# P2X7 receptor/NLRP3 inflammasome complex and a-synuclein/parkin balance in neo-diagnosed, treatment-naïve Parkinson disease: a prospective study

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**Research**

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## Abstract

# Background

Neuro, and likely systemic inflammation, with abnormal α-synuclein deposition, participate in the development of Parkinson's disease (PD). The P2 × 7 receptor/NLRP3 inflammasome complex is upregulated in the brain of PD patients. Aim of this study was to explore whether the systemic activation of such complex might participate in the pathogenesis of PD.

# Methods

Systemic expression and functional activity of the inflammasome were measured in 25 newly diagnosed PD patients and 25 controls at baseline and after twelve months of pharmacologic treatment, exploring the involved intracellular signalling and its epigenetic regulation. A putative mechanistic explanation of the results was validated in a murine model of neuroinflammation.

# Results

De-novo PD patients were characterized by a systemic hyper-expression of the P2 × 7R/NLRP3 inflammasome platform, likely modulating circulating and lymphomonocyte $\alpha$-synuclein, whose deposits represent the main pathogenetic factor of PD. A reduced JNK phosphorylation might be the involved intracellular signalling. miR-7 and miR-30, implied in the pathogenesis of PD and in the post-transcriptional control of α-synuclein and NLRP3 expression, were also increased in PD. After one year of usual anti-Parkinson treatments, such inflammatory platform was significantly reduced. In the substantia nigra of P2 × 7R KO mice, a neuroinflammatory stimulus induced a strong expression of parkin, a protective protein, suggesting that P2 × 7R activation might participate in the inflammatory damage occurring in specific brain areas also by inhibiting parkin expression.

# Conclusion

Newly-diagnosed PD subjects display a systemic hyper-expression of the P2 × 7R/NLRP3 inflammasome platform that seems to modulate circulating and lymphomonocyte $\alpha$-synuclein; a reduced JNK phosphorylation might be the intracellular signalling mediating this effect, undergoing an epigenetic regulation by miR-7 and miR-30.

# Trial registration

ClinicalTrials.gov (NCT03918616).

# Introduction

Parkinson's disease (PD) is a chronic degenerative disease characterized by a progressive loss of dopaminergic neurons in the substantia nigra [1]. Its pathophysiological mechanisms are still partially unknown; a main role seems to be played by chronic neuroinflammation [2], with an excess of microglia and astrocytes activation and increased expression of pro-inflammatory mediators like TNF-α, IL-1β, IL-6, and interferon-γ [3, 4], able to rapidly induce neuronal degeneration. Among these mediators, IL-1β appears of interest: it is abundant in the microglia surrounding Lewy bodies in experimental models and in PD patients [5]; however, it should be pointed out that, in PD, Lewy bodies are not limited to substantia nigra, being also located in other sites of central and peripheral nervous system.

The release of the mature form of IL-1β is mediated by the NLRP3 inflammasome. It consists of intracellular multi-protein compartments whose assembly can be induced by a wide spectrum of danger signals, playing a pivotal role in the host defence against harmful threats [6]. It is highly expressed in microglia [7] and essential to the process of neuroinflammation [8]. Downstream the inflammasome cascade, Caspase-1 cleaves the inactive precursor pro-IL-1β to mature IL-1β in the cytosol. NLRP3 can be activated by several "danger signals" like urate crystals, bacterial toxins or beta-amyloid aggregates [*9, 10]. NLRP3 inflammasome can be activated through P2 × 7 receptor (P2 × 7R), one of the most studied and controversial receptor, due to its atypical pharmacological and structural features. It is rapidly activated by extracellular ATP (eATP) in the millimolar concentration, while its more prolonged stimulation regulates additional events like mitochondrial membrane depolarization, production of reactive oxygen species, formation of plasma membrane pores and, ultimately, apoptosis and cell death [11].

The first gene associated to PD was *SNCA*, encoding for α-synuclein, main component of Lewy bodies in several degenerative disorders [12]; such protein can also form aggregates able to damage pre-synaptic terminations and determining a sort of "synaptotoxicity" by interfering with mitochondrial or microtubular function and axonal protein transport [13]. Recently, a role of fibrillar α-synuclein, through interaction with Toll-like receptor 2 (TLR2) has been reported [14, 15]. Alpha-synuclein is mainly transported by red blood cells and, besides the β-amyloid and Tau proteins, is a useful tool to better understand the pathophysiology of PD [16]; to measure it, as well as other biomarkers in circulating cells, often a mirror of molecular processes occurring in different organs and tissues, is a nice opportunity to gain insights mechanisms and progression of the disease by a non- invasive and relatively inexpensive approach.

A few reports have addressed the possible involvement of the inflammasome in PD, just describing the protective effect of P2 × 7R blockers in murine models of the disease [17, 18] and in microglial cells, where NLRP3 is activated by α-synuclein, triggering a neuroinflammation that contributes to degeneration of dopaminergic neurons [19]. Additionally, the inflammasome activation might undergo an epigenetic regulation by short, non-coding RNA species, potentially influencing several genes involved in neuroinflammation and chronic neurodegenerative diseases.

It is still unclear whether, in addition to the increased brain expression and function of the NLRP3 inflammasome platform, a systemic activation of such complex might participate in the pathogenesis of PD, which could be the role of the P2 × 7R in this scenario, and whether such patterns undergo any specific epigenetic regulation. The present study has been designed to address these issues.

# Subjects And Methods

*Participants* The study consecutively enrolled 25 newly diagnosed, treatment-naive PD individuals among those referring in the years 2015–2016 to the Centre for PD, Neurology Unit, University Hospital in Pisa, Italy. Inclusion criteria were onset of suggestive symptoms not later than 12 months, age < 80 years, no previous specific treatment, no previous personal history of any neurological disease, including ischemic stroke, no previous history of systemic inflammatory or immunological disease, no anti-inflammatory drugs assumed in the three months preceding the enrolment. Diagnosis matched the UK Parkinson's Disease Society Brain Bank [20]. All patients performed cerebral SPECT with tracer for Dopamine Transporter, that showed a nigro-striatal degeneration, and brain MRI in order to exclude cerebrovascular complications. An age- and sex-matched control group (CTL, n = 25) was formed, on a volunteer basis, by the spouse of the probands participating in the study.

The day of the study (T0) patients underwent a complete clinical evaluation and a blood routine analysis. Motor symptoms were evaluated by the Unified Parkinson's Disease rating scale (UPDRS), the cognitive state by Mini Mental State Examination (MMSE) and the disease was staged according to the HoehnYahr scale [21]. Blood samples were collected from an antecubital vein to assess serum/plasma aliquots (frozen at -20 °C until required for quantitation) and circulating lymphomonocytes isolation.

*Biochemistry and Mononuclear cells analysis* Biochemical parameters (fasting glucose, lipid profile, serum creatinine, uric acid) were measured by standard methods in the biochemistry laboratory of the University Hospital in Pisa.

Lymphomonocytes were isolated from fresh blood samples by density gradient centrifugation (Fycoll Paque, GE Healthcare, Uppsala Sweden), according to the manifacturer's standard protocol. The resulting cells were immediately frozen (for RNA/protein extraction) or plated $400 \times 10^3$ in two-chamber slides and left to adhere for 1 h in incubator; after a fixation step (10 min) in PFA 4% and 3 wash with PBS, the cells were ready for the immunofluorescence experiments.

