using Mann-Whitney test. The Holm-Sidak method was applied to take into account multiple comparisons. The significance threshold was adjusted to p values < 0.01. Accuracy of the sample size was checked by performing a power analysis (power = 0.8). Significant values are shown in bold. GAPDH differential expression was also analysed using a Student's t test (p-value < 6.8.10<sup>-3</sup>) after verification of normal distribution by a Shapiro-Wilks test.

**Table 2:**

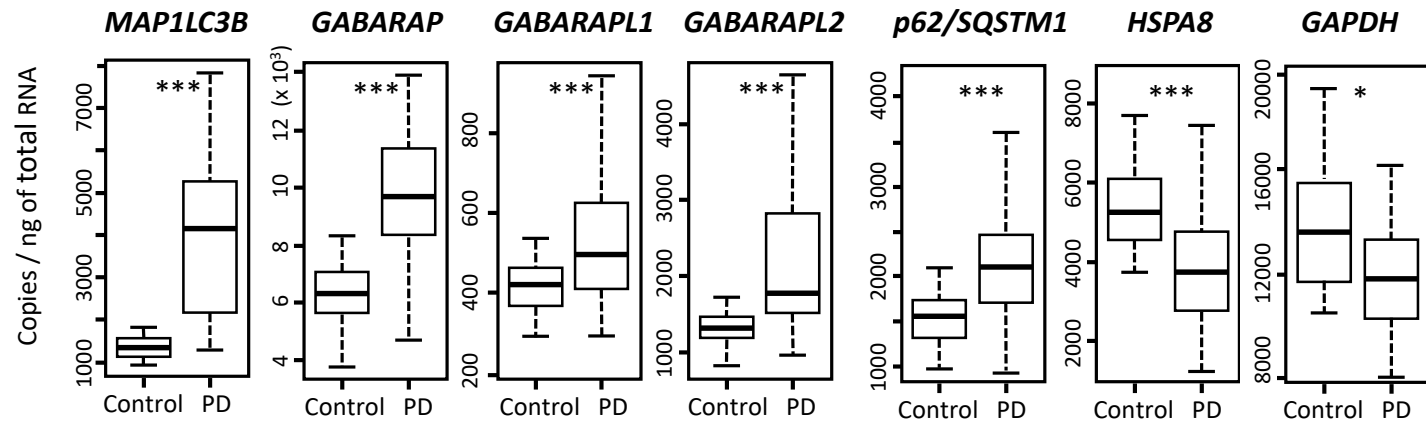
Predictive performance of gene expression levels using the supervised machine learning algorithm Support Vector Machine (SVM)

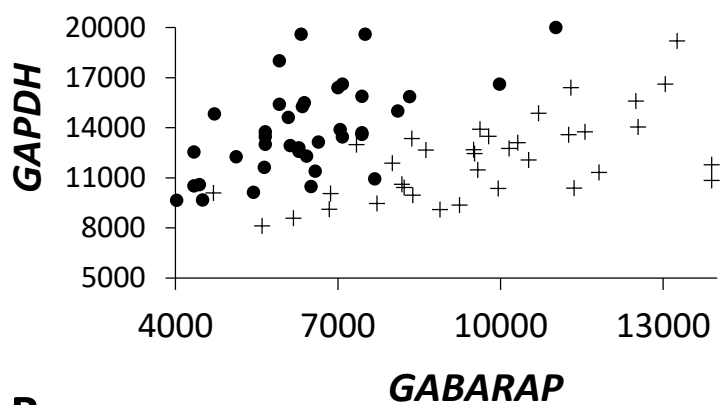
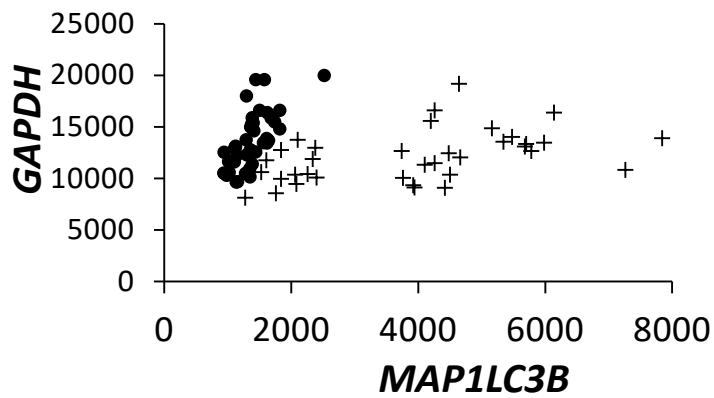
Gene	1 gene	<i>MAP1LC3B</i>	<i>GABARAP</i>
	mean± SD	+ 1 gene mean± SD	+ 1 gene mean ± SD
<i>GALIG</i>	0.447 ± 0.187	0.829 ± 0.216	0.792 ± 0.302
<i>SNCA</i>	0.513 ± 0.053	0.816 ± 0.176	0.845 ± 0.243
<i>GAPDH</i>	0.590 ± 0.223	<b>0.948 ± 0.099</b>	<b>0.947 ± 0.100</b>
<i>GABARAP</i>	<b>0.832 ± 0.213</b>	0.829 ± 0.198	
<i>GABARAPL1</i>	0.645 ± 0.230	0.815 ± 0.158	0.791 ± 0.204
<i>GABARAPL2</i>	0.766 ± 0.360	0.936 ± 0.137	0.897 ± 0.147
<i>MAP1LC3B</i>	<b>0.842 ± 0.218</b>		0.829 ± 0.198
<i>HSPA8</i>	0.707 ± 0.322	0.895 ± 0.063	0.817 ± 0.123
<i>P62/SQSTM1</i>	0.753 ± 0.208	0.882 ± 0.177	0.871 ± 0.190
<i>BECN1</i>	0.527 ± 0.027	0.908 ± 0.198	0.818 ± 0.211
<i>ATG9a</i>	0.500 ± 0.103	0.868 ± 0.085	0.818 ± 0.163

Mean accuracy and SD of classification predictions with StratifiedKFold cross validation (with k=5) over standardized gene expression (Standard Scaler) for target attribute PD vs control subjects. Predictions were scored taking into account data from each individual gene (column 1 gene) or data combining *MAP1LC3B* gene plus one additional gene (column *MAP1LC3B* + 1 gene) or *GABARAP* plus one additional gene (column *GABARAP* + 1 gene).

**Fig. 1** Statistical comparisons between PD patients and controls. Box plots exhibit expression levels and statistical comparisons of the genes from the Table 1 list but only genes with a significantly different expression between PD patients and controls were reported on the figure. Mann Whitney tests were performed except for *GAPDH* which was also analysed by a Student test. The box plots represent the interquartile range with the median values shown as horizontal lines inside the boxes; the whiskers correspond to the minimal and maximal values within the samples. \*\*\*: $p < 0.001$ , \*: $p < 0.00851$  (see Table 1)

**Fig. 2.** Distribution of MAP1LC3B and GAPDH transcripts (upper graph) and GABARAP and GAPDH gene expression (lower graph) in PD patients (crosses) vs control subjects (circles). **A** X and Y axes are expressed as numbers of copies per ng of input reverse transcribed RNA (cDNA) in RT reactions. **B** Distribution of MAP1LC3B/GAPDH ratios vs GABARAP/GAPDH ratios.



**A****B**