Insulin-Like Growth Factor 2 (IGF2) and Autophagy Gene Expression Alteration in Parkinson's Disease: Potential Predictor Markers.

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Abstract

Insulin-like growth factor 2 (IGF2) and autophagy-related genes have been proposed as interesting biomolecules related to idiopathic Parkinson's disease (PD). The objective of this study was to determine the IGF2 and IGF1 levels in plasma and peripheral blood mononuclear cells (PBMCs) from patients with moderately advanced PD and explore the potential correlation with autophagy-related genes in the same blood samples. IGF1 and IGF2 levels in patients' plasma were measured by ELISA, and the IGF2 expression levels were determined by real-time PCR and Western blot in PBMCs. The expression of autophagy-related genes was evaluated by real-time PCR. The results show a significant decrease in IGF2 plasma levels in PD patients compared with a healthy control group. We also report a dramatic decrease in *IGF2* mRNA and protein levels in PBMCs from PD patients. In addition, we observed a downregulation of key components of the initial stages of the autophagy process. Although IGF2 levels were not directly correlated with disease severity, we found a correlation between its levels and autophagy genes expression from the same samples, in a sex-dependent manner. To further explore this correlation, we treated mice macrophages cell culture with α -synuclein and IGF2. While α -synuclein treatment decreased levels of Beclin1 and Atg5, IGF2 treatment reverted these effects. Our results suggest a relationship between IGF2 levels and the autophagy process in PD and its potential application as a multi-biomarkers to determine the PD patients' stages of the disease.

Introduction

Parkinson's disease (PD) is one of the most frequent neurodegenerative diseases and prevalence of 1% in persons over 60 years and 3% in people over 80 years (Lee and Gilbert, 2016). There are no research data about the prevalence of PD in Chile, but it is estimated in 40,000 patients (Chana et al., 2013). PD is characterized by motor dysfunction with both cardinal symptoms as bradykinesia, rigidity, tremor at rest (Jankovic, 2008), and axial symptoms such as gait disturbances and postural instability (Fasano et al., 2015; Hely et al., 2005). Tremor at rest is the classic manifestation, with a 4-6 Hz frequency, and can be absent in 25% of the patients at diagnosis (Jankovic, 2008). Additionally, non-motor symptoms in PD include smell disorders, constipation, sleep disturbances like rapid-eye-movement (REM) behavioral disorder, among others (Navarro-Peternella and Marcon, 2012; Schapira et al., 2017), which are manifested in the early stages of the pathology. At the molecular level, the accumulation of the synaptic protein α -synuclein in Lewy bodies is a histopathological hallmark of idiopathic and familial PD cases (Schulz-Schaeffer, 2010; Spillantini et al., 1998). The expression of α -synuclein is not limited to the nervous system, also is present in the cerebrospinal fluid (CSF), in plasma (El-Agnaf et al., 2003; Forland et al., 2018), as well as is expressed in the erythropoietic lineage cells (Nakai et al., 2007) and peripheral lymphocytes (Kim et al., 2004). However, the definitive diagnosis of PD is based on motor impairment, when the neurodegenerative process is extremely advanced. The finding of biomarkers for an accurate diagnosis and progression monitorization of PD remains unsolved.

An ever-increasing number of humoral growth factors active in different tissues have been implicated in brain physiology. Insulin and its related peptides, insulin-like growth factors 1 and 2 (IGF1 and IGF2), have

been recognized as neuroactive peptides, influencing neuronal homeostasis through different mechanisms of action (Vincent and Feldman, 2002). The IGF system comprises hormonal ligands, insulin, IGF1, and IGF2 and their receptors, IR, IGF1R, and IGF2R (Cohen et al., 1991). Studies in cellular and preclinical models of neurodegenerative diseases have validated the neuroprotective effects of IGFs to prevent disease progression (Jarvis et al., 2007), including PD, particularly considering both their roles as (i) neurogenic agents and (ii) their apparent neuroprotective effect (Garcia-Huerta et al., 2016; Hakuno and Takahashi, 2018; Martin-Montanez et al., 2021). In the case of the neurotrophic factor IGF1, it has a role in brain development, neuroprotection, and neurogenesis (Torres-Aleman, 2000). In PD, IGF1 has shown a neuroprotector effect, reducing apoptosis levels in the toxicity induced by dopamine in neuronal cultures (Offen et al., 2001). Also, it reduces α -synuclein toxicity **and protein aggregates formation in neuroblastoma cell lines (Kao, 2009). In rat models of PD, IGF1 treatment has shown beneficial effects in preventing dopaminergic neuronal loss in the substantia nigra (Ebert et al., 2008) and improving motor deficits (Guan et al., 2000; Krishnamurthi et al., 2004).** Accumulating evidence suggests that a progressive decline in the levels of serum IGF1 may also contribute to age-associated brain disorders (Carro et al., 2002; Torres-Aleman, 2000; Trejo et al., 2004).