*RNA extraction and quantitative real-time PCR* RNA extraction from human cells and animal tissues was performed with QIAcube (Qiagen, Hilden, Germany), a robotic workstation for automated purification of nucleic acids, using RNeasy mini kit (Qiagen) and following the manufacturing protocol. Real Time PCR was performed on an Eco real time instrument (Illumina Inc., San Diego, CA, USA) according to the standard procedure. Transcripts were quantified by TaqMan Gene Expression Assays (TermoFisher-LifeTechnologies). The following primers were used: for human samples, P2 × 7R: Hs00175721_m1; NRLP3: Hs00918082_m1; Caspase-1: Hs00354832_m1; NF-kB: Hs00765730_m1; Amplifications were

normalized by GAPDH (Hs02758991_g1); for mouse samples, Parkin: Mm01323528_m1; α-synuclein: Mm01188700_m1; NRLP3: Mm00840904_m1; GAPDH: Mm99999915_g1.

*Immunofluorescence and Quantitative analysis of immunofluorescence images* The immunofluorescence experiments in human lymphomonocytes and mouse brain tissues were performed overnight at 4 °C with the following antibodies: P2 × 7R (1:100, APR-004 Alomone, Jerusalem, Israel), NRLP3 (1:200, AG-20B-0014 Adipogene, Liestal, Switzerland), α-synuclein (1:100, ab212184 abcam, Cambridge, UK), GFAP (1:500, ab7260 abcam), Iba1 (1:100, sc-32725 S.Cruz), TH (1:200, ab75875 abcam); the next day, specific immunoreactivity was revealed with Alexa Fluor 594-goat anti rabbit and 488-goat anti mouse antibodies (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The images used for the quantitative analysis of immunofluorescence were acquired with a Leica TCS SP8 confocal microscope. After a preliminary analysis of different samples to establish the best conditions for the instrument, the confocal setting was held constant within all experimental sessions. All image analyses were performed using ImageJ (public domain software developed at the NIH). For human lymphomonocytes, stacks of optical sections were collected in three different fields for each patients, and 30 immuno-positive cells were assessed for the quantification. Each single cell area was traced (avoiding the background) and the mean value of signal intensity was calculated; one hundred immuno-positive cells were assessed for each experimental group. In the mouse brain, similar stacks of optical sections were acquired for each animal. Threshold area was established on the base of average background signal. Briefly, for each image transformed in grey scale, the grey value in 3 different non-signal area was measured, and the average value was used to set the threshold; in the obtained binary image, the percentage area of positive pixel was calculated. In the case of not specific green signal in vessels, these parts were masked before calculating the threshold area.

*Cytokine levels* Plasma levels of IL-1β, IL-18 and α-synuclein were measured by high sensitivity Quantikine enzyme-linked immunosorbent assay (ELISA) Kits (R&D Systems Inc, Minneapolis, MN, USA) following manufacturer's instructions. The sensitivity of the assay was < 1 $pg/ml^{-1}$, with an interassay variability of 4.5%.

*Circulating miRNAs* Circulating miR-7 and miR-30, likely involved in the pathogenesis of PD [22, 23] were isolated by the robotic workstation QIACUBE (Qiagen) loaded with miRNeasy Serum/Plasma Kit (cat. 217184, Qiagen, Hilden, Germany). After an equilibration period at room temperature, samples were centrifuged to remove cryoprecipitates, and 200 µl of thawed serum were processed following the manufacturer's instructions. For each patient, the cDNA templates was assessed from 2 µl of sample eluent; we used the TaqMan Advanced miRNA cDNA Synthesis kit (A28007, Applied Biosystems, Foster City, CA, USA). miR-7 and miR-30 expression was measured by TaqMan Advanced MicroRNA Assays (cat. A25576, Applied Biosystems); PCR reactions were run in triplicate, diluting 1:10 3–5 µl of cDNA template, and miRNAs levels were expressed as 2-ΔCt using two references miRNAs (miR-484 and miR-191-5p) selected on the basis of the scientific literature and checking their low variability in our samples.

*Intracellular signalling* Total proteins were extracted from lymphomonocytes of a subset of PD and CTL individuals to measure total and phosphorylated ERK 1/2, p38 and JNK by WB analysis. Twenty µg

protein extracted from lymphomonocytes were diluted in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and heated at 100 °C for 5 min. Samples were separated on Any kD Mini -Protean TGX gels (Bio -Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking and repeated washing, blots were incubated overnight with primary antibodies (Cell Signaling, Leiden, The Netherlands) against ERK 1/2 (9102/9101), p38 MAPK (9212/9211) and JNK (9252/9251), and their relative phosphorylated isoforms. After treatment with secondary antibodies, bands were identified by enzymatic chemiluminescence.

*Follow up* After baseline evaluation, patients underwent a personalized anti-Parkinson treatment on the basis of clinical indications and international guidelines; in detail, 13 patients received levodopa, 8 started dopamine-agonists (rotigotine, pramipexole, or ropinirole), 7 were treated with MAO-B inhibitors (rasagiline or selegiline). All participants were checked after one year of pharmacologic treatment, evaluating levodopa equivalent dose (LED: 319.4 ± 90.6), current medications, disease severity and cognitive status, and repeating the measurement of P2 × 7R, NLRP3 and Caspase-1 expression.

*Studies in WT and P2 × 7 receptor KO mice* Eight WT (strain C57BL6J, Charles River, Lecco, Italy) and eight P2 × 7R KO mice (Jackson Laboratory, through Charles River were used for the experiments, all carried out in accordance with the EU Directive 2010/63. The study protocol was approved by the Italian Minister for Animal Care (#943/2015-PR). Mice, housed in a germ-free stabularium, were treated for 16 weeks with a high fat diet (HFD) (PF4215, 60% of total calories from fat, from Research Diets Mucedola, Settimo Milanese, Italy), already shown to be able to induce PD [24, 25]. At the end of the 16-week period, mice were deeply anaesthetized with sevoflurane; brains were removed from the skull, and sagittally cut in two part; half part was fixed by immersion in 4% paraformaldehyde, rinsed in phosphate buffer containing 30% sucrose at 4 °C overnight, frozen and sectioned with a cryostat at 50 μ; the other one was immediately frozen on dry ice for RNA isolation.

*Substantia Nigra analysis in mice* Coronal brain sections (50 μm thick) cut in the cryostat and mounted on non-charged slides were immediately observed with a microscope and sections containing midbrain were recognized on the base of mouse brain atlas. For the subsequent RNA extraction, the ventral midbrain was dissected out from each section using a needle and the tissue was used to quantify NLRP3, Parkin and α-synuclein gene expression by realtime PCR (see *RNA extraction and quantitative real-time PCR*). A free-floating immunostaining was performed for P2 × 7R, NRLP3, Iba1, GFAP, TH: coronal sections through the substantia nigra were collected in PBS; after a post-fixation step (1 h at room temperature) in PFA 4% and three washes with PBS, serial sections were treated with specific antibodies (see Immunofluorescence).

*Availability of data and materials* Data shown and analysed in this paper are available from the corresponding author on reasonable request.

# Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was carried out using one-way analysis of variance with *post-hoc* Bonferroni correction, the Kruskal-Wallis test for non-parametric data and paired *t*-test for comparison between groups. A value of $p < 0.05$ was considered statistically significant.

# Results

*Systemic expression and activity of the complex P2 × 7R/inflammasome, and related pathways* Clinical characteristics of the participants are shown in **Suppl. Table 1**. CTL and PD patients resulted adequately matched for age, sex and prevalence of chronic comorbidities.

