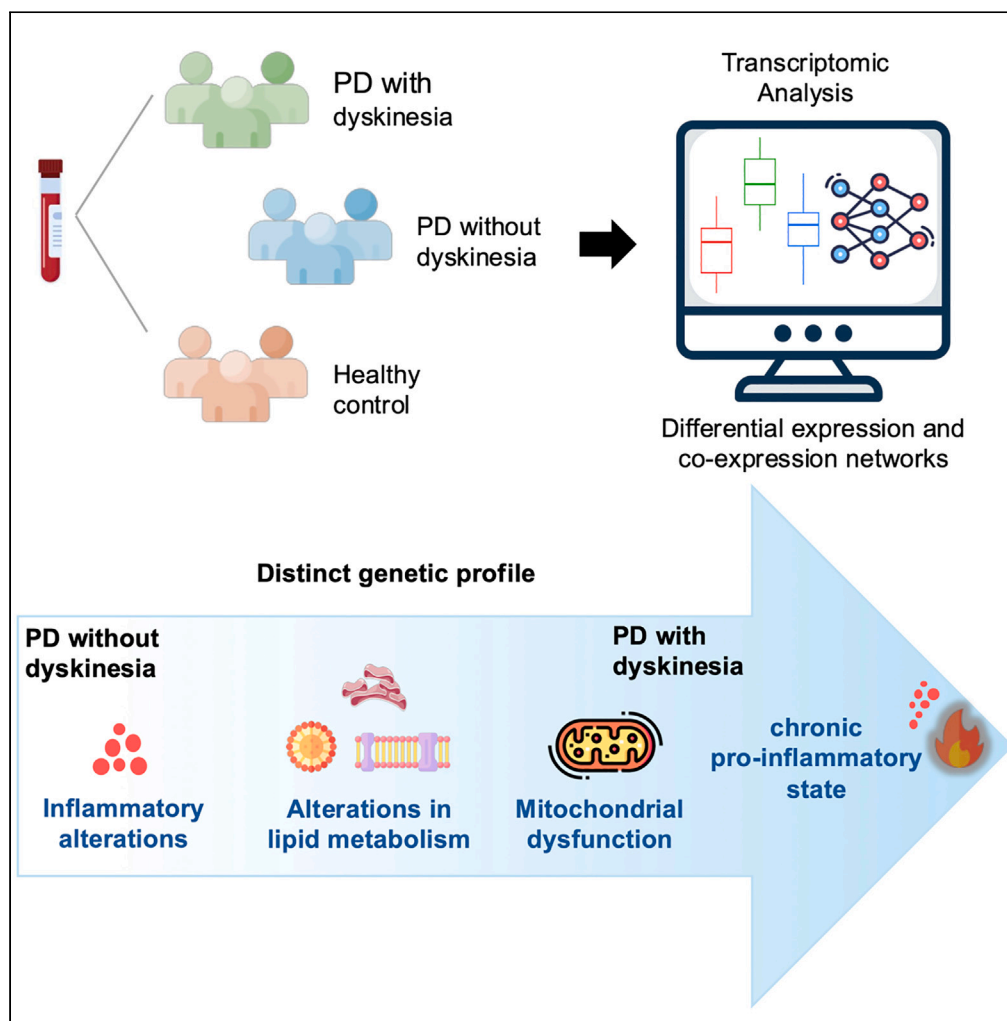


Article

# Unveiling differential gene co-expression networks and its effects on levodopa-induced dyskinesia



Tatiane Piedade de Souza, Gilderlano Santana de Araújo, Leandro Magalhães, ..., Giordano Bruno Soares-Souza, Bruno Lopes Santos-Lobato, Ândrea Ribeiro-dos-Santos

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**Highlights**

Differential expression analysis revealed ten new transcripts altered in PD patients

Dyskinesia patients showed an exclusive gene co-expression network

Alterations in mitochondrial genes may contribute to the pro-inflammatory state in PD

Inflammatory pathways may contribute to the onset of levodopa-induced dyskinesia

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## Article

## Unveiling differential gene co-expression networks and its effects on levodopa-induced dyskinesia

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## SUMMARY

**Levodopa-induced dyskinesia (LID) refers to involuntary motor movements of chronic use of levodopa in Parkinson's disease (PD) that negatively impact the overall well-being of people with this disease. The molecular mechanisms involved in LID were investigated through whole-blood transcriptomic analysis for differential gene expression and identification of new co-expression and differential co-expression networks. We found six differentially expressed genes in patients with LID, and 13 in patients without LID. We also identified 12 co-expressed genes exclusive to LID, and six exclusive hub genes involved in 23 gene-gene interactions in patients with LID. Convergently, we identified novel genes associated with PD and LID that play roles in mitochondrial dysfunction, dysregulation of lipid metabolism, and neuroinflammation. We observed significant changes in disease progression, consistent with previous findings of maladaptive plastic changes in the basal ganglia leading to the development of LID, including a chronic pro-inflammatory state in the brain.**

## INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease and the one among movement disorders, affecting 1–2% of the population over the age of 60. PD is the fastest-growing neurological condition and may become the primary neurodegenerative disease in the next two decades.<sup>1,2</sup> PD is a complex and multisystemic disease, characterized by widespread alpha-synuclein aggregates, mitochondrial and lysosomal dysfunction, synaptic transport, and neuroinflammation, which lead to the death of neurons, especially dopaminergic.<sup>3,4</sup> There are currently no specific diagnostic biomarkers to detect its early stages, and diagnosis occurs decades after the onset of neuronal degeneration.<sup>3</sup>

The available treatments for PD are effective in alleviating the symptoms, moreover, does not modify the neurodegenerative process. The main treatment with levodopa significantly improves motor symptoms of people with PD.<sup>5,6</sup> Levodopa can cross the blood-brain barrier and restores the dopamine levels. Despite its effectiveness in the early stages of treatment, disease progression coupled with the need for increased levodopa dosage can lead to the development of levodopa-induced dyskinesia (LID), characterized by involuntary movements.<sup>5,7</sup> LID can affect approximately 30–51.2% of people with PD after only five years of levodopa treatment, becoming more prevalent as the disease progresses, particularly in female individuals and those with a younger onset age of PD.<sup>5,8,9</sup>

Several known molecular mechanisms contribute to the development of LID, such as the dysregulation of dopamine storage and release control in the synaptic cleft, increased D1 receptors in post-synaptic neurons, release of dopamine by serotonergic and noradrenergic neurons as a replacement of the nigrostriatal degeneration, and greater spontaneous glutamate release, all of which lead to overstimulation of the direct pathway in the basal ganglia, resulting in further movement dysregulation.<sup>7,10–13</sup> Shifts on gene expression at various levels may affect neurotransmitters, but there is still much to explore about the underlying molecular processes involved in the onset and pathophysiology of LID to seek better treatment strategies.<sup>5,10</sup>

System biology approaches such as differentially expressed genes (DEGs) and differentially co-expressed gene network (DCGNs) in LID is still unexplored, that may help identifying altered gene-gene interactions between conditions.<sup>14</sup> The present study aimed at investigating the