On the other hand, IGF2 is a single-chain polypeptide expressed by most tissues during fetal life, being found predominantly within the brain and spinal cord during adulthood (Russo et al., 2005; Soares et al., 1985). Several pieces of evidence highlight the physiological function of IGF2 in cognition, neuronal differentiation, and neuronal survival (Allodi et al., 2016; Dikkes et al., 2007; Garcia-Huerta et al., 2020; Stern et al., 2014; Suh et al., 2013). For instance, *Igf2* is a target of the transcription factor C/EBP β that is engaged during memory consolidation in rats (Chen et al., 2011), and IGF2 overexpression rescues working memory deficits in a mouse model of schizophrenia (Ouchi et al., 2013). Also, IGF2 has been described as a regulator of adult hippocampal neurogenesis (Bracko et al., 2012) and a neuron survival factor in *in vitro* studies (Haselbacher et al., 1989; Knusel et al., 1990). It has also been shown that IGF2 participates in neuromuscular synapse formation (Ishii, 1989). Interestingly, IGF2 expression is decreased in the hippocampus of Alzheimer's disease (AD) patients and a mouse model of this disease (Mellott et al., 2014). The IGF2 overexpression reduced amyloid- β plaques in the hippocampus of transgenic AD mice (Mellott et al., 2014; Pascual-Lucas et al., 2014), which was mediated by the IGF2R (Pascual-Lucas et al., 2014). Recently, we described the protective role of IGF2 in Huntington's disease (HD) (Garcia-Huerta et al., 2020). IGF2 administration diminished the load of mutant huntingtin protein in HD preclinic models and iPSCs from HD patients. We determined a significant decrease in plasmatic IGF2 levels in blood samples from HD patients (Garcia-Huerta et al., 2020). IGF2 also showed a substantial expression reduction in peripheral blood mononuclear cells (PBMCs) and brain tissues from HD patients, arising as an interesting biomarker for neurodegenerative diseases. Remarkably, genetic linkage studies have associated IGF2 with PD. A single-nucleotide polymorphism (SNP) in the *IGF2* gene was identified as a potential modifier of the susceptibility to develop idiopathic PD in a Caucasian group from Australia (Sutherland et al., 2008). This polymorphism was associated with reduced IGF2 expression levels, lower body weight, and low levels of tyrosine hydroxylase enzyme (TH) expression, the limiting enzyme in dopamine production (Gaunt et al., 2001). PD patients usually present a low body mass index, potentially

reflecting a broader metabolic disorder (Bachmann et al., 2009; Kistner et al., 2014). However, the molecular mechanism involved in IGF2 neuroprotection is until under study.

IGF2/IGF2R signaling participates in the transport of lysosomal hydrolases from the Golgi apparatus to lysosomes (Killian and Jirtle, 1999; Kornfeld, 1992), suggesting a potential connection between IGF2 with the autophagy-endolysosomal pathway. It has been demonstrated that IGF2 is overexpressed in colorectal cancer cells and that this phenomenon is associated with increased autophagy activity (Gao et al., 2020). Moreover, the overexpression of IGF2 in pancreatic b cells is associated with increased ER stress, autophagy activation, and b cells dedifferentiation (Casellas et al., 2015). Macroautophagy (hereafter referred to as autophagy) is the primary intracellular process responsible for cargos' bulk or selective degradation by delivering them to lysosomes by vesicle traffic. This process is highly regulated by several ATG (autophagy-related genes) protein complexes (Filimonenko et al., 2010). The autophagy process is an important pathway for the clearance of misfolded proteins involved in neurodegenerative diseases such as tau, mutant huntingtin, ataxin-3, and mutated SOD1 (Frake et al., 2015; Vidal et al., 2014). It has been described that selective chaperone-mediated autophagy (CMA) is involved in α -synuclein degradation in PD *in vivo* models and PD patients (Alvarez-Erviti et al., 2010; Cuervo et al., 2004).