Figure 1 shows the expression of P2 × 7R and NLRP3 inflammasome components in lymphomonocytes of PD and CTL. PD displayed an almost two-fold higher P2 × 7R gene expression; accordingly NLRP3, and Caspase-1 were more expressed in PD. These data were corroborated by those obtained in immunohistochemistry: as shown in Fig. 2, P2 × 7R and NLRP3 proteins were both abundantly expressed, and colocalize, in lymphomonocytes from PD patients, while they were virtually absent in CTL cells.

The activation of the P2 × 7R/NLRP3 inflammasome is expected to drive the release of IL-1β and IL-18, pro-inflammatory markers of PD [26]. Therefore, we measured plasma levels of these cytokines in CTL and PD patients, and we found that both were within the normal range, and no difference emerged between the two groups (IL-1β: 12.3 ± 8.9 in CTL and 12.6 ± 9.4 pg/ml in PD, p = 0.88; IL-18: 344.4 ± 181.2 in CTL and 323.8 ± 63.0 pg/ml in PD, p = 0.59). However, a weak direct correlation was found between P2 × 7R expression and IL-18 levels in the whole study population (R = 0.279, p = 0.05).

Activation of the P2 × 7R/NF-kB signalling stimulates the expression of the upstream IKK gene and the p65 subunit of NF-kB, both of which play a role in inflammatory responses and form an alternative pathway able to trigger IL-1β release; therefore, to confirm the P2 × 7R/NLRP3 axis as main pathway mediating this cytokine release, we also measured the expression of NF-kB. No difference emerged between PD and CTL in the expression of such gene (normalized values: PD, 0.60 ± 0.37; CTL, 0.63 ± 0.24, p = 0.85).

To gain insights of the mechanism linking P2 × 7R to PD, we explored by immunofluorescence

α-synuclein (the main constitutive factor of plaques accumulating in the substantia nigra) protein expression in lymphomonocytes and, as a putative negative control, in red blood cells from PD and CTL, and we measured circulating levels of α-synuclein. As shown in Fig. 3, PD patients had a different pattern of α-synuclein expression with respect to CTL; in detail, erythrocytes displayed a quite similar amount of α-synuclein in the two groups, while PD lymphomonocytes were strongly enriched by the protein, with a main localization in the perinuclear area (Fig. 3a); α-synuclein accumulation was scarce in immune cells from CTL (Fig. 3b). In face of that, circulating α-synuclein levels did not differ between CTL and PD (Fig. 3c); interestingly, a significant direct correlation between P2 × 7R and α-synuclein levels was observed in PD subjects (R = 0.400, p = 0.043), but not in CTL (R = 0.198, p = 0.344).

*Follow up* After twelve months of standard anti-Parkinson therapies, PD subjects showed an amelioration of symptoms (UPDRS III: from 21.2 ± 8.9 to 20.5 ± 6.8); accordingly, a reduction of lymphomonocytes expression of P2 × 7R (-40 ± 10%), NLRP3 (-32 ± 8%) and Caspase-1 (-25 ± 7%; all $p < 0.05$) was observed. Individual changes in P2 × 7R and NLRP3 expression are reported in **Suppl. Figure 1.**

*Extracellular signalling* We next tried to possibly identify the intracellular signalling supporting the activation of the P2 × 7R/inflammasome complex in lymphomonocytes, and to verify a possible epigenetic regulation of such pathway. To this aim, we compared the lymphomonocyte expression of total and phosphorylated isoforms of extracellular signal-regulated kinases (ERK 1/2), p38 MAP kinase and c-Jun N-terminal kinase (JNK). As shown in Fig. 4, we found that JNK phosphorylation was reduced by approximately 60% in PD ($p = 0.03$); a coherent trend was also observed for p38.

Lastly, searching for a putative epigenetic regulation of such complex scenario, we explored two miRNAs (miR-7 and miR-30) involved in the post-trascriptional control of α-synuclein and NLRP3 expression. We found that circulating levels of these miRNA are both increased in PD patients vs CTL (T/R for miR-7: 3.0 ± 3.8 in PD and 0.8 ± 0.7 in CTL, $p = 0.012$; T/R for miR-30: 5.3 ± 3.9 in PD and 2.2 ± 1.3 in CTL, $p = 0.001$).

*Studies in animal models* The second part of our study design was to better explore such complex system in the brain, in the attempt to link our results to an organ- and tissue- specific damage. To this aim we used an animal model, *i.e.* WT and P2 × 7R KO mice treated with normal chow and a high fat diet (HFD), a stimulus able to induce an inflammatory damage at the level of substantia nigra, the brain area which is considered the anatomical target of PD. We measured gene and protein expression of NLRP3, α-synuclein and parkin (the latter is protective toward the synaptic dysfunction related to the early symptom of PD). Results are shown in Fig. 5. As expected, HFD induced a tendentially higher expression of α-synuclein in the substantia nigra, which was actually similar in the two strains. Intriguingly, parkin expression did not vary in WT animals treated with HFD, while it was strongly increased in P2 × 7R KO mice, while NLRP3 inflammasome followed exactly an opposite trend, being upregulated in the substantia nigra of WT animals treated with HFD. To confirm substantia nigra as target of the specific inflammatory damage induced by HFD, we also looked at tyrosine hydroxylase (TH)-positive neurons. In WT treated with HFD, these dopaminergic cells show a reduced diameter, and the immunoreactivity signal is also less intense. In such animals, double staining experiments show the activated microglia (Iba1-positive cells) mostly expressed in TH neurons, while in KO mice, HFD does not induce any difference (Fig. 6). In WT animals, in the same brain area, P2 × 7R and NLRP3 proteins were upregulated (Fig. 7), reinforcing the hypothesis of its participation in the neuroinflammatory process. Interestingly, P2 × 7R co-localizes with microglia (identified by Iba1 positive cells), and NLRP3 does not colocalize with astrocytes (identified by GFAP) (Fig. 7).

# Discussion

This paper offers a novel, relevant contribution in clarifying the role played by P2 × 7R in the pathogenesis of PD. We show here for the first time: *i*) a systemic hyper-expression of the P2 × 7R/NLRP3

inflammasome platform in newly-diagnosed treatment-naive PD patients; *ii*) that such complex seems to modulate circulating and lymphomonocyte *α*-synuclein, whose deposits represent the main pathogenetic factor of PD; *iii*) that a reduced JNK phosphorylation might be the intracellular signalling mediating this effect; *iv*) that circulating levels of miR-7 and miR-30, epigenetic modulators of neuroinflammation responses, are enhanced in PD patients; *v*) that in P2 × 7R KO mice, a neuroinflammatory stimulus induces a strong expression of parkin, a protective protein, suggesting that a P2 × 7R activation might participate in the inflammatory damage occurring in specific brain areas also by inhibiting parkin expression.

P2 × 7R is expressed in dopaminergic areas affected by PD, where its activation promotes death of nigrostriatal dopaminergic neurons, and its inhibition is neuroprotective in rat models of PD [27, 17, 18]. On the other hand, the components of the NLRP3 inflammasome (NLRP3, apoptosis-associated speck-like protein containing a Caspase activating recruitment domain [ASC] and Caspase-1) assembly to react to several stimuli and promote secretion of IL-1β and IL-18, key cytokines in the neuroinflammation process [28]. Such assembly is known to occur via TLR4/NF-*κ*B activation, as shown in astrocytes and in microglia [29–31]. We show here an increased mRNA and protein expression of the P2 × 7R/NLRP3 inflammasome in lymphomonocytes of PD patients, suggesting that extra-neural P2 × 7R might be relevant in the early phase of the disease, while systemic NF-*κ*B activation does not.