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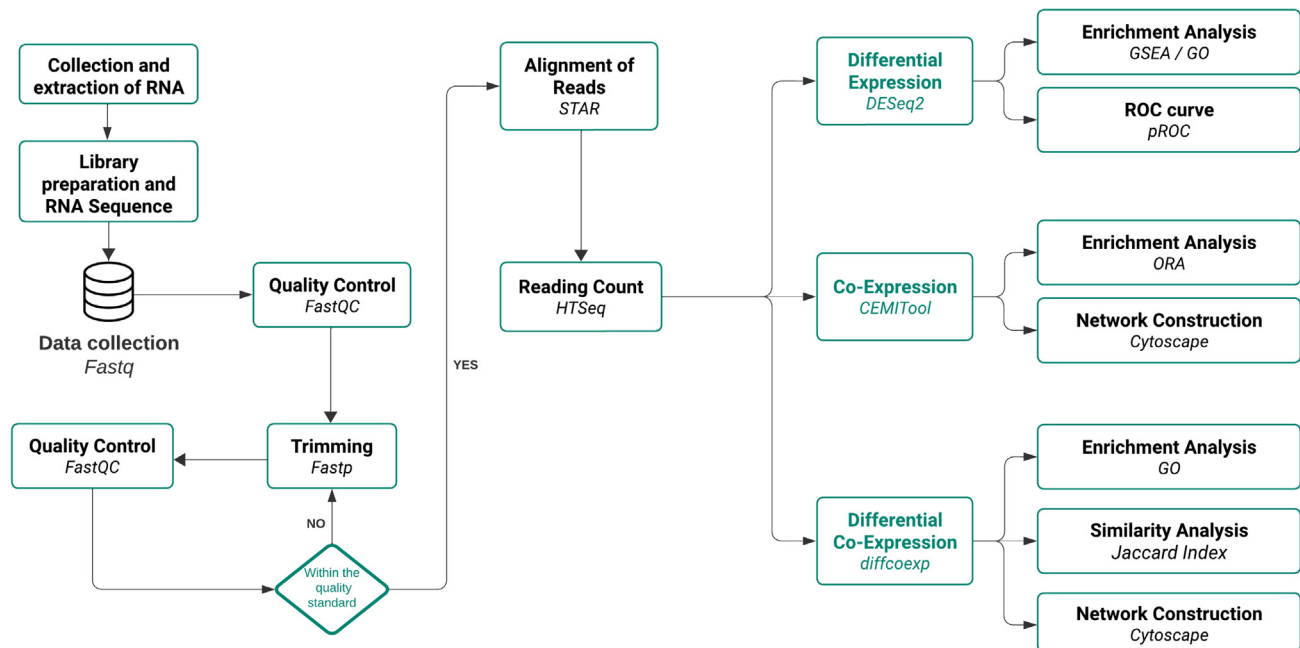
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**Figure 1. Flowchart for RNA-Seq data analysis to investigate differential expression, co-expression, and differential co-expression**

gene expression, co-expression, and differential gene co-expression based on the transcriptome analysis of peripheral blood in controls (CT) and people with PD with LID (PD-D group) and absence of LID (PD-ND group).

## RESULTS

### Epidemiological and clinical data

Our data analysis flowchart is depicted in [Figure 1](#). A comprehensive analysis of clinical and epidemiological data revealed that the PD-D group exhibited lower age at disease onset, longer disease and levodopa use durations, and higher levodopa doses when compared to the PD-ND group. Sex and age at evaluation were matched between groups and the analysis is represented in the [Table 1](#). (See also the [Table S1](#); [Figure S1](#) for general data of samples and RNA-sequencing).

### Differential gene expression reveals new blood biomarkers in levodopa-induced dyskinesia

We conducted individual comparative analysis of the PD-D and PD-ND groups among themselves and with the CT group. No DEG was observed between PD-D vs. PD-ND samples. When comparing each PD group individually with the CT, we found 13 DEGs in PD-ND and six DEGs in PD-D group. We conducted an exclusion analysis considering all the results and identified ten exclusive DEGs in PD-ND and three exclusive DEGs in PD-D. (DEGs are represented in [Table 2](#); [Figures 2A](#) and [2B](#)).

Gene Ontology analysis of the 6 DEGs between PD-D vs. CT primarily enriched for nucleotide biosynthesis and energy metabolism, highlighting the genes *ACAT1* and *TK2* ([Figure 2C](#)). When investigating the 13 DEGs between PD-ND vs. CT, the genes *HBA1*, *HBA2*, *CYTB*, *ND5*, and *ND6* were related to gas transport, response to reactive oxygen species (ROS), and mitochondrial metabolism ([Figure 2D](#)).

Our research identified ten new transcripts associated with PD and two of these new transcripts (*HBBP1* and *ENSG00000281383*) have not been thoroughly studied in terms of their function. We also accessed the potential of prediction of PD for these genes through the ROC curve. The *HBA2*, *HBBP1*, and *ENSG00000281383* genes were commonly altered in both PD-ND and PD-D groups and exhibited an area under the curve (AUC) over 0.8 ([Figure S2A](#)).

The gene set enrichment analysis (GSEA) for PD-ND group revealed that the main exclusive enriched pathways for genes with high expression levels were DNA methylation, RNA polymerase I promoter opening, assembly of the ORC complex at the origin of replication, SIRT1 negatively regulates rRNA expression and HDMs demethylate histones. Pathways with lower enrichment score were *RUNX1* regulates transcription of genes involved in differentiation of hematopoietic stem cells, DNA double-strand break repair, metabolism of carbohydrates, heme signaling, and binding and uptake of ligands by scavenger receptors ([Figure S2B](#)).

In PD-D group, GSEA revealed that the set of over-expressed genes was particularly associated with the fatty acid metabolism pathways, NCAM1 interactions, integrin cell surface interactions, extracellular matrix organization, and neutrophil degranulation. The main pathways enriched for the under-expressed genes were PD-1 signaling, mitochondrial translation, mitochondrial metabolism, RNA processing, and immune system. Details of these pathways and the involved genes are represented in [Figure S2C](#).

**Table 1. Epidemiological and clinical data and inclusion/exclusion criteria of patients with PD and control group**

General Characteristics	CT (n = 20)	PD-ND (n = 11)	PD-D (n = 15)	p value
Sex (Male/Female)	13/7	8/3	11/4	0.839 <sup>a</sup>
Age at clinical evaluation	59.50 (53–64)	54 (49.5–60)	53 (50–60)	0.228 <sup>c</sup>
Age at onset of PD <sup>b</sup>	–	50 (46–55)	41 (38.5–49)	0.042 <sup>d</sup>
Disease duration (years) <sup>b</sup>	–	4 (2–7)	10 (6–11.5)	0.0006 <sup>d</sup>
LEDD (mg/day) <sup>b</sup>	–	550 (300–600)	1200 (737.5–1562)	0.001 <sup>d</sup>
Dose of levodopa (mg/day) <sup>b</sup>	–	300 (250–475)	700 (450–1188)	0.003 <sup>d</sup>
Levodopa duration (years)	–	3 (1–4.5)	10 (6.5–12)	0.0001 <sup>d</sup>

CT, control group; NA, not applied; PD-D, people with Parkinson's disease and levodopa-induced dyskinesia; PD-ND, people with Parkinson's disease without levodopa-induced dyskinesia.

<sup>a</sup>Chi-squared test comparing frequencies between CT, PD-ND and PD-D groups.

<sup>b</sup>Values in median (interquartile range).

<sup>c</sup>Kruskal-Wallis test comparing medians between CT, PD-ND and PD-D groups.

<sup>d</sup>Mann-Whitney test comparing medians between PD-ND and PD-D groups.

### Distinct gene co-expression patterns in levodopa-induced dyskinesia

Gene co-expression analysis was performed for 15,372 transcripts regarding CT, PD-ND, PD-D groups, and a pool of samples (CT + PD-ND + PD-D). Six modules of co-expressed genes were identified in the PD-D group and three modules in the PD-ND group. In the overall analysis by exclusion gene modules and pathways we identified 92 gene-gene interactions in PD-D group and 98 in PD-ND group (all results of co-expression analysis can be observed in the [Table S2](#)). The complete network of co-expressed genes in PD-ND and PD-D groups can be found in [Figures S3A and S3B](#).

To focus on exclusive interactions, we systematically compared the interaction results across different groups ([Figure S3C](#)). Notably, 23 interactions involving 25 genes were presented in the PD-ND group and 12 exclusive interactions involving 12 genes were found in the PD-D group ([Figures 3D and 3E](#)).

The over representation analysis (ORA) results revealed significantly enriched pathways for the co-expressed gene modules in all investigated groups (FDR-adjusted *p* value <0.05). Specifically, 54 enriched pathways were found in the PD-D modules, two in PD-ND, two in CT, and 31 in the pooled sample group. The analysis of exclusive pathways was conducted to identify those specifically associated with PD-ND or PD-D groups. We identified 24 exclusive pathways enriched with PD-D ([Figure 3F](#); [Table S3](#)) and the main of these pathways associated with LID were showed in [Figure 3G](#). No metabolic pathway was exclusively represented in the PD-ND group.

### Exclusive dyskinesia-specific differential co-expression network

We conducted a comprehensive analysis of differential co-expression patterns in people with PD with and without LID. An analysis of 15,372 transcripts highlighted differential co-expressed links (DCLs) and differentially co-expressed genes (DCGs). In the PD-D group, we identified 79 gene-gene interactions, while in the PD-ND group, 3,055 interactions were detected. All interactions were specific to each investigated group, and six DCGs were identified in the PD-D group and 200 in the PD-ND group. Upon examining the similarity between the two investigated groups using the Jaccard index, we confirmed that the gene sets represented in the DCLs of PD-D and PD-ND do not show similarity (Jaccard = 0.0), implicating in distinct molecular mechanisms in each condition.