Lysosomes and autophagosome vesicles were reported together with Lewy bodies and α -synuclein in postmortem PD brain samples (Shahmoradian et al., 2019). The expression of autophagy genes was also explored in blood samples from patients in moderate to advanced PD. It was reported a downregulation in mRNA levels of six core regulators of autophagy *ULK1*, *ATG5*, *ATG2A*, *ATG4B*, *ATG6L1*, and *HDAC6* in PBMCs from PD patients (Miki et al., 2018). Interestingly, protein levels of ULK1, BECLIN1, and AMBRA1, proteins participating at initial steps of the pathway, were increased in PBMCs samples obtained from PD patients in another study (Miki et al., 2018). Although some links have been proposed between IGFs and the lysosomal-autophagy pathway, IGF1 or IGF2 levels and the expression of autophagy genes in PBMCs from PD patients were not yet associated. These data could contribute to a multi-biomarker approach to monitor disease progression and diagnosis.

In the present work, we evaluated IGF1 and 2 levels and the transcriptional expression of key genes from the autophagy pathways in blood samples from moderate to severe male and female sporadic PD patients and their association with the pathology's severity. We observed a significant reduction in IGF2 mRNA and protein levels in PD patients, with no differences in IGF1 instead. We also found a decrease in *ATG5*, *ULK1*, and *BECLIN1* transcriptional levels in PBMCs samples from sporadic PD patients, associated with the reduction of IGF2 mRNA and protein levels. To assess the effect of IGF2 on autophagy genes expression in a PD context, we treated primary mice macrophages with IGF2 after preincubation with α -synuclein fibers. IGF2 treatment reversed the downregulation of *ATG5* and *Beclin1* caused by α -synuclein, indicating a potential IGF2 positive effect on autophagy activity in a PD model. Our results show decreased serum IGF2 levels as an easy-going outcome for PD. It is associated with the levels of autophagy genes, particularly from the initial steps of autophagosome formation.

Materials And Methods

Study participants and clinical evaluation

These experiments were performed in the Neurology department of *Hospital Clínico Fuerza Aérea de Chile* (FACH), and the Center for Integrative Biology (CIB) of Universidad Mayor in Santiago, Chile, in accordance with the Declaration of Helsinki and was approved by the local ethical committee of the FACH hospital.

All the participants, 43 PD patients, and 41 healthy controls were recruited and signed written informed consent.

PD patients were evaluated with clinical history, physical examination and received a score from the Hoehn and Yahr modified (Goetz et al., 2004) and UPDRS (Movement Disorder Society Task Force on Rating Scales for Parkinson's, 2003; Palmer et al., 2010) scale (Tables 1, 2). We considered as inclusion criteria subjects aging over 18 years, having a history of attention in Hospital FACH, subjects with a diagnosis of PD in stage 1-4 of Hoehn and Yahr modified scale, signed informed consent. On the other hand, exclusion criteria were age under 18 years, and subjects diagnosed with PD in stage 5 of Hoehn and Yahr modified scale.

Plasma and PBMCs isolation

The blood sample was 30 mL of venous blood extracted at the FACH Hospital and stored in EDTA tubes. Plasma and PBMCs fractions are obtained based on centrifugation and using-ficoll protocols (Biddison, 2001; Garcia-Huerta et al., 2020).

Western blot and ELISA

For western blot analysis, cells were homogenized in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100) containing protease and phosphatase inhibitors (Roche). After sonication, protein concentration was determined in all experiments by micro-BCA assay (Pierce), and 20-30 µg of total protein was loaded onto 8 to 15 % SDS-PAGE mini gels (Bio-Rad Laboratories, Hercules, CA) prior to transfer onto PVDF membranes. Membranes were blocked using PBS, 0.1% Tween-20 (PBST) containing 5% milk for 60 min at room temperature and then probed overnight with primary antibodies in PBS, 0.02% Tween-20 (PBST) containing 5% skimmed milk. The following primary antibodies and dilutions were used: anti-IGF2 1:1000 (Abcam, Cat. nº Ab9574), anti-GAPDH 1:2000 (Santa Cruz, Cat. nº SC-365062). Bound antibodies were detected with peroxidase-coupled secondary antibodies incubated for 2 h at room temperature and the ECL system. The plasma levels of IGF1 and IGF2 were measured by ELISA (ALCO, 22-IGFHU-E01, and 22-IG2HU-E01) according to the manufacturer's instructions.