This potentiated system does not translate, in our study, in different level of systemic inflammation, as shown by the similar IL-1β and IL-18 levels in PD and CTL, even in the presence of a trend for IL-18. This resonates with the recent publication by White and colleagues [32], even in a stimulated setting, and could be due to the short duration of the disease of our patients, the very good matching of our controls (same age and biochemical profile), or to the relatively small number of studied subjects. Matter of fact that a weak but significant correlation between IL-18 and P2 × 7R was found. Alternatively, we might hypothesize that the systemic activation of the P2 × 7R-NLRP3 complex has much more to do with other signal pathways.

In concomitance with a clinical and symptomatic improvement, we show here for the first time as the usual anti-Parkinson treatments reduce the systemic expression of P2 × 7R, NLRP3 and Caspase-1 in PD individuals. This suggests, on one hand, that the peripheral expression of such platform might be regarded as a marker of improvement of this chronic neurologic disease, thus serving as potential diagnostic and therapeutic biomarker, and on the other hand, that the therapeutic benefits of usual anti-Parkinson treatments, even not primarily mediated by anti-inflammatory effects, might concur to the downregulation of specific pro-inflammatory proteins.

In PD, NLRP3 has been previously related to α-synuclein [13, 33]; P2 × 7R also participates in microglial activation by extracellular α-synuclein, thus inducing oxidative stress and accelerating PD [34]. However, this piece of knowledge comes from studies performed in cell and animal models. We confirm the functional link between the two molecules in human beings, firstly by the finding of higher lymphomonocyte levels of α-synuclein in PD than in CTL, so far described only in the cerebrospinal fluid

and in brain plaques [35, 36], and secondly by the strict linear correlation between P2 × 7R and α-synuclein expression only in PD subjects. Even more, the different enrichment in α-synuclein of circulating immune cells suggests for the first time a participation of infiltrating macrophages in the process of α-synuclein accumulation in the CNS, never described before. On the other hand, red blood cells, recognized as the main source of circulating α-synuclein [37], appear similar at immunofluorescence, thus likely explaining the lack of difference occurring in plasma levels between PD and CTL.

Lymphomonocyte phosphorylation of JNK appears significantly reduced in PD; a trend was evident also for p38. Intriguingly, this could be linked to the increased α-synuclein expression, as already reported in some neuronal cell lines [38, 39]. Several studies have pointed out as α-synuclein might either induce toxic or neuroprotective effects, depending on its expression levels and its conformational structure [40, 41]; we cannot exclude that α-synuclein acts as a competitive substrate for such kinase, whose activation is required for calcium-dependent dopamine release, thus influencing dopaminergic neurons degeneration [42]. Taken as a whole, our results suggest that, at least in an early phase of the disease, α-synuclein might exert a sort of systemic compensatory role, by counteracting inflammation via JNK inhibition. This is indirectly confirmed by the same level of systemic inflammation (as from IL-1β e IL-18 plasma levels) in PD and CTL.

Searching for putative mechanisms at the level of CNS supporting this role of P2 × 7R in the pathophysiology of PD, we explored such pathways in WT and P2 × 7 KO mice treated with high fat diet, able to induce neuroinflammation specifically at the level of dopaminergic neurons, as shown by their morphologic alterations. To this aim, beside α-synuclein, we evaluated the effect of such pro-inflammatory stimulus on parkin, whose main function is to ligate ubiquitin to lysine residues, an essential post-translational modification involved in numerous cellular pathways. Mutations of parkin gene have been related to familial and sporadic forms of PD [43, 44]. In our murine model of P2 × 7R KO, we describe a marked increase in brain parkin expression induced by the neural inflammatory stimulus (high fat diet); at the same time, as expected on the basis of our working hypothesis of a functional link between P2 × 7R and NLRP3, the latter is upregulated in WT animals where P2 × 7R is present and is likely promoting inflammation. Such relationship between P2 × 7R and parkin has been never described before, even though parkin was shown to potentiate ATP-induced currents that result from activation of P2X receptors [45], suggesting a relationship between parkin and neurotransmitter receptors involved in synaptic activity. Differently, an inverse relation between parkin and NLRP3 inflammasome has been already described, with the former inhibiting the latter via NF-kB [46, 47]. It might be hypothesized that this increased parkin expression induced by high fat diet in P2 × 7R KO mice might protect toward the formation of α-synuclein aggregates, rather than on the α-synuclein amount, that - in fact - does not differ in the substantia nigra of WT and P2 × 7R KO animals. Such shield is lack in WT animals, in which P2 × 7R is hyper-expressed and hyper-functioning; we should also point out that high fat diet induces specific structural alterations in dopaminergic neurones, likely attributable to the presence of activated microglia.

Lastly, searching for a putative epigenetic regulation of such complex scenario, we explored miR-7 (regulating neuro-inflammation in animal models of Parkinson’s disease by repressing α-synuclein

expression) and miR-30, that - together with miR-7 - post-transcriptionally controls NLRP3 activation [48, 49], and we found as circulating levels of both these markers are increased in PD individuals. Such results are at odds to that observed in the brain of animal models, and even of PD patients, where a decreased miR-7 has been described;[50] we may hypothesize that, at the onset of the disease, these two markers could be released in excess by the injured brain, where they might be abundantly synthetized in the attempt to counter the onset of the disease.

We should acknowledge some limitations of our study: the relatively small number of study subjects, the lack of availability of CSF samples to confirm our results in a biological fluid specifically involved in Parkinson's disease, the absence of a pure animal model of Parkinson's disease where exploring the P2 × 7R/inflammasome axis, the technical inability to validate our hypothesis in murine circulating cells.

# Conclusions

Neo-diagnosed PD displays a systemic hyper-expression of the P2 × 7R/NLRP3 inflammasome complex that seems to modulate lymphomonocyte *α*-synuclein; a reduced JNK phosphorylation might be the intracellular signalling mediating this effect, undergoing to an epigenetic regulation by miR-7 and miR-30. A role of such inflammasome in the modulation of the balance α-synuclein/parkin in PD can be reasonably hypothesized.

# Abbreviations

CTL control group

eATP extracellular ATP

ELISA enzyme-linked immunosorbent assay

GFAP *Glial fibrillary acidic protein*

HFD high fat diet

IBA1 ionized calcium-binding adapter molecule 1

MAO-B Monoamine oxidase-B

miRNA MicroRNA

MMSE Mini Mental State Examination

MRI Magnetic Resonance Imaging

NLRP3 nucleotide-binding oligomerization domain-like receptor [NLR] family pyrin domain-containing 3

PBS Phosphate-Buffered Saline

PD Parkinson disease

PFA paraformaldehyde

PVDF polyvinylidene difluoride

P2 × 7R P2 × 7 receptor

SPECT Single Photon Emission Computed Tomography

TH *Tyrosine Hydroxylase*

TLR2 Toll-like receptor 2

UPDRS Unified Parkinson's Disease rating scale

# Declarations

### Ethics approval and consent to participate

The protocol was approved by the Ethics committee of the University of Pisa (#14282) and registered on ClinicalTrials.gov (NCT03918616); all participants signed an informed consent. We also followed all the international and national guidelines for the care and use of animals (Italian Ministry for Animal Care #943/2015-PR).

### Consent for publication

Not applicable

### Availability of data and materials

Data shown and analysed in this paper are available from the corresponding author on reasonable request.

### Competing interests

A.S. reports research support from Astra Zeneca, Boehringer Ingelheim, Lilly, Mundipharma, Novo, Sanofi. U.B. declares fees for speech from Zambon, UCB Pharma, Boehringer Ingelheim, Lusofarmaco, FB Health. R. C. declares fees for speech from Zambon, UCB Pharma, General Electric, Medtronic, Lusofarmaco, AbbVie. All the above-reported supports are outside of the submitted work. The other authors declare no competing interests.