We used the DCGs from each group to filter their respective DCLs to investigate specific interactions involving these hub genes. In PD-ND group, 200 hub genes are involved in 48 gene-gene interactions among themselves ([Figure 4A](#)), and in PD-D we observed that six hub genes are involved in 23 gene-gene interactions ([Figure 4B](#)). Gene Ontology analysis for the biological processes in PD-D group was conducted, but no enriched processes were identified through the STRING, g:Profiler, or ToppGene platforms.

The Gene Ontology analysis was performed for the complete DCLs of the PD-D and PD-ND groups and found that all enriched pathways were also exclusive to each condition. We identified 152 enriched biological processes in the PD-D group and 220 in the PD-ND group. Subsequently, we performed the exclusion analysis, revealing 110 biological processes exclusive to PD-D group and 178 exclusives to PD-ND group. With a focus on LID, the top 10 most significant processes enriched in the PD-ND and PD-D groups were represented in [Figures 4C and 4D](#), respectively.

Finally, we analyzed the overall distribution of genes identified in the DCLs and DCGs and intersected with the results of co-expression. No overlap was observed between the co-expressed and DCGs in the PD-ND group ([Figure 4E](#)) and the PD-D group ([Figure 4F](#)). Overall, the analysis revealed a network of DCGs specific to the PD-D group, which helps elucidate the genetic background involved in the LID.

## DISCUSSION

In our study, we detected DEGs, gene co-expression networks, and differential co-expression in the blood of people with PD, indicating subsequent molecular adaptations throughout the disease progression. To better discuss our results, we divide into sections offering new insights into molecular changes in PD development and dyskinesia.

**Table 2. Characterization of differentially expressed genes between people with Parkinson's disease and control subjects, according to the presence of levodopa-induced dyskinesia**

PD-ND vs. CT				
Gene	Log2 Fold-Change	p value	p. adjusted (FDR)	Regulation
MT-CYB	-0.74	<0.001	0.005	Downregulated
CWC27 <sup>a</sup>	-0.77	<0.001	0.018	Downregulated
MT-ND5	-0.85	<0.001	0.005	Downregulated
RAPH1 <sup>a</sup>	-1,11	<0.001	0.003	Downregulated
HBA1	-2,10	<0.001	0.005	Downregulated
MT-ND6	-2,15	<0.001	0.008	Downregulated
MT-TY <sup>a</sup>	-2,38	<0.001	0.040	Downregulated
MT-TF <sup>a</sup>	-2,55	<0.001	0.002	Downregulated
MT-TQ <sup>a</sup>	-2,62	<0.001	0.012	Downregulated
MT-TE <sup>a</sup>	-2,71	<0.001	0.040	Downregulated
HBA2	-2,75	<0.001	<0.001	Downregulated
HBBP1 <sup>a</sup>	-3,89	<0.001	<0.001	Downregulated
ENSG00000281383 <sup>a</sup>	-4,23	<0.001	<0.001	Downregulated
PD-D vs. CT				
BTNL8 <sup>a</sup>	0.98	<0.001	0.005	Upregulated
TK2	0.84	<0.001	0.009	Upregulated
ACAT1 <sup>a</sup>	-0.65	<0.001	0.045	Downregulated
HBA2	-1.84	<0.001	0.011	Downregulated
ENSG00000281383 <sup>a</sup>	-2.66	<0.001	0.003	Downregulated
HBBP1 <sup>a</sup>	-2.84	<0.001	0.003	Downregulated

CT, control group; PD-D, people with Parkinson's disease with levodopa-induced dyskinesia; PD-ND, people with Parkinson's disease without levodopa-induced dyskinesia; FDR, false discovery rate.

<sup>a</sup>New transcripts associated with Parkinson's disease.

### Differential gene expression analysis reveals novel biomarkers in Parkinson's disease

Our results showed no significant difference in gene expression between the PD-D and PD-ND groups. However, as observed in the co-expression analysis results, a composition of different genes was co-expressed within their respective groups. This finding suggests that the fundamental molecular mechanisms distinguishing these groups may involve complex interaction and regulation of multiple genes, rather than isolated changes in gene expression.

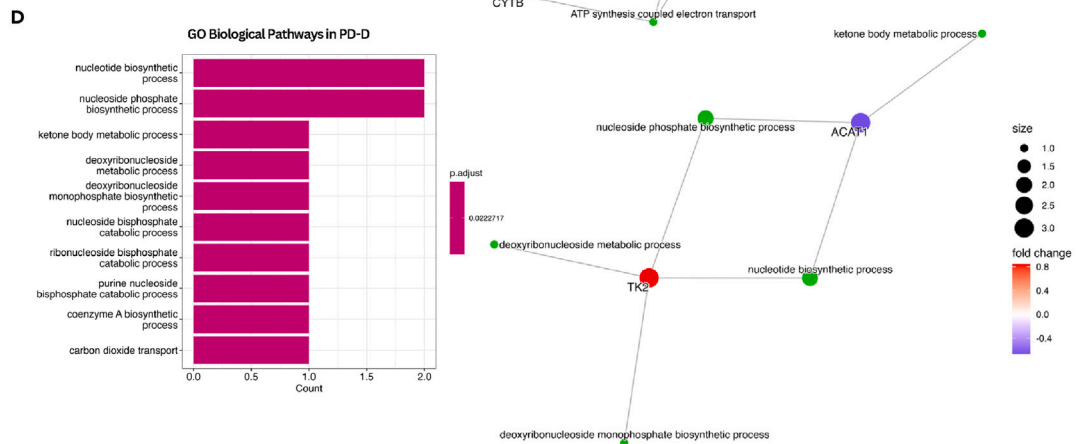
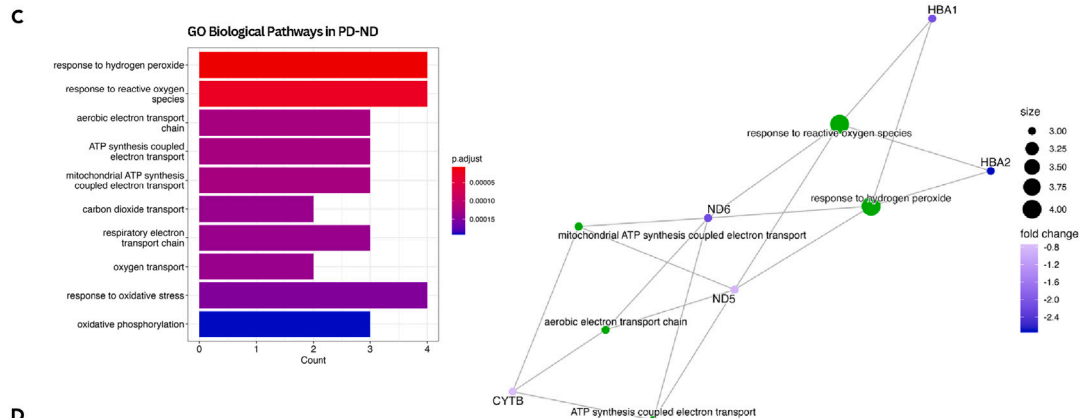
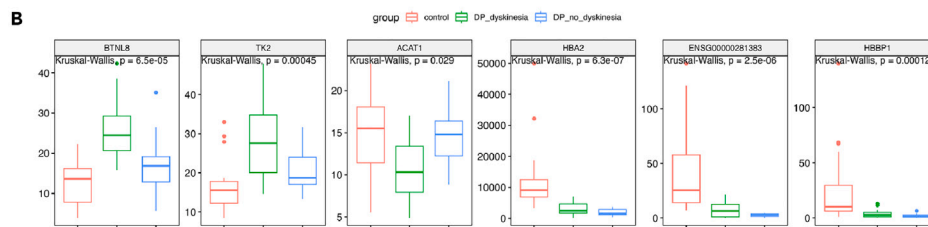
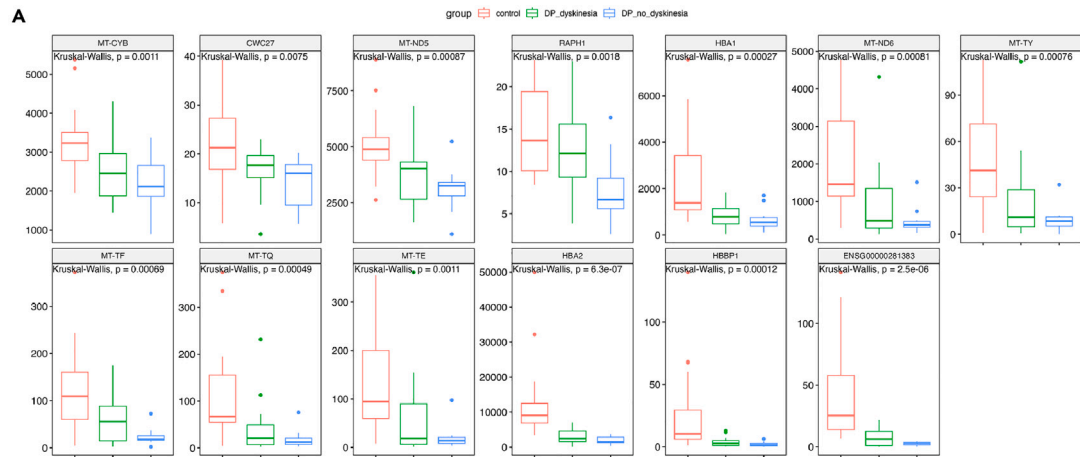
DEG analysis revealed ten new genes associated with PD. This finding highlights the complexity of the genetic architecture of PD, which is still not well understood, particularly in admixed populations from the Amazon.<sup>15</sup> Of these, three were downregulated in PD overall with AUC > 80% (*HBA2*, *HBBP1*, and *ENSG00000281383*).