Bone marrow macrophages isolation.

α -synuclein overexpressing (ASO) mice at 3 months old (Rockenstein et al., 2002) were euthanized by isoflurane overdose. Bone marrows from the femur cavity were extracted as previously described (Zhang et al., 2008). Briefly, bone marrows were extracted with a needle using a sterile 15-ml tube. Cells were centrifuged for 5 min at 500×g, at room temperature. Cell pellets containing macrophages were resuspended with complete medium (MCS-F) by tapping the tube, pipetting up and down, then transferring them to a 100 mm sterile plate. Macrophages were maintained at 37°C, 5% CO₂ incubator for seven days. To determine the effect of IGF2 on the autophagy genes expression, macrophages were treated with recombinant IGF2 (5 ng/mL, Sigma) for 1, 3, and 5 days (Du et al., 2019). After the IGF2 treatment, macrophages were stimulated with 1 μ g of recombinant mouse α -synuclein PFFs were generated as previously described (Mahul-Mellier et al., 2015) or PBS for 72 h.

Human PBMCs extraction and mice macrophage gene expression analysis.

RNA extraction from PBMCs and cDNA synthesis were performed to evaluate the IGF2 and autophagy-related mRNA levels by real-time PCR. The human primers used were: *IGF2*: 5'-GTG CTG TTT CCG CAG CTG-3' and 5'-AGG GGT CGA CAC GTC CCT C-3' *ULK-1*: 5'-CAG ACG ACT TCG TCA TGG TC-3' and 5'-AGC TCC CAC TGC ACA TCA G-3', *P62*: 5'-TGC CCA GAC TAC GAC TTG TG-3' and 5'-AGT GTC CGT GTT TCA CCT TCC-3', *LC3*: 5'-CAT GAG CGA GTT GGT CAA GA-3' and 5'-CCA TGC TGT GCT GGT TCA-3', *RUBICON*: 5'-CTGGCAGTTCGTGAAAGACA-3' and 5'-TTAGCAGGAAGGCAGCATCT-3', *ATG5*: 5'-TTT GCA TCA CCT CTG CTT TC-3' and 5'-TAG GCC AAA GGT TTC AGC TT-3', *BECLIN*: 5'-GGA TGG TGT CTC TCG CAG AT-3' and 5'-TTG GCA CTT TCT GTG GAC AT-3'; *SDHA*: 5'-GAG GCA GGG TTT AAT ACA GCA-3' and 5'-CCA CCA CTG GGT ATT GAG TAG AA-3'. The murine primers used were as following: *mATG5*: 5'-GCC TAT ATG TAC TGC TTC ATC CA-3' and 5'-CAT TTC AGG GGT GTG CCT TCA-3'; *mBeclin1*: 5'-GTG CGC TAC GCC CAG ATC-3' and 5'-GAT GTG GAA GGT GGC ATT GAA-3'; *mLC3*: 5'-CGG AGC TTT GAA CAA AGA GTC-3' and 5'-TCT CTC ACT CTC GTA CAC TTC-3'; *mp62*: 5'-TGA AAC ATG GAC ACT TTG GCT-3' and 5'-ACA TTG GGA TCT TCT GGT GGA-3'; and *mActin1*: 5'-GGC TGT ATT CCC CTC CAT CG-3' and 5'-CCA GTT GGT AAC AAT GCC ATG T-3'.

Statistical analysis

Outliers test was performed in each measurement, resulting in differences in samples number in each experiment. Pearson statistical analysis was performed to correlate plasma levels of IGF1 and 2 with the clinical stage of PD, and after that, it was analyzed with PRISM 8 software. Mann-Whitney test was performed to analyze the differences between controls and patient groups for genic expression and protein levels. For example, for the hypothesis that the average IGF2 plasma value (ng/mL) in patients is less than in controls, a Shapiro-Wilk (Shapiro and Wilk, 1965) normality test was performed, obtaining SW-W=0.821, p = 0.00001 for control, and SW-W=0.798, p=0.00001 for patients, it is rejected that both samples have an approximately normal distribution. Since both variables do not have a normal distribution, a non-parametric U Mann-Whitney test is used. For the $H_0 : \mu_c = \mu_p$ vs $H_1 : \mu_c > \mu_p$ hypothesis, the following are obtained: $T_c=3240$, $T_p=2755$, $U_p=1914$, $z=2.64$, as this calculated z value is greater than the critical z value for $\alpha=0.05$, H_0 is rejected, then it is concluded that the mean of IGF2 in controls is more

significant than in patients, p value=0.004. mRNA expression levels in macrophage cell culture were analyzed using ordinary one-way ANOVA.