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### Author's contribution

A.S. conceived the study, proposed the study protocol for granting, analysed data and wrote the manuscript. C.R. conceived and performed all the experiments in animals and several determinations in humans, and gave an essential contribution in writing the paper. E.S. assessed the P2X7R/inflammasome complex in humans. F.R. performed the extracellular signalling experiments. M.G., E.D.P. recruited participants and provided clinical data. F.P. and E.B. helped to recruit patients and to perform the experimental work. U.B. reviewed the paper. R.C. recruited and managed all the patients and contributed to the discussion.

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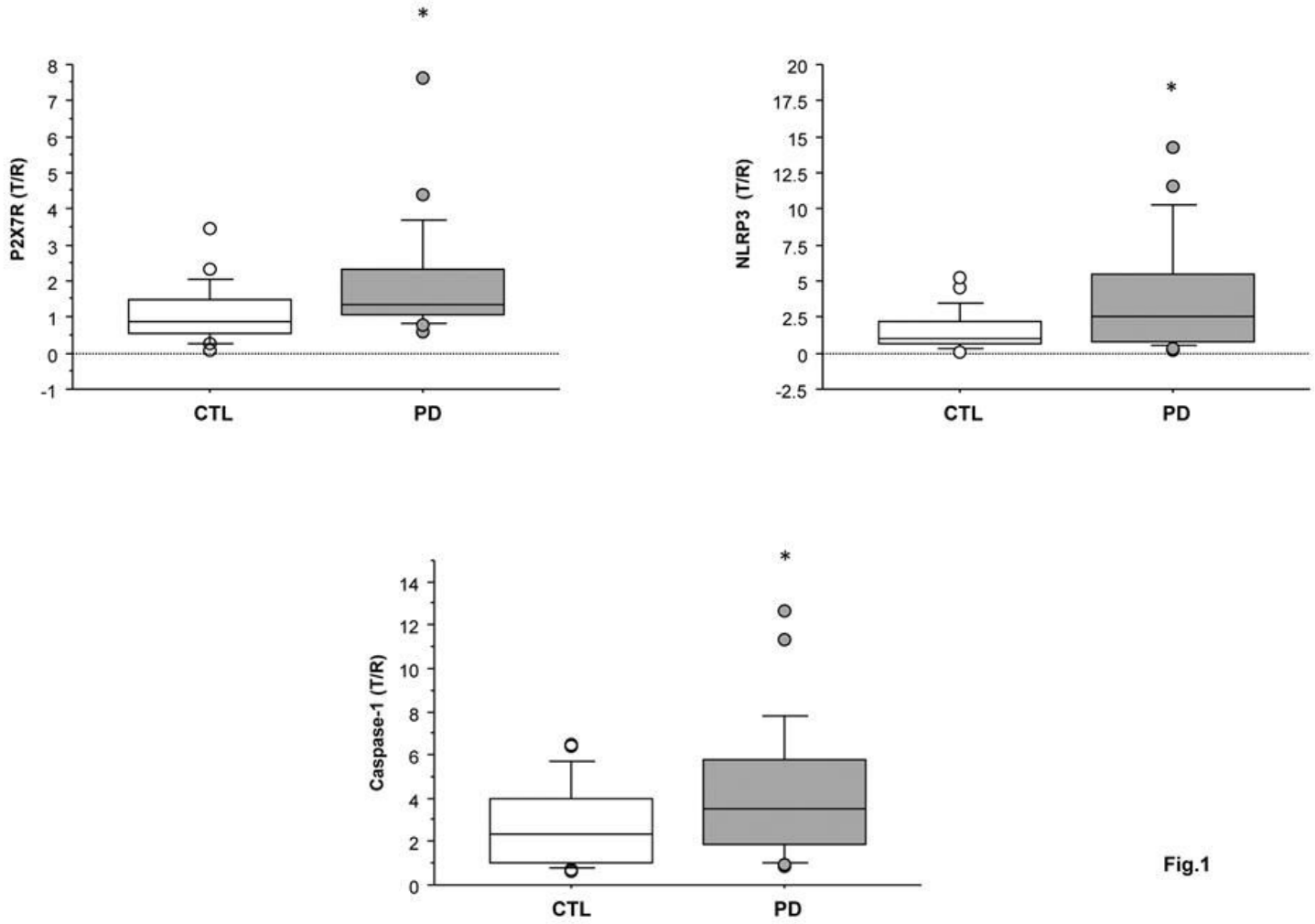
# Figures




## Figure 1

P2X7 receptor, NLRP3 and Caspase-1 gene expression in lymphomonocytes of twenty-five CTL (white plots) and twenty-five PD patients (gray plots). * p=0.013 for P2X7R; p=0.0086 for NLRP3; p=0.045 for Caspase-1.