Currently, there is no available information regarding the function of the long non-coding RNAs (lncRNA) *ENSG00000281383* and its interactions with other genes. There is also no information on the role of *HBBP1* in PD, but studies have associated its downregulation with reduced hemoglobin levels and the development of beta-thalassemia.<sup>16,17</sup> There are no previous data associating these genes with PD, and a potential regulatory role in neurodegenerative mechanisms of PD needs further clarification through functional investigations.

*HBA1* and *HBA2* are genes encoding the alpha subunit of hemoglobin, which is expressed in blood cells and dopaminergic neurons of the mesencephalon, where its under-expression is linked to disease severity in postmortem brain tissue from people with PD.<sup>18–21</sup> Furthermore, the downregulation of *HBA2* in the blood observed in our results may indicate a reduction in oxygen transport in the body and, consequently, a depletion of its availability in neurons, contributing to oxidative stress and mitochondrial damage, a hallmark characteristic in PD. Another evidence of mitochondrial damage observed in our results is the reduction in the heme signaling metabolic pathway, a molecule produced in the mitochondria that is essential for gas transport in hemoglobin in blood cells and in dopaminergic neurons susceptible to degeneration in PD, as well as in other protein complexes such as the cytochrome family and peroxidase, which are essential for the cellular response to oxidative stress.<sup>22</sup>

### Dysregulation of mitochondrial function and stress response occurs in early and intermediate stages of PD

Mitochondria are organelles that play a crucial role in cellular homeostasis and survival. Mitochondrial dysfunction is described in PD, where genetic and environmental risk factors contribute to the dysregulation of its bioenergetics, biogenesis, transport, and internal quality





**Figure 2. Differentially expressed genes and enrichment analysis**

(A) Boxplot of DEG in PD-ND.

(B) Boxplot of DEG in PD-D.

(C) GO for differentially expressed genes of PD-ND group, with enriched pathways (left) and representative genes for the main pathways (right).

(D) GO for differentially expressed genes of PD-D group, with enriched pathways (left) and representative genes for the main pathways (right). CT, control group; PD-D, people with Parkinson's disease with levodopa-induced dyskinesia; PD-ND, people with Parkinson's disease without levodopa-induced dyskinesia; DEG, differentially expressed genes; GO, Gene Ontology. p.adjust, p-value adjusted by FDR.

control.<sup>23</sup> Exclusive to people with PD before the onset of motor complications, such as LID (PD-ND group), we observed downregulated mitochondrial genes involved in the electron transport chain (*MT-CYB*, *MT-ND5*, and *MT-ND6*) and mitochondrial transfer RNAs, as *MT-TY*, *MT-TF*, *MT-TQ*, and *MT-TE*. Alterations in *MT-CYB*, *MT-ND5*, and *MT-ND6* have been linked to the dysregulation of ATP synthesis and an increase in ROS, which contribute to the elevated damage to both mitochondrial DNA and nuclear DNA.<sup>24–27</sup> Furthermore, the disruption of tRNA metabolism becomes critical for ATP production and protein synthesis in people with PD.<sup>28</sup>

The gene co-expression analysis for the PD-ND group resulted in a network of genes involved in mitochondrial functions, such as the regulation of apoptosis, response to stress (*LYN*, *PAG1*, and *MAP3K1*), regulation of glucose levels (*GAB1* and *IRS2*), and neurodegeneration (*ROCK1* and *CXCR4*).<sup>29–35</sup> Also, the *PIK3CB* gene showed a direct or secondary interaction with all genes involved in these processes. A GWAS study conducted in multi-ethnic populations identified new genes associated with PD, including *IRS2* and a gene from the *PIK3CA* family (related to the *PIK3CB* gene).<sup>36</sup> The *PIK3CB* gene generates a kinase protein primarily associated with immune signaling and apoptosis regulation.<sup>37,38</sup> Regarding *IRS2*, there is an increase in phosphorylation and a simultaneous reduction in the *IRS2* gene, along with a decrease in insulin signaling in the basal ganglia of an animal model of PD.<sup>31</sup> Furthermore, studies have shown that the dysregulation of insulin pathways, as seen in type II diabetes mellitus, may be a risk factor for PD. These pathways are typically altered in the brains of people with PD, contributing to neurodegeneration and affecting various mitochondrial and autophagy processes.<sup>39</sup>

The differential gene co-expression analysis revealed over 3,000 statistically significant interactions and 178 unique metabolic processes for the PD-ND group, converging toward mitochondrial functions such as cellular respiration, oxidative phosphorylation, and the electron transport chain, along with methylation and ribosomal RNA metabolic processes. All these factors contribute to the persistent oxidative stress and mitochondrial energy crisis observed in PD, which suggest a series of responses involving the activation of the immune response, response to ROS, DNA repair systems, and selective mitophagy. As mitochondrial damage and cellular stress persist, a cascade of molecular signals leads to programmed neuronal death.<sup>40</sup>