Results

IGF2 is downregulated in the blood of PD patients.

Since IGF2 is a soluble secreted factor and its plasma levels have been suggested as a possible biomarker of cancer (Huang et al., 2010; Zanella et al., 2015), we decided to measure the quantity of IGF2 in plasma from PD patients using ELISA. This analysis revealed a slight but significant decrease in circulating IGF2 present in the plasma samples derived from PD patients compared to control subjects (Fig. 1A), which may be related to the different contributions of tissues and cell types to plasmatic IGF2 levels. Controversially, the soluble IGF1 plasma levels were similar in PD patients compared to healthy control (HC) (Fig. 1B).

Different studies suggest that the changes observed in cellular homeostasis in the brain can be reflected in variation in the concentration of different plasma components, such as secreted factors or even changes in the mRNA expression levels. Therefore we evaluate the presence of IGF2 in PBMCs from PD patients. Although control PMBCs presented a clear expression of IGF2, PD-derived cells had a significant decrease in IGF2 levels, observing a nearly 70% decrease in its protein levels using Western blot analysis (Fig. 1C). These results were confirmed when mRNA levels of *IGF2* were measured in the same samples, observing a near 90% decrease in its levels (Fig. 1D). Taken together, these results suggest that IGF2 levels are drastically reduced in plasma and blood cells of PD patients.

Demographic and clinical characteristics of PD patients and HC are shown in Table 1. The average medical history is 6.6 ± 5.6 years in PD patients. The evaluation with Höehn and Yahr scale showed the majority of PD patients cohort were in stage III (58%), and 92-95% of the patients had a mild to moderate state of disease (between I to III of Höehn and Yahr scale) which corresponds to a state of self-valence of patients with postural stability disorder. The average motor UPDRS was 34.6 ± 12.5 , categorizing the patients in an intermediate state of the disease concerning their motor condition. There was no significant difference in participants' age or gender ratio in both groups (PD and HC). However, plasma IGF1 or IGF2 levels were not correlated with clinical scores (UPDRSIII and H&Y) in PD patients (Fig. 2).

Autophagy components are altered in PBMCs from PD patients.

Considering previous reports describing a positive correlation between IGF2 and autophagy pathway in colorectal cancer cells and pancreatic β cells (Casellas et al., 2015; Gao et al., 2020), we determined the levels of autophagy-related genes in PBMCs from PD patients and HC. We observed a significant downregulation of *ATG5*, *ULK1*, and *BECLIN1*, genes involved at initial stages in the autophagy pathway, in PD patients compared to HCs (Fig. 3A). Moreover, we observed an upregulation of *RUBICON* and *LC3* mRNA levels in PD patients compared to HCs (Fig. 3B). However, we did not observe significant changes in *P62* levels, a selective autophagy receptor (Fig. 3B).

We generated a heat map to determine each autophagy component's correlation with the different subjects in the healthy and PD groups. We observed a clear difference in the gene expression profile between the HC group (Fig. 4A, upper) and the PD patients' group (Fig. 4A, bottom), showing a decrease in the expression of genes from the initial step of the autophagy pathway, excepting *LC3* and *RUBICON*. Then, we performed a principal component analysis (PCA) (Pang et al., 2021; Seki et al., 2019; Wang et al., 2021) by using the Statistics 10.0 software (Hair et al., 2013). This analysis revealed a clear separation profile for a group of patients (PC1). The variance of the PC1 explains 28.11% of the variation, and the variance of the PC2 explains 18.89% within the data (Fig. 4B). The PCA showed that the primary variables to explain these variances are *ULK1*, *BECLIN1*, and *ATG5* transcriptional levels. Notably, patients in Q1 are strongly correlated with *IGF2* and *BECLIN1* gene expression profiles, most represented by female patients (green circles). Alternatively, male patients were more related to *LC3*, *ATG5*, and *RUBICON* expression profiles (blue circles) in Q4 (Fig. 4B).