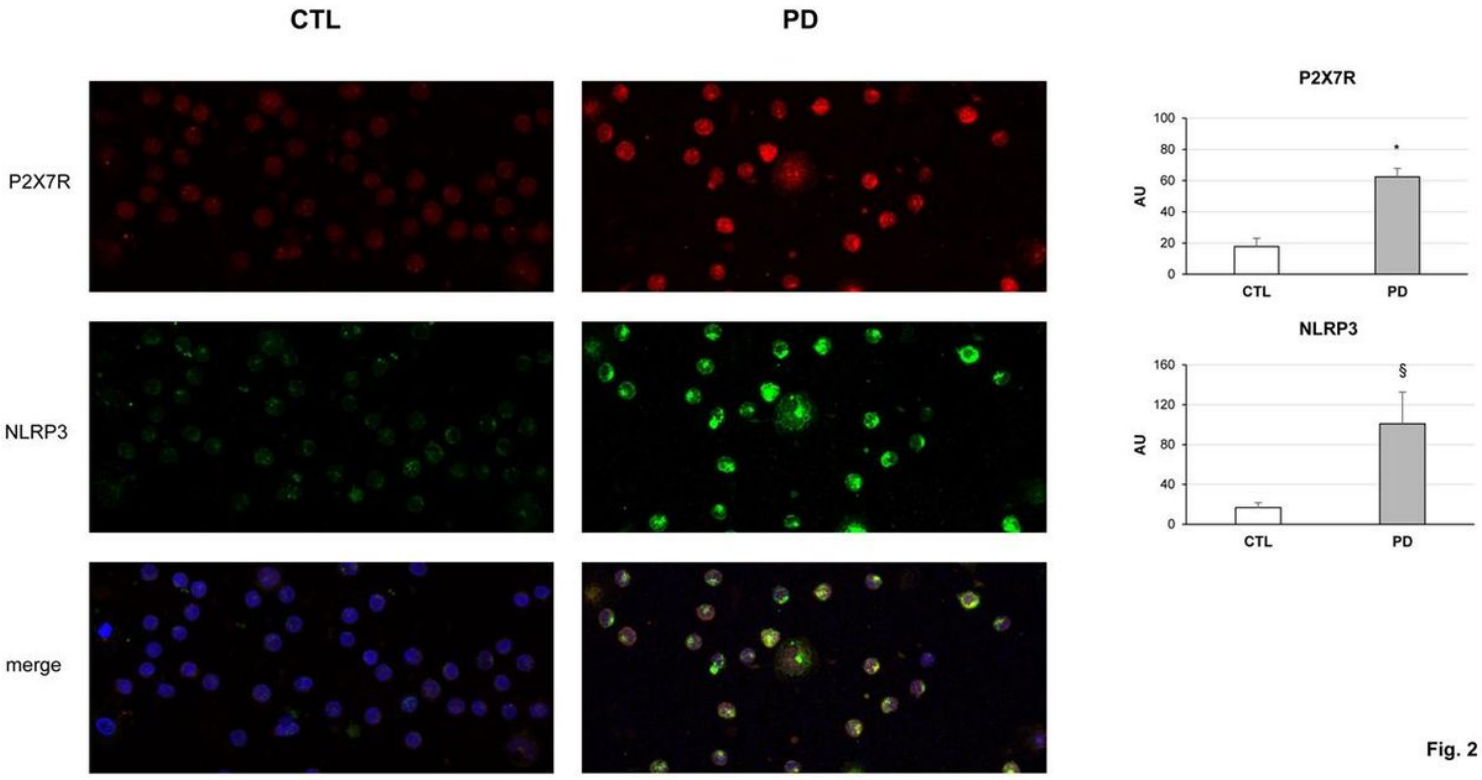



Fig. 2

## Figure 2

P2X7R and NLRP3 protein expression in lymphomonocytes of CTL and PD patients. A representative immunofluorescence image shows as both proteins are highly expressed, and colocalize, in PD lymphomonocytes, while they are virtually absent in CTL cells. Graphs show mean±SD of all study subjects. * p<0.005 vs CTL; § p<0.01 vs CTL

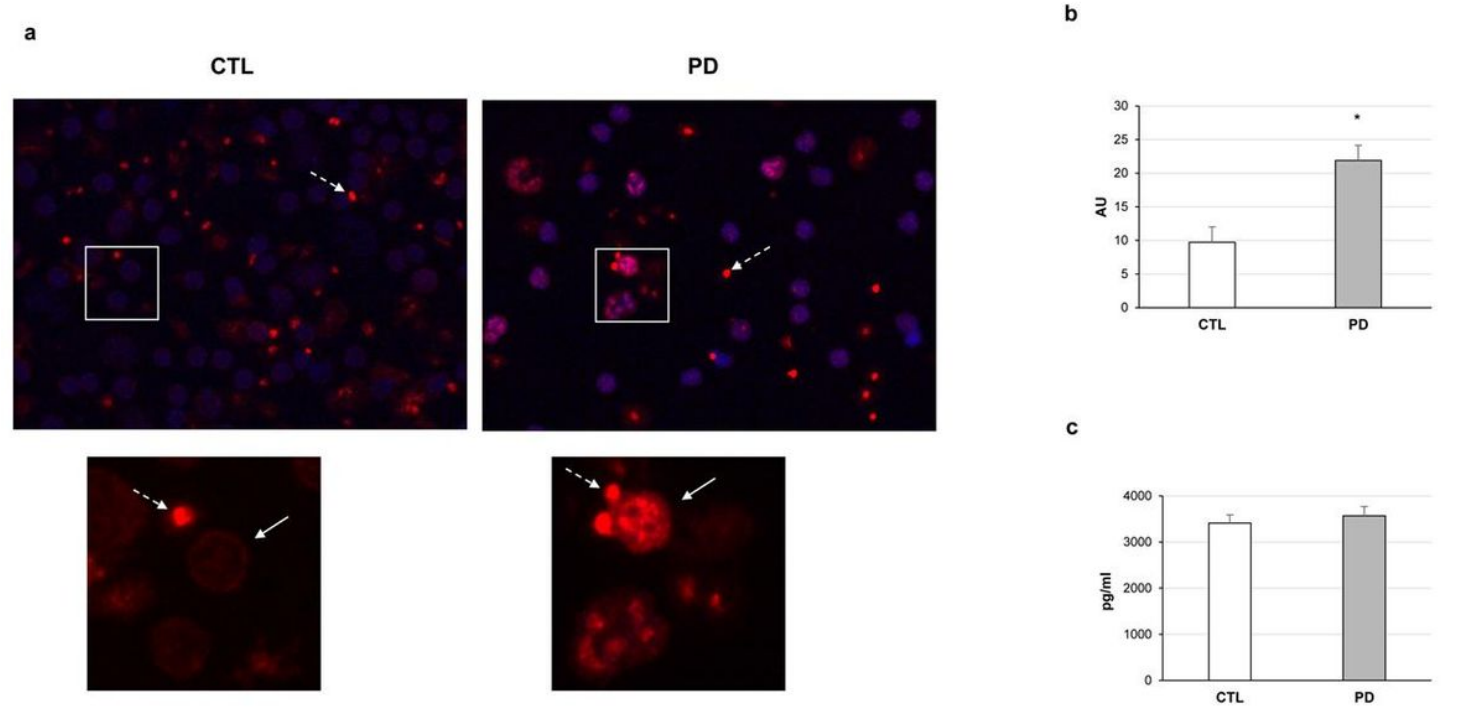



Fig. 3

## Figure 3

Panel a: Intracellular α-synuclein protein in erythrocytes and lymphomonocytes of CTL and PD patients. Upper panels: representative immunofluorescence image; lower panels: magnification of the areas of interest (white squares). Red signal: α-synuclein; blue signal: DAPI staining for nuclei. In red blood cells, the immunoreactivity for α-synuclein protein is the same in CTL and PD (dashed white arrows), while it is much higher in lymphomonocytes of PD (continuos white arrows) vs CTL. Panel b: mean±SD of α-synuclein protein immunoreactivity measured in lymphomonocytes of all study subjects. * p<0.001 vs CTL Panel c: circulating α-synuclein levels in all study participants evaluated by ELISA.