**Dysregulation of lipid homeostasis, endoplasmic reticulum stress, and immune signaling may be related to LID**

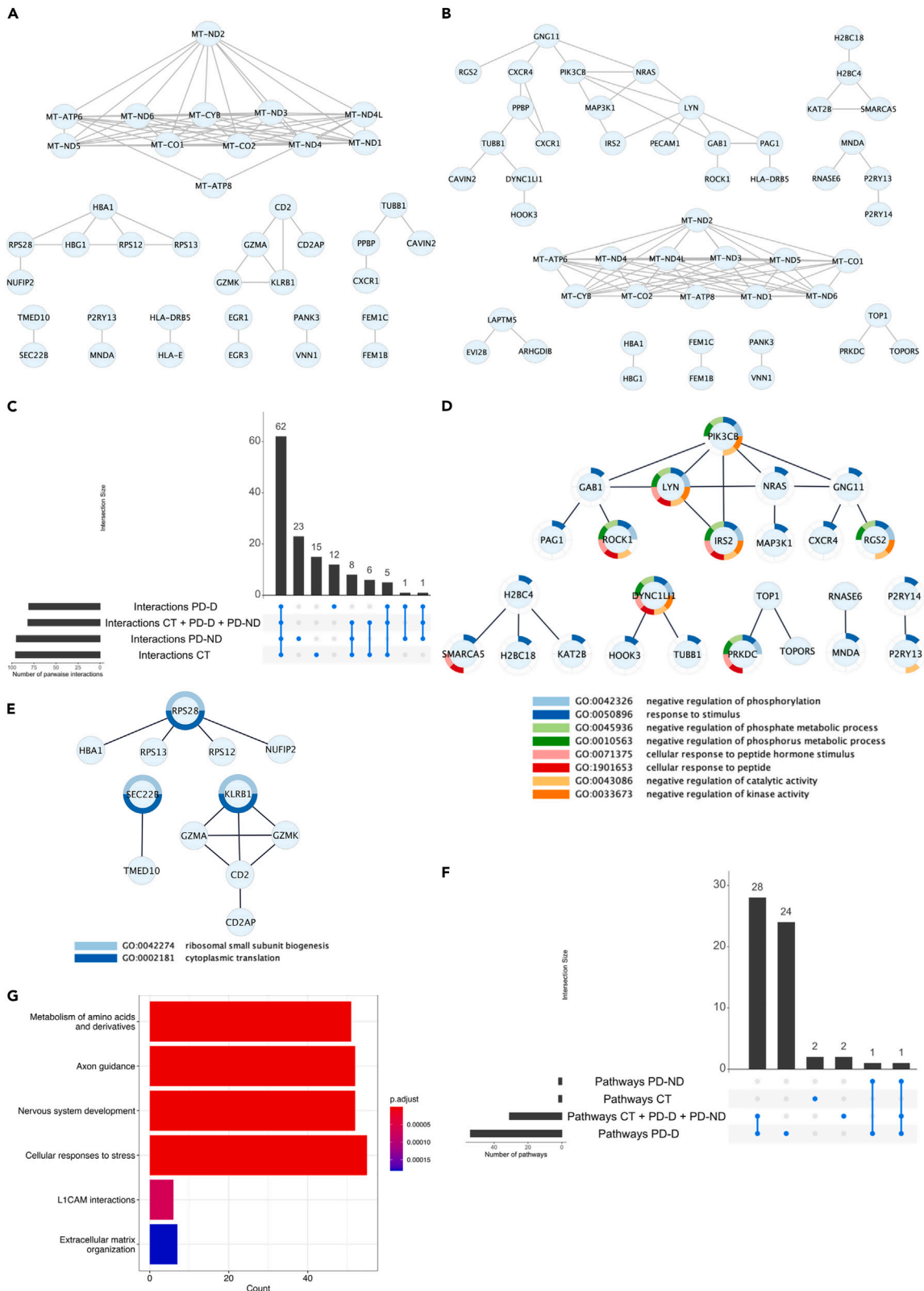
DEG analysis demonstrated an increased expression of *TK2*, which is produced in the nucleus and plays role in nucleotide synthesis and the maintenance of mitochondrial DNA. Considering LID usually emerges in advanced stages of the disease, this overexpression is likely to occur in response to frequent oxidative stress and mitochondrial damage.<sup>41</sup> Variants in this gene have already been identified in individuals with mitochondrial maintenance disorders, and a comprehensive proteomic analysis observed high expression of this protein in synaptosomes of the substantia nigra in the brains of people with PD.<sup>42,43</sup>

*ACAT1* gene was under expressed and is linked to alterations in other neurological phenotypes, and its inhibition led to the stimulation of autophagy and lysosomal activity in an Alzheimer's animal model.<sup>44–49</sup> This gene is typically enriched in the mitochondria-associated endoplasmic reticulum membrane, regulating the connection between these organelles.<sup>45</sup>

In the co-expression analyses of the PD-D group, *SEC22B* and *TMED10* are hub genes identified in our analysis. These genes are responsible for the regulation of protein and vesicle trafficking associated with the endoplasmic reticulum and the Golgi complex.<sup>50,51</sup> Other co-expressed genes, such as *HBA1* and *RPS/RPL* family genes involved in oxygen transport and protein synthesis regulation, respectively, were observed in the PD-D group. A module containing the genes *KLRB1*, *GZMA*, *GZMK*, *CD2*, and *CD2AP*, which mediate immune response, neuronal survival, and membrane trafficking, was also observed.<sup>52–55</sup> Furthermore, the enrichment analysis of the co-expression network in LID pointed to important pathways that may be involved in the uptake and metabolism of levodopa, such as amino acid metabolism, axon guidance, L1CAM interactions, and extracellular matrix organization. This is noteworthy since these processes could lead to increased BBB permeability,<sup>56,57</sup> potentially influencing the bioavailability of levodopa in the brain and further contributing to dopamine imbalance.

Similarly, the results of differential co-expression between the PD-D group and control subjects also converged on modifications in genes associated with the following functions: endoplasmic reticulum stress and lipid and protein homeostasis (*SIL1*, *BORCS6*, *SWT1*, *TTK*, *TBC1D1*, *CDC42BPA*, and *DCUN1D1*)<sup>58–61</sup>; DNA repair and cell survival (*ATAD5*, *APLF*, *PARBP*, and *ZNF280B*)<sup>62–65</sup>; mitochondrial metabolism, regulation of cell death, immune signaling, and neurogenesis (*CISD1*, *ALAS1*, *TLR4*, and *CDC27*)<sup>66–69</sup>; and reorganization of the cytoskeleton and formation of the mitotic spindle, cilia and flagella, microtubules, and centrioles (*CFAP45*, *CEP57L*, *KIF11*, and *TBC1D31*).<sup>70–74</sup> Except for *CFAP45* and *RPL17P39*, the remaining DCGs do not have a described function. They are classified as long intergenic non-coding RNAs (lincRNA) such as *LINC02596* and *ENSG00000279082*, or lncRNAs like *ENSG00000261416* and *STX16-NPEPL1*. Functional studies aiming to identify the regulatory role of these non-coding RNAs should be conducted for a better understanding of their involvement in PD.

Supporting our results, the dysregulation of lipid metabolism and neuroinflammation have also been identified in the plasma and cerebrospinal fluid metabolic profiles of patients with LID.<sup>75</sup> Furthermore, a recent study conducted a transcriptomic analysis in the peripheral blood of PD patients and observed several DEGs involved in protein synthesis and ribosomal function.<sup>76</sup> Finally, some studies have demonstrated that the use of tetracyclines has significant anti-inflammatory therapeutic potential. For instance, doxycycline has been shown to





**Figure 3. Networks of co-expressed genes**

- (A) Complete Network of co-expressed genes in PD-ND.
- (B) Complete Network of co-expressed genes in PD-D. Network of exclusive co-expressed genes in PD-ND group.
- (C) Number of intersections of gene-gene interactions across all groups.
- (D) Network of exclusive co-expressed genes in PD-ND group and biological process enriched to each gene.
- (E) Network of exclusive co-expressed genes in PD-D group and biological process enriched to each gene.
- (F) Number of intersections of enriched pathways for co-expressed genes.
- (G) The main pathways enriched in the group of patients with dyskinesia. CT, control group; PD-D, people with Parkinson's disease with levodopa-induced dyskinesia; PD-ND, people with Parkinson's disease without levodopa-induced dyskinesia.

protect dopaminergic neurons from 6-hydroxydopamine (6-OHDA) induced degeneration and reduce LID in hemi parkinsonian rats, indicating that there may be a pro-inflammatory state contributing to the onset of LID.<sup>77–79</sup>

**Transcriptome reveals altered molecular plasticity in the developmental progression of PD and LID**

According to these results, we suggest that the disease progression, from the early to the advanced stage when the onset of LID occurs, is crucially influenced by mitochondrial damage. Initial mitochondrial changes promote a series of immune and cellular survival responses that enhances oxidative stress, leading to a cycle of mitochondrial dysfunction across the PD neurodegenerative process. Simultaneously, other mechanisms (alterations in nucleotide and lipid metabolism, inflammation, protein synthesis, endoplasmic reticulum stress, modifications in the membrane, and extracellular matrix) are influenced by multiple dysfunctions in mitochondria and enhances mitochondrial damage. Together, these pathogenic processes may lead to a chronic pro-inflammatory state in the basal ganglia, which has been associated with the development of LID.<sup>80–87</sup> See the details of these processes in the [Figure 5](#).

In conclusion, we have identified novel genes and gene-gene interaction networks associated with the progression of PD and the onset of LID. Our findings align with previous research on the pathophysiology of LID, indicating that dynamic maladaptive plastic changes contribute to motor complications. Furthermore, genes such as *HBA2*, *HBBP1*, and *ENSG00000281383* are of particular interest, as they seem to play a significant role in PD progression and the development of LID. In individuals with PD but without motor complications, we noticed downregulation of mitochondrial genes involved in the electron transport chain (*MT-CYB*, *MT-ND5*, and *MT-ND6*). These alterations are associated with dysregulation of ATP synthesis and an increase in reactive oxygen species (ROS). Furthermore, gene co-expression network analysis in the PD-ND group revealed a network of genes associated with mitochondrial functions, apoptosis regulation, stress glucose level regulation, and neurodegeneration. Our findings suggest that mitochondrial damage plays a crucial role in the progression of PD and neuroinflammatory chronic stages in PD. Our study highlights the importance of addressing mitochondrial dysfunction as a potential therapeutic target in managing PD progression and the development of LID.