IGF2 treatment increases autophagy genes expression in macrophages

Mutation in the *SCNA* gene encoding α -synuclein is one of the causes of PD cases. However, the accumulation of intraneuronal α -synuclein proteins is a neuropathological hallmark of PD (Schulz-Schaeffer, 2010; Spillantini et al., 1998). The expression of α -synuclein is not limited to the CNS. This protein is present in the cerebrospinal fluid (CSF), plasma (El-Agnaf et al. 2003, Forland et al. 2018), is expressed in the erythropoietic lineage cells (Nakai et al. 2007), and peripheral lymphocytes (Kim et al. 2004). To evaluate the IGF2 effect on autophagy genes in a PD context, we prepared primary mice macrophages cultures from bone marrow from ASO mice, which express human α -synuclein in the CNS (Rockenstein et al., 2002). Macrophages cells were treated with recombinant IGF2 (5 ng/mL) for 1, 3, and 5 days. After the IGF2 treatment, macrophages were stimulated with α -synuclein fibrils per 72 hours or PBS. We then evaluated a similar panel of autophagy genes as utilized for PBMCs from PD patients (Fig. 5). Notably, we observed that α -synuclein treatment caused a decrease in *Beclin1* and *Atg5* transcriptional expression, which were reversed by the treatment of IGF2 (Fig. 5A and B). Levels of *p62* were unchanged in any condition (Fig. 5C). *Rubicon* mRNA levels presented a tendency to decrease under α -synuclein treatment, but it was not statistically significant (Fig. 5D). *LC3* levels decreased after α -synuclein, with no differences in cells also treated with IGF2 (Fig. 5D). Thus, our results show a positive link between IGF2 treatment and the expression of two key genes from the initial steps of the autophagy pathway in a cellular context of PD, reversing the α -synuclein effect.

Discussion And Conclusion

Currently, extensive evidence supports the contribution of autophagy in the pathogenesis of PD (Cerri and Blandini, 2019; Ho et al., 2020; Kett et al., 2015; Mak et al., 2010; Xilouri et al., 2008; Zhang et al., 2015). In particular, both α -synuclein and LRKK2, proteins present in Lewy Bodies, have been described as CMA substrates (Cuervo et al., 2004; Zhang et al., 2015). Additionally, alteration of autophagy and CMA was reported in PD postmortem brain tissue due to the accumulation of misfolded protein such as α -synuclein in dopaminergic neurons (Alvarez-Erviti et al., 2010; Anglade et al., 1997; Dehay et al., 2010). However,

fewer reports are available describing autophagy impairment in blood cells from PD patients and the possible mirror effect of proteostasis phenomenon on the brain tissue. In addition, few publications have studied IGF2 signaling and its relationship with autophagy impairment in blood cells.

IGF2 signal transduction and function are highly dependent on the type of target cells. For instance, in pancreatic β cells, the overexpression of IGF2 disrupted islet structure, promoted islet hyperplasia, and a pre-diabetic state in mice. IGF2 overexpression caused increased ER stress, autophagy activation, and β cells dedifferentiation (Casellas et al., 2015; Steinmetz et al., 2018). In osteosarcoma (Shimizu et al., 2014) and colorectal cancer cells (Gao et al., 2020), IGF2 is upregulated and is associated with autophagy activity induction, potentiating the tumor's growth. In embryonic skeletal muscles, IGF2R acts as an IGF2 negative regulator and induces its degradation through lysosomes, modulating the systemic IGF2 levels (Spicer and Aad, 2007; Torrente et al., 2020). Indeed, IGF2 has essential roles in the development process of fast myofibers in youth and aging (Deschenes, 2004; Pedemonte et al., 1999). Of note, the reduced plasma levels of IGF2 observed in PD patients could negatively impact the skeletal muscle function as well as the central nervous system (CNS) cells.

Our data and other works show that IGF2 overexpression has a neuroprotective effect in different brain disease contexts (Garcia-Huerta et al., 2020; Martin-Montanez et al., 2017; Mellott et al., 2014; Pascual-Lucas et al., 2014). In addition, a recent study showed that the systemic treatment of a mice model of autism with IGF2 reverted the negative social behaviors characteristic of autism spectrum disorder (Steinmetz et al., 2018). These improvements were associated with reversing abnormal levels of the AMPK-mTOR-S6K pathway in hippocampus samples (Steinmetz et al., 2018). mTOR is one of the most significant inhibitory kinases of autophagy activity. Moreover, a study using the pharmacologic 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity in cell cultures and mice nigrostriatal dopaminergic neurons found neuroprotection by IGF2 related to antioxidant effects and improvement on mitochondrial function (Martin-Montanez et al., 2021). In the former study, researchers found an increase in the mTOR phosphorylation in cell culture treated with IGF2. However, autophagy activity was not evaluated.