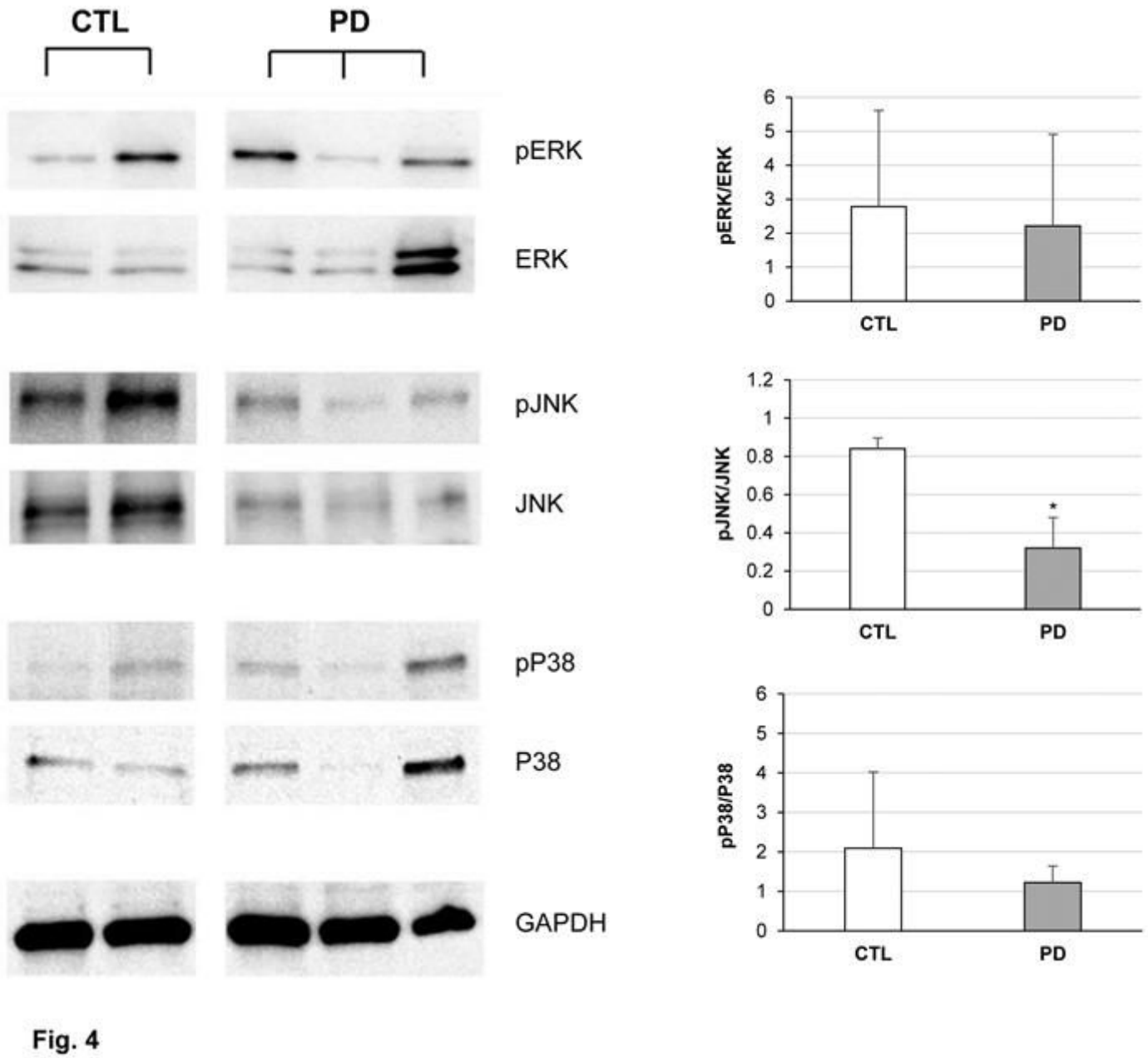



Fig. 4

## Figure 4

Total and phosphorylated isoform expression of ERK, JNK and p38 MAPK in CTL and PD. Representative immunoblots of two CTL and three PD are shown on the left; on the right, quantification of all the determinations are reported. * p<0.01 vs CTL

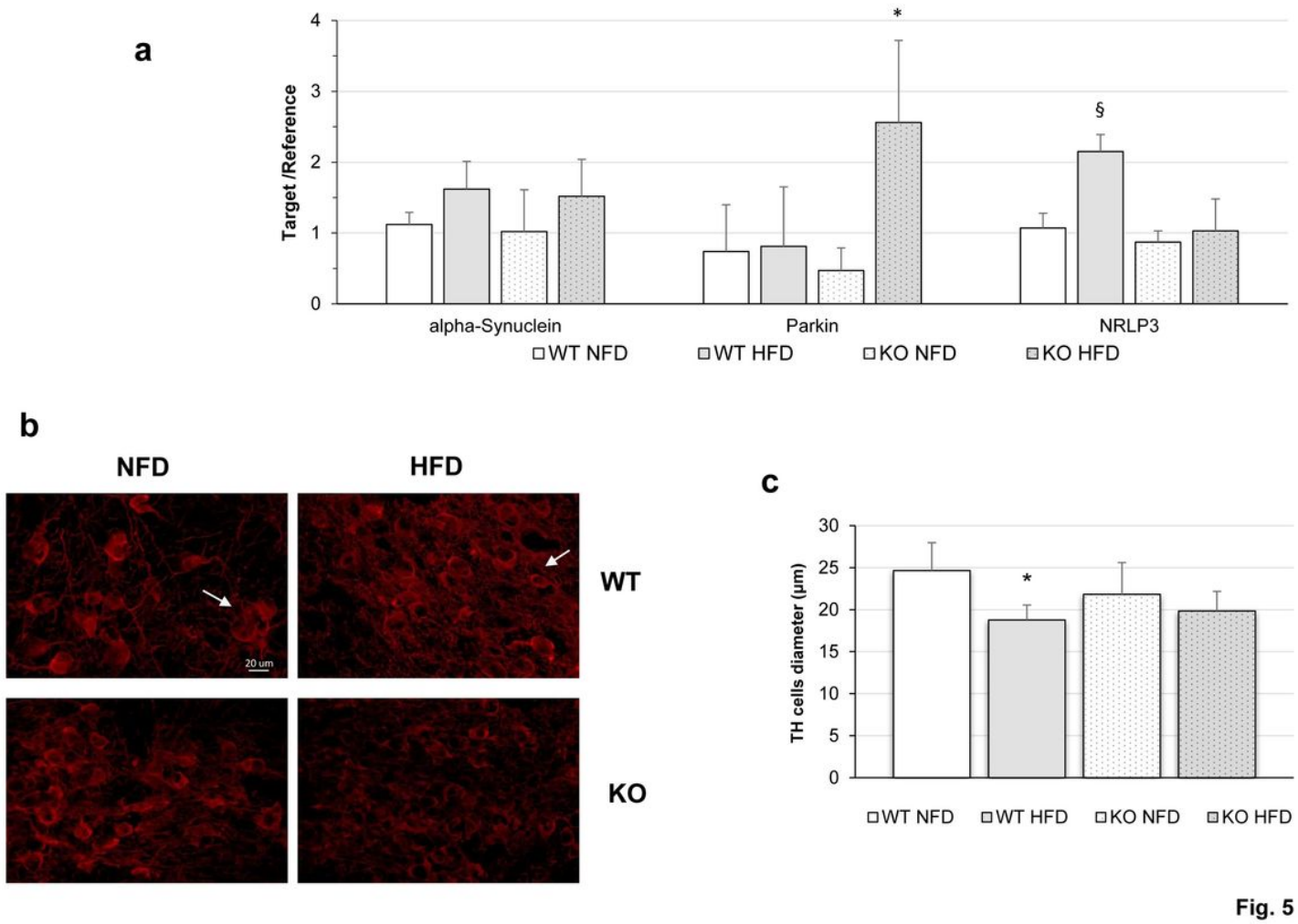



Fig. 5

## Figure 5

Panel a: α-synuclein, parkin and NLRP3 gene expression in substantia nigra of WT and P2X7R KO mice treated with normal (NFD) or high (HFD) fat diet. Data are reported as mean±SD. *p<0.001 vs KO NFD. §p<0.005 vs WT NFD. Panel b: Immunofluorescence images of the tyrosine hydroxylase (TH)-positive neurons in the substantia nigra. In WT animals, the TH cell diameter is significantly reduced by HFD (white arrows), while it is relatively preserved in P2X7 KO animals. Panel c: quantification of IF in the whole study group. *p<0.001 vs WT NFD