**Limitations of the study**

We acknowledge certain limitations that could be addressed in future research. While blood serves as an excellent tissue for investigating systemic alterations in individuals, it is known that the administration of levodopa can cause changes in gene expression in inflammatory pathways in the central nervous system (CNS) and blood, as dopamine participates in the regulation of the immune system through its binding to DAR receptors, which are also present in the blood cells.<sup>88,89</sup> Thus, our findings related to inflammatory pathways may be more linked to peripheral events than to CNS events. To study this more precisely, cerebrospinal fluid (CSF) samples or the isolation of extracellular vesicles from blood derived from the CNS are good strategies, representing more specifically molecular changes in the CNS that occur during neurodegeneration in PD.

Other limitations include the absence of experimental validations through *in vitro* or *in vivo* models of the identified genes and pathways; the lack of a prodromal PD group, which would be crucial for better distinguishing the CT; the small number of samples, which could be expanded to improve the study's statistical power; and the lack of functional studies to confirm the role of the identified transcripts in PD and LID.

Among the strengths, we highlight that by pairing the groups for age and gender, controlling for various other variables, we present a controlled analysis of gene expression. Also, we explored the data using various methodologies and identified a convergence of results, confirming the pathways and mechanisms involved in PD and LID. We emphasize the importance of genetically characterizing a highly diverse and underrepresented population on the global stage, so that the molecular mechanisms described here can serve as a foundation for further studies aiming to improve the quality of life for patients and explore new therapeutic strategies.

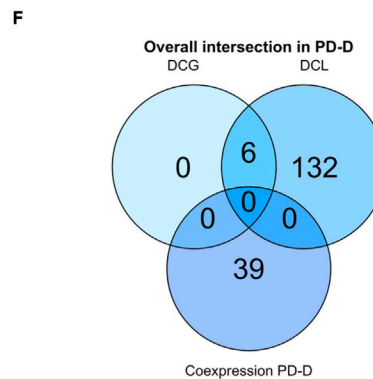
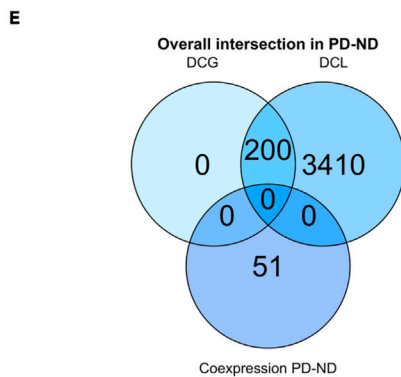
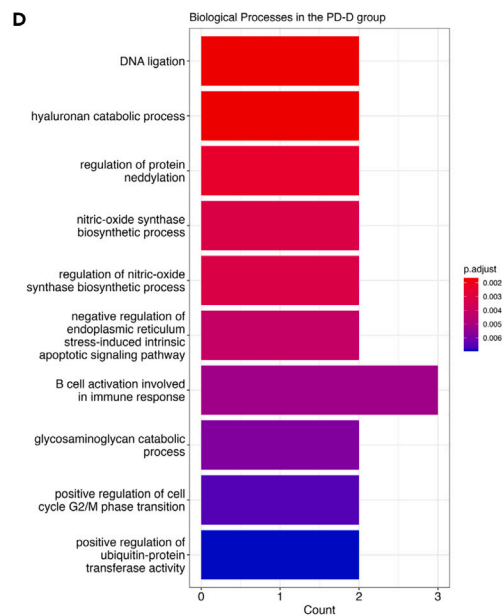
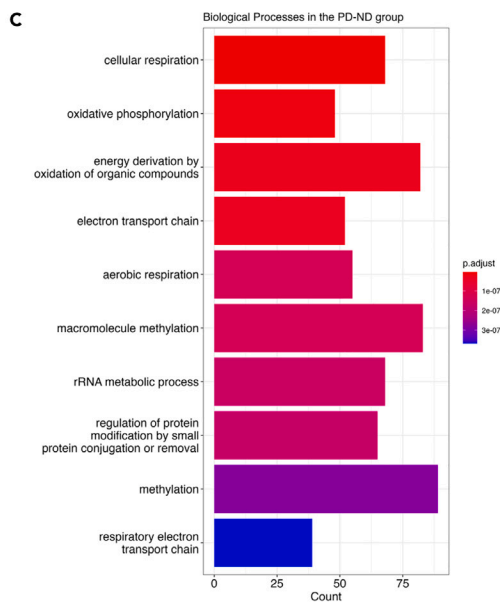
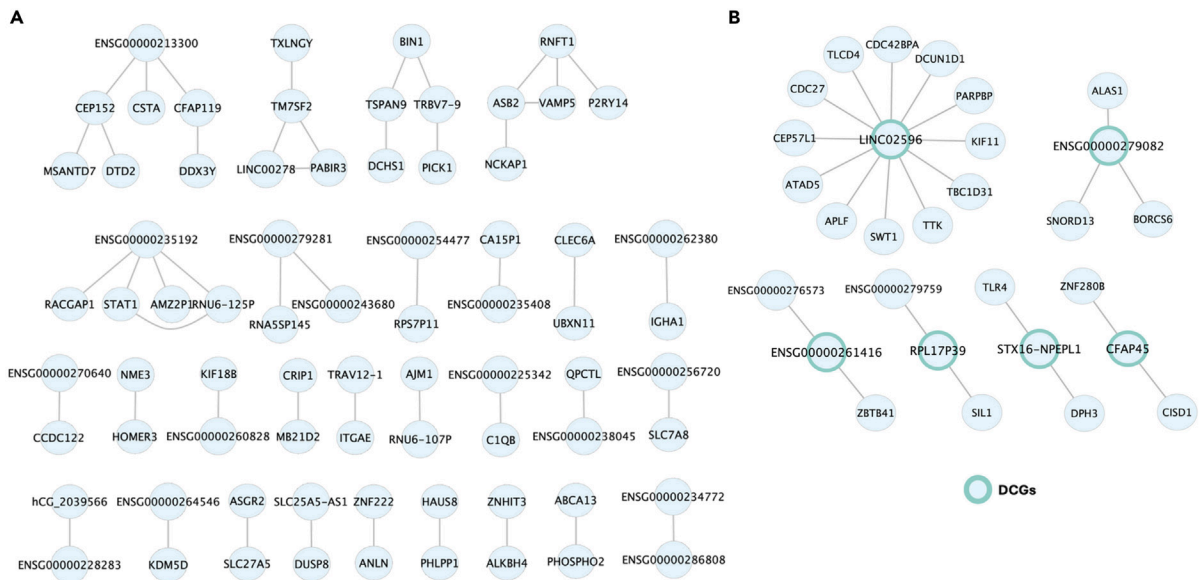
**RESOURCE AVAILABILITY**

**Lead contact**

Any additional information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ândrea Ribeiro-dos-Santos ([akelyufpa@gmail.com](mailto:akelyufpa@gmail.com)).

**Materials availability**

This study did not generate new unique reagents.