In the present research, we found a strong decreased transcriptional and protein levels of IGF2 in PMBCs from PD patients, suggesting a relationship between the downregulation of IGF2 and neurodegenerative pathology. We also report a downregulation of genes involved in the initial steps of the autophagy pathway, *ULK1*, *ATG5*, and *BECLIN1*, in the same samples. ULK1 engages the initiation of the autophagosome formation induced by amino acid deprivation, accumulation of protein aggregates, or under mitophagy activation (selective autophagy of dysfunctional mitochondria) (Kim et al., 2011; Wu et al., 2014). ATG5 is a protein essential for forming autophagosomes, acting in a complex with ATG12 and ATG16L downstream of the ULK1 complex (Itakura and Mizushima, 2010). Recently, an SNP in the gene encoding ATG5 was reported to increase the susceptibility to PD with cognitive impairment in Chinese patients (Han et al., 2021). This SNP results in reduced ATG5 plasma levels in early-onset PD patients, suggesting a role for deficient autophagy in PD pathology. BECLIN1 is a protein involved in at least two pathways complexes, acting as a positive member from the class III PI3K (Itakura et al., 2008). The reduction of these genes from initial complexes of the pathway suggests a decrease in the initiation of

autophagy. Recently, an ultrastructural study in PBMCs obtained from PD patients demonstrated a reduction in the number of autophagy vacuoles per cell (Biagioni et al., 2021), supporting our results. These results align with our observation on the reduced transcriptional levels of *Beclin1* and *Atg5* in macrophages in culture treated with α -synuclein. Notably, IGF2 reverted the α -synuclein effect, increasing the transcriptional levels of both genes. However, IGF2 treatment did not change levels of Rubicon or LC3.

We also evaluated a potential gender difference in the expression of autophagy genes in patients. PD is more prevalent in men than women, including differences in severity and progression. In a work that studied the gene expression profile of dopaminergic neurons from substantia nigra pars compacta in men and women, researchers found an increased expression of α -synuclein in men (Cantuti-Castelvetri et al., 2007), which could partially explain the prevalence of PD. In our study, women showed a strong correlation between reduced levels of *IGF2* and *BECLIN1* mRNA. Alternatively, men's patients were more related to *LC3*, *ATG5*, and *RUBICON* expression profiles. A possible explanation of these differences could be the levels of the hormones. 17β -estradiol were shown to influence autophagy (Coto-Montes et al., 2009), while Beclin1 was reported to downregulate 17β -estradiol signaling, proposing an unexplored interaction between the two pathways (Cahill, 2006; Cantuti-Castelvetri et al., 2007). However, more details are needed to search for more specific diagnoses and treatments for PD depending on the patients' sex.

The signaling transduction that mediates the IGF2 effect in PD and its relationship to the autophagy pathway needs further study. However, a recent study showed that IGF2R deletion in cervical cancer cells disrupted the Golgi-to-lysosome transport of M6P-tagged cathepsins, decreasing lysosomal activity, impairing autophagy and mitophagy activity (Takeda et al., 2019). It has also been reported that there is a reduction of lysosomal activity in PBMCs cells from PD patients (Papagiannakis et al., 2019). These data allow a hypothesis that deficient levels of IGF2 reduce hydrolases transport to the lysosomal lumen, generating negative feedback on autophagy genes from the initial stages of autophagy and mitophagy pathways to cope with lysosomal failure. This theory aligns with the increased *RUBICON* levels observed in PBMCs from PD, an inhibitory protein from the autophagosome maturation (Matsunaga et al., 2009; Zhong et al., 2009). IGF2 treatment should restore lysosomes luminal hydrolases and initiate autophagosome formation, observed by increased Beclin1 and Atg5 in macrophage cell culture treated with IGF2.

Taken together, our results suggest a change of the proteostasis network associated with reducing IGF2 and autophagy in PD patients. Our results can contribute to elucidating the potential of IGF2 and the autophagy process on PD's pathogenesis and their possible use as multi-predictor biomarkers for PD early diagnosis and disease progression.