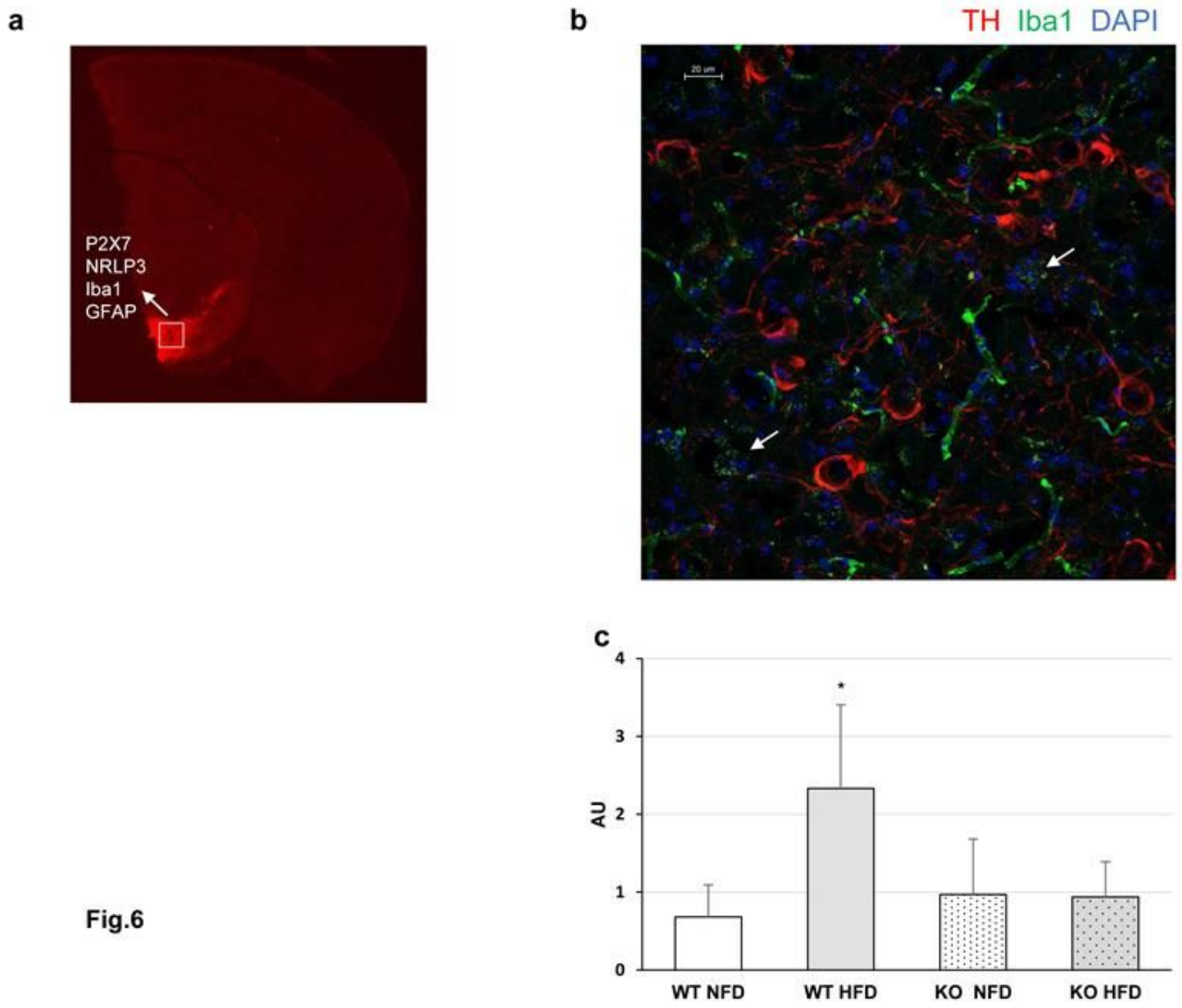



Fig.6

## Figure 6

Panel a: section of murine half brain showing TH-immunostaining (red) that identifies the substantia nigra. The immunofluorescence experiments executed in serial sections were performed with the indicated antibody. White square indicates the area where the confocal images were acquired. Panel b: confocal image of WT mouse brain treated with HFD: red staining labels TH neurons; activated microglia (green) is labeled with Iba1 antibody; nuclei are blue (DAPI). Panel c: quantification of Iba1 immunoreactivity

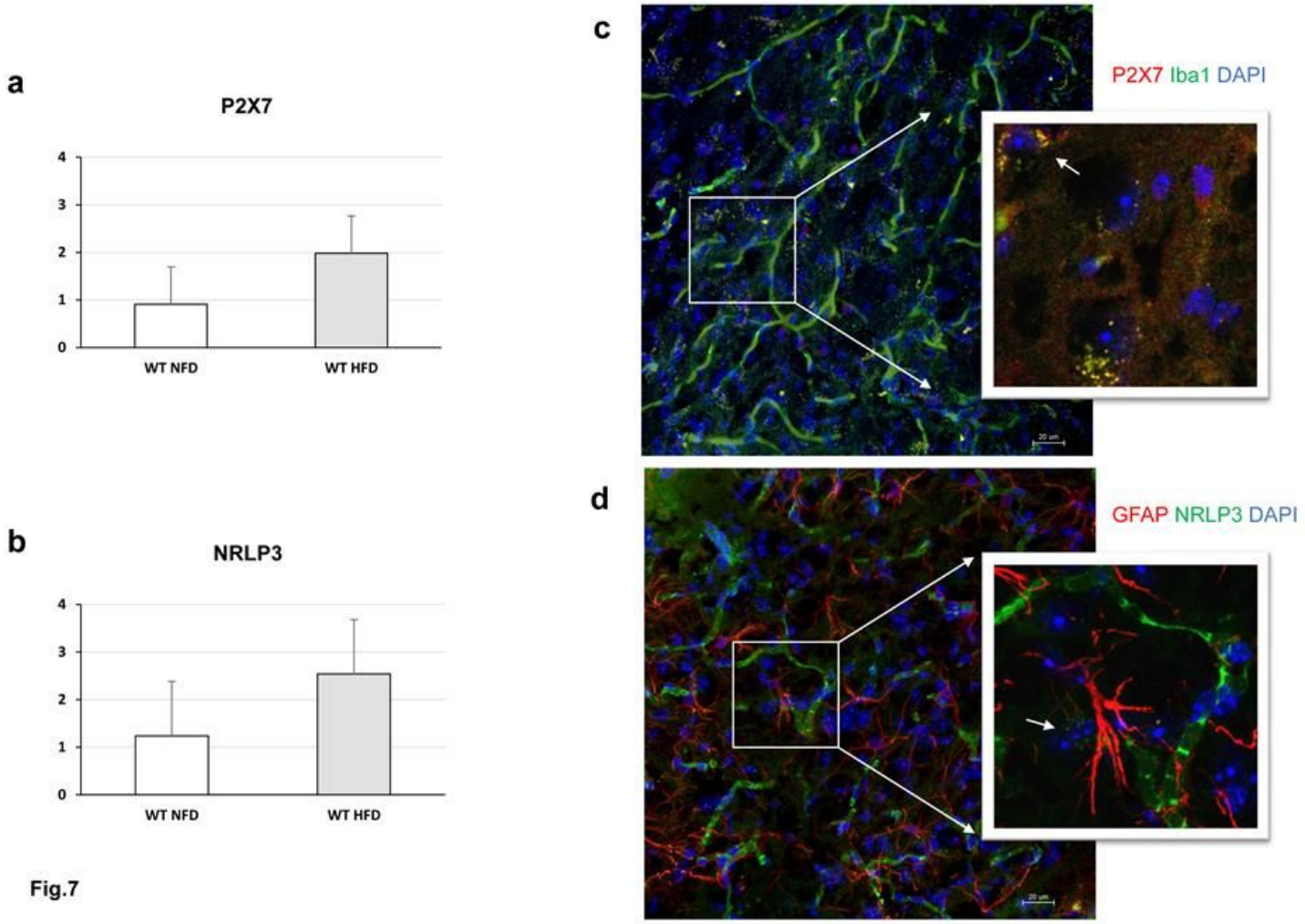



**Figure 7**

Quantification of P2X7 (panel a) and NRLP3 (panel b) immunoreactivity in WT mice treated with normal (NFD) and high fat diet (HFD). Panel c) representative image of P2X7R (red) and Iba1 (green) colocalization; nuclei are blue (DAPI). Panel d) representative image of NRLP3 (green) and GFAP (red) double staining, that does not show any colocalization; nuclei are blue (DAPI). Both inserts are single focal planes of enlarged area.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplFigure1.tif
- SupplTable1.docx