**Figure 4. Networks of differentially co-expressed genes**

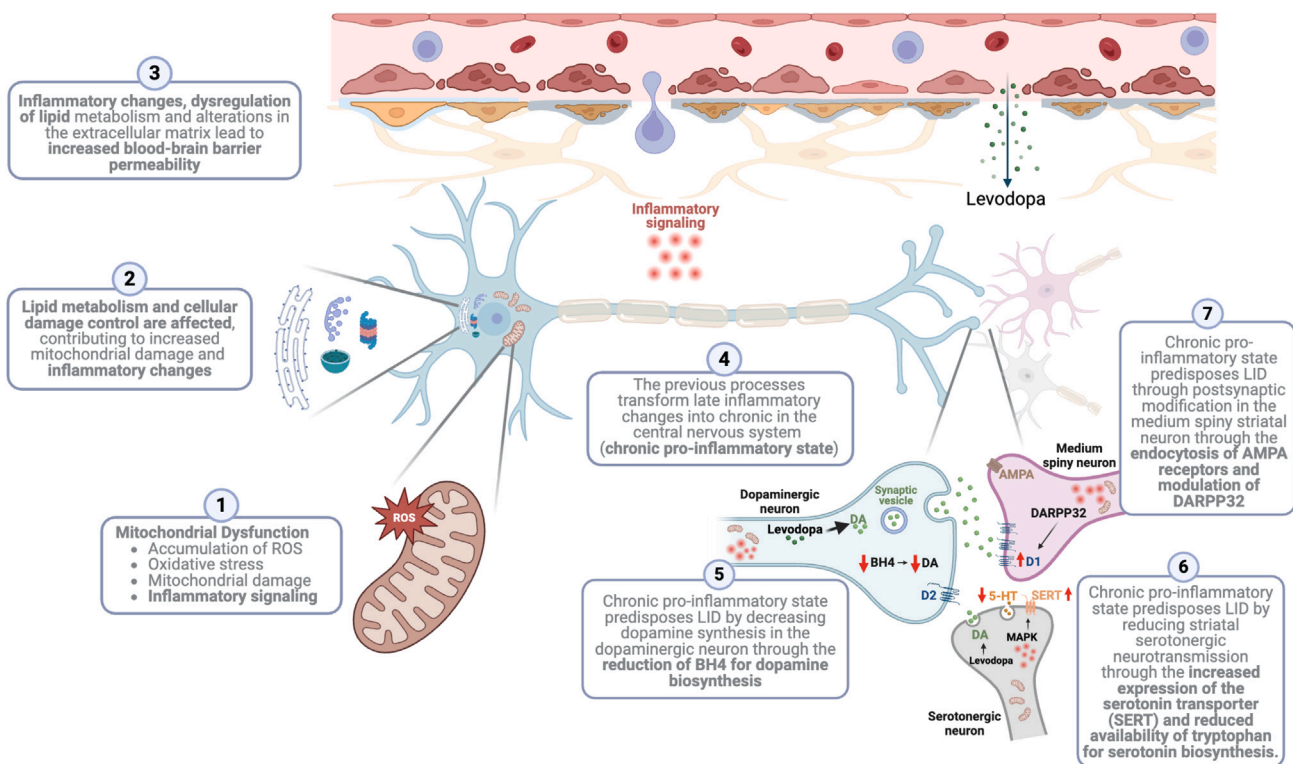
- (A) Network of DCLs of hub genes involved in 48 gene-gene interactions among themselves in PD-ND group, filtered by DCGs.  
 (B) Network of DCLs of hub genes involved in 23 gene-gene interactions exclusive co-expressed genes in PD-D group, filtered by DCGs.  
 (C) Top 10 most significant biological processes enriched for differentially co-expressed genes in the PD-ND.  
 (D) Top 10 most significant biological processes enriched for differentially co-expressed genes in the PD-D.  
 (E) Overall intersection of co-expression and differential co-expression results in PD-ND group.  
 (F) Overall intersection of focused LID co-expression and differential co-expression results. PD-D, people with Parkinson's disease with levodopa-induced dyskinesia; PD-ND, people with Parkinson's disease without levodopa-induced dyskinesia; DCGs, differentially co-expressed genes; DCLs, differentially co-expressed links.

**Data and code availability**

- All raw sequences are deposited at the European Nucleotide Archive (ENA), with the accession number PRJEB72411 and are publicly available as of the date of publication.
- All data analysis codes are available in the GitHub repository: <https://github.com/tati-souz/RNA-Seq-data-analysis>.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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We are immensely grateful to everyone who participated in this research. And we emphasize the importance of characterizing the genetic mechanisms present in a highly diverse and underrepresented population on the global scene, such as the Amazon. We hope that the results can serve to improve patients' quality of life and explore new therapeutic strategies.



**Figure 5. Mitochondrial damage leads to levodopa-induced dyskinesia (LID) through the chronic pro-inflammatory stage and other processes**

(1) Mitochondrial dysfunction is observed during Parkinson's disease (PD), reinforcing a pro-inflammatory state; (2) initial mitochondrial damage and inflammation contribute to the dysregulation of lipid metabolism and protein synthesis/regulation; (3) these changes affect the permeability of the blood-brain barrier, leading to increased flow of levodopa and immune cells and molecules; (4) the ongoing dysregulation in these systems increasingly reinforces inflammation to a chronic pro-inflammatory stage; (5) chronic neuroinflammation can negatively regulate dopamine (DA) biosynthesis through the reduction of tetrahydrobiopterin (BH4), which is essential for DA synthesis<sup>31,82</sup>; (6) chronic neuroinflammation reduces serotonin (5-HT) levels in the striatum and contributes to LID by dysregulating DA release through stimulation of the MAPK pathway<sup>83</sup> and increasing of serotonin transporter (SERT) expression<sup>84,85</sup>; (7) Chronic neuroinflammation induces modifications in  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 32 kDa cyclic AMP-regulated phosphoprotein (DARPP32), affecting corticostriatal glutamatergic synapses and post-synaptic D1 dopamine receptor stimulation.<sup>86,87</sup> Created with [BioRender.com](https://BioRender.com). Abbreviations: DA, Dopamine; BH4, Tetrahydrobiopterin; 5-HT, Serotonin; MAPK, Mitogen-Activated Protein Kinase; SERT, Serotonin Transporter; AMPA,  $\alpha$ -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid; DARPP32, 32 kDa Cyclic AMP-Regulated Phosphoprotein.

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## AUTHOR CONTRIBUTIONS

Recruitment of participants and collection of biological material, T.P.S., C.S.S., and G.L.E.; clinical assessment of participants, A.F.E. and B.L.S.L.; definition of the methodology, A.R.S., T.P.S., G.S.A., and G.B.S-S; extraction, quality control, library preparation, and sequencing, T.P.S., C.S.S., G.C.C., and L.M.; article writing, T.P.S.; data analysis, T.P.S., A.R.S., G.B.S.S., and G.S.A.; illustration, T.P.S. and B.L.S.L.; review and coordination, A.R.S., G.S.A., and B.L.S.L.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
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## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Peripheral blood (whole blood) from patients with Parkinson's disease with dyskinesia	Movement Disorders Clinic of the Hospital Ophir Loyola	N/A
Peripheral blood (whole blood) from patients with Parkinson's disease without dyskinesia	Movement Disorders Clinic of the Hospital Ophir Loyola	N/A
Peripheral blood (whole blood) from healthy donors	Movement Disorders Clinic of the Hospital Ophir Loyola	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
TRIZol LS Reagent	Thermo Fisher Scientific by Invitrogen™	Cat#10296010
<b>Critical commercial assays</b>		
TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold kit	Illumina Inc., Chicago, IL, USA	Cat# RS-122-2301, RS-122-2302, and RS-122-2303
<b>Deposited data</b>		
Raw sequences of RNA-Seq	This study	ENA: PRJEB72411
<b>Software and algorithms</b>		
Code of RNA-Seq Data analysis	This study	<a href="https://github.com/tati-souz/RNA-Seq-data-analysis">https://github.com/tati-souz/RNA-Seq-data-analysis</a> .
Fastp tool	Chen et al. <sup>90</sup>	
Star Aligner Software	Dobin et al. <sup>91</sup>	<a href="https://doi.org/10.1093/bioinformatics/bts635">https://doi.org/10.1093/bioinformatics/bts635</a>
HTSeq software	Anders et al. <sup>92</sup>	<a href="https://academic.oup.com/bioinformatics/article/31/2/166/2366196">https://academic.oup.com/bioinformatics/article/31/2/166/2366196</a>
DESeq2 package	Love et al. <sup>93</sup>	<a href="https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html">https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html</a>
RStudio 4.2.3 software	R Core team (2023) <sup>94</sup>	<a href="https://www.R-project.org/">https://www.R-project.org/</a>
pROC package	Robin et al. <sup>95</sup>	<a href="https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-12-77">https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-12-77</a>
ClusterProfiler package	Yu et al. <sup>96</sup>	<a href="https://www.liebertpub.com/doi/10.1089/omi.2011.0118">https://www.liebertpub.com/doi/10.1089/omi.2011.0118</a>
Ggplot2 package	Wilkinson et al. <sup>97</sup>	<a href="https://academic.oup.com/biometrics/article/67/2/678/7381027?login=false">https://academic.oup.com/biometrics/article/67/2/678/7381027?login=false</a>
CEMitoool package	Russo et al. <sup>98</sup>	<a href="https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-018-2053-1">https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-018-2053-1</a>
Cytoscape software	Shannon et al. <sup>99</sup>	<a href="https://genome.cshlp.org/content/13/11/2498">https://genome.cshlp.org/content/13/11/2498</a>
Diffcoexp package	Wei et al. <sup>100</sup>	<a href="https://github.com/hidelab/diffcoexp">https://github.com/hidelab/diffcoexp</a>
<b>Other</b>		
Qubit 2.0 Fluorometer	Thermo Fisher Scientific	Cat#32866
NanoDrop ND1000	Thermo Fisher Scientific	Cat# ND2000CLAPTOP
2200 TapeStation System	Agilent	Cat#G2964AA
NextSeq Sequencing 550 System	Illumina	Cat# SY-415-1002