Declarations

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Author contributions

Conceptualization, R.L.V., D.S., and P.T.-E.; Methodology, R.L.V., D.S., C.A., A.V., P.V. and F.G.; Investigation, R.L.V., C.A., R.V., P.V., A.V., M.C., D.S., and P.T.-E. Resources, R.L.V., R.V., and C.A.; Writing – Original Draft, R.L.V., D.S., MN, and P.T.-E.; Project Administration, R.L.V.; Funding Acquisition R.L.V.

Competing of interests

The authors declare having no conflict of interests.

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Tables

Table 1: Demographics and clinical characteristics of Parkinson's disease patients (PD) and healthy controls (HC).

	PD (n=43)	HC (n=41)	P value
Age (years)	71.2 ± 7.87	67.5 ± 7.82	No significant
Gender (male/female)	22/21	17/24	No significant
Years of disease	6.6 ± 5.6	-	
Hoehn and Yahr stage	3 (1-4)	-	
UPDRS-III score	34.6 ± 12.5	-	

Table 2: Distribution by Hoehn and Yahr stage for Parkinson's disease (PD) patients.

Hoehn and Yahr stage	Number of patients	Percentage
1	1	2.3%
2	14	32.5%
3	25	58.1%
4	3	6.9%

A normality test was achieved, for the ages of both groups, using the Shapiro-Wilks test (Shapiro and Wilk, 1965), obtaining normality in both controls subjects (SW-W = 0.955, $p = 0.1052$) and PD patients (SW-W = 0.9767, $p = 0.5227$). Test for equality of variances was performed, resulting in both being equal. t-Student test showed no difference between patients and controls. A χ^2 test was performed, resulting in no difference between gender.

Figures

Figure 1

IGF2 is downregulated in the blood of PD patients. (A and B) IGF1 and IGF2 content in plasma obtained from PD patients and healthy control (HC) subjects were measured by ELISA. (C) Total protein extracts were generated from isolated peripheral blood mononuclear cells (PBMCs) from PD patients and HC subjects. GAPDH was determined as a loading control (left panel). IGF2 levels were quantified and normalized to GAPDH levels (right panel). (D) IGF2 mRNA levels were measured and normalized to SHDA mRNA levels by real-time PCR in PBMCs obtained from PD patients and HC subjects. (PD, N = 40; HC, N=40). Statistically significant differences were detected by two-tailed unpaired t-test (***: $p < 0.001$; *: $p < 0.05$).

Figure 2

Plasma IGF1 and IGF2 levels in PD patients do not correlate with motor scores of PD patients. (A and B) Pearson correlation analysis between IGF2 levels in PD patients with H&Y ($r = 0.1371$) and UPDRSIII ($r = -0.01401$) scores. (C and D) Correlation analysis between IGF1 levels in PD patients with H&Y ($r = 0.1498$) and UPDRSIII ($r = -0.07823$) scores.

A

B

Figure 3

Autophagy components are altered in PBMCs from PD patients. (A and B) mRNA levels were measured by real-time PCR in PBMCs obtained from PD patients and HC subjects. (PD, N=40; HC, N=40). Autophagy-related gene mRNA levels were quantified and normalized to SHDA mRNA levels. Statistically significant differences were detected by two-tailed unpaired t-test (***: $p < 0.001$; *: $p < 0.05$).

Figure 4

Analysis of IGF2 and autophagy markers of patients with Parkinson’s disease and healthy controls. (A) Gene expression measured by real-time PCR shows distinct mRNA expression patterns in healthy controls (HC, upper) and Parkinson’s disease patients (PD, bottom). (B) Principal component analysis (PCA) considers all variable measures in blood samples from PD patients. Orange circles represent the variables measured in blood samples from HC and PD patients. Female patients are represented in green circles. Male patients are represented in blue circles. (N = 40/40 HC/PD).

Figure 5

Autophagy genes expression under α -synuclein and IGF2 treatment in macrophages. mRNA levels from (A) Beclin1, (B) Atg5, (C) p62, (D) Rubicon, and (E) LC3 were measured by real-time PCR in macrophages cultures obtained from bone marrow from ASO mice treated with IGF2 and/or α -synuclein PFF or PBS as control for 72 hours. Autophagy-related gene mRNA levels were quantified and normalized to Actin mRNA levels. Statistically significant differences were detected by ordinary one-way ANOVA (**: $p < 0.01$; *: $p < 0.05$).