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

### Human participants

The use of human subjects was approved by the Hospital Ophir Loyola Ethics Committee (Number 82848118.5.0000.5550), and all participants provided written informed consent. We conducted an observational cross-sectional study to profile gene and gene-gene co-expression networks in people with PD with LID (PD-D), people with PD without LID (PD-ND), and non-PD controls (CT). Participants with PD diagnosed according to the UK Parkinson's Disease Society Brain Bank were recruited in the Movement Disorders Clinic of the Hospital Ophir Loyola, in Belém, Brazil, between January 2018 and December 2021. People with PD and control subjects were recruited using a 1:1 ratio matching for sex and age within four years (See the detailed information of clinical characteristics in [Table S1](#)).

Brazilian populations have a high rate of miscegenation, reflecting the different ethnic contributions throughout our history. Consequently, our genomes and/or biological markers represent mosaics of European, African, indigenous, and other ancestries. In the Amazon region, we observed an average contribution of European ancestry genes at 60%, indigenous (original peoples) at 28%, and African ancestry at 12%.<sup>101</sup> It is worth noting that the Amazon region has the largest contribution of genes from indigenous peoples. Additionally, previous work by our group on Parkinson's patients did not observe statistically significant differences in the average contribution of genomic ancestry genes (59% European, 26% indigenous, and 15% African).<sup>102</sup>

### Inclusion and exclusion criteria for participants

As inclusion criteria, we enrolled participants with age at evaluation over 40 years and, for people with PD, a stable levodopa dose in the previous month. CT samples were obtained from volunteers who were invited to participate in the research. Participants with a history of autoimmune, inflammatory, cancer and other neurological diseases were excluded. In addition, PD patients with severe psychotic symptoms (score >2 in item 1.2 of the Movement Disorder Society – Unified Parkinson's Disease Rating Scale, MDS-UPDRS) and clinical diagnosis of dementia were removed or control subjects with a family history of PD.

## METHOD DETAILS

### Clinical evaluations

All participants with PD were evaluated by the same movement disorders specialist (B.L.S.-L.) using the MDS-UPDRS in the ON-stage. We defined the presence of LID by a score  $\geq 1$  on item 4.1 of the MDS-UPDRS Part IV (time spent with dyskinesia) and confirmed if the patient presented abnormal movements at evaluation. Blood samples were collected on the same day of clinical evaluation.

### Blood collection and total RNA extraction

Peripheral blood was collected on the same day and period, between 09:00 and 11:00 a.m., without fasting. All people with PD were instructed to take their regular morning antiparkinsonian drugs before peripheral blood intravenous puncture. Blood samples were stored with TRIzol LS (Thermo Fisher Scientific) following the manufacturer's instructions at  $-20^{\circ}\text{C}$  until further use. Total RNA extraction from the blood samples followed the nucleic acid extraction protocol using TRIzol LS (Thermo Fisher Scientific), in accordance with the manufacturer's instructions. After extraction, the total RNA was assessed for integrity, purity, and concentration using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific), NanoDrop ND1000 (Thermo Fisher Scientific), and 2200 TapeStation System (Agilent).

### Library preparation, sequencing and quality control

For library preparation (total transcriptome), 1  $\mu\text{g}$  of total RNA per sample was used. A total of 46 libraries were constructed (20 controls, 15 PD-D, 11 PD-ND) using the TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold kit (Illumina Inc., Chicago, IL, USA). The libraries were normalized and sequenced on the NextSeq Sequencing System (Illumina) using the NextSeq High Output 150 cycles (Illumina). After sequencing, quality control was performed, where low-quality reads (phred-score = Q30) were discarded, the 3' ends of sequences were trimmed, and contaminants were removed using the *fastp* tool.<sup>90</sup> For alignment, the *STAR* software<sup>91</sup> was used with the human genome reference GRCh38. Counting of messenger RNAs (mRNAs) and long non-coding RNAs (lncRNAs) was performed using HTSeq.<sup>92</sup>

### Differential gene expression analysis

Differential expression analysis was performed for the PD-D, PD-ND, and CT groups using the *DESeq2*<sup>93</sup> package in RStudio software (v.4.2.3).<sup>66–69</sup> *DESeq2* normalizes counts by library size and adjusts gene expression, taking gene size into consideration. To compare CT group vs. general PD group (PD-D + PD-ND) and the subgroups with and without dyskinesia, the controlled covariates were age, sex, and sequencing batch. Furthermore, when we compared PD-D vs. PD-ND, the covariates controlled were age, sex, sequencing batch, duration of disease, and levodopa equivalent daily dose (LEDD) to avoid confounding factors and false-positive results. Adjusted *p*-values (FDR) < 0.05 were considered statistically significant. To identify potential biomarkers associated with PD, we investigated the sensitivity and specificity of DEGs using the ROC curve through the pROC package in software (v.4.2.1).<sup>66–69</sup> Thus, we selected those genes with an area under the curve (AUC) > 80% as potential disease biomarkers. We conducted Gene Set Enrichment Analysis (GSEA) to identify enriched metabolic pathways for all genes in PD-D and PD-ND groups. Consequently, pathways with high and positive enrichment scores are presumed to be associated with over-expressed genes, while those with high and negative scores are linked to under-expressed genes. Furthermore, Gene Ontology

analyses for DEGs were carried out using the *ClusterProfiler* package<sup>96</sup> in R software (v.4.2.1). The graphics are constructed with ggplot2 package<sup>97</sup> in RStudio.

### Gene co-expression analysis

Gene co-expression analysis was performed using the *CEMitoool*<sup>98</sup> package in RStudio, for PD-D, PD-ND and CT groups. Correlation was computed to assess the similarity of gene expression within each group. This method allows us to infer modules of co-expressed genes that may play the same biological process roles. We also performed the joint co-expression analysis of CT + PD-D + PD-ND to select genes exclusively co-expressed in the PD-D and PD-ND groups. Finally, co-expression networks were drawn using Cytoscape software (v.3.10).<sup>99</sup> The *CEMitoool* package also performs Over Representation Analysis (ORA) to assess the pathways enrichment.

### Differential co-expression gene network analysis

The differential co-expression gene network analysis aims to identify modules of Differential Co-expressed Links (DCLs), representing pairs of genes that exhibit statistically differences on correlations between two distinct conditions. Moreover, it identifies Differentially Co-expressed Genes (DCGs) referring to genes with a higher number of DCLs than expected by chance (gene hubs). We employed the *diffcoexp* package<sup>100</sup> in the RStudio, setting for spearman correlation method for gene pairwise similarity between the groups (PD-D x CT and PD-ND x CT). Subsequently, we constructed differential co-expression networks using Cytoscape software (v.3.10). Significance was determined by adjusted *p*-values (FDR) < 0.05, and genes with a correlation coefficient (*r*) > 0.8 were selected. To assess network specificity for each group, a similarity analysis utilizing the Jaccard index was conducted. This helped to determine whether the networks of DCGs were exclusive to each group. For insights into the biological processes involving DCGs and genes in DCLs, GO analysis was performed through the *ClusterProfiler* package<sup>96</sup> in RStudio.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses detailed in the results (Table 1) were performed using RStudio. The selection of tests was based on the data types, number of samples, and data distribution pattern (see Table S1). To compare the frequency of each sex between groups, the chi-square test was used. The Kruskal-Wallis test was employed to compare the age at clinical evaluation, and the Mann-Whitney test was conducted to compare the means of age at disease onset, levodopa dosage, and treatment duration.