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A combined panel of salivary biomarkers in *de novo* Parkinson's Disease

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Number of characters in the title: 70

Number of characters in the running head: 42

Abstract word count: 248

Introduction word count: 440

Discussion word count: 1589

Manuscript word count: 4332

Numbers of figures: 8

Numbers of color figures: 8

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ana.26550

Summary for Social Media

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Current knowledge of the topic: The presence of clinical subtypes of Parkinson's Disease is supported by several evidences, but very few studies have reported the correlation between clinical and molecular subtypes. Saliva is an easily accessible biofluid and is emerging as a promising source for molecular biomarkers in PD.

Question addressed by the study: can we use saliva to detect molecular biomarkers of alpha-synuclein and tau aggregation, autophagy and inflammation in *de novo* Parkinson's Disease?

Knowledge added by the study: we demonstrate that biomarkers targeting different molecular pathways involved in Parkinson's Disease can be measured in saliva. These biomarkers can potentially distinguish different molecular subtypes of Parkinson's disease.

Potential impact on the practice of neurology: the detection of molecular biomarkers in an easily accessible biofluid, as saliva, could improve the diagnosis of. Parkinson's Disease at the early stages. Molecular subtyping could drive future studies for individualized disease modifying therapies.

Abstract

Objective: To investigate molecular biomarkers of a-synuclein and tau aggregation, autophagy, and inflammation in the saliva of de novo Parkinson's disease (PD) patients in comparison to healthy subjects (HS), and to correlate molecular data with clinical features of PD patients, in order to establish whether abnormalities of these parameters are associated with specific clusters of de novo PD patients, and their potential diagnostic power in differentiating PD patients from HS.

Methods: We measured total and oligomeric a-synuclein, total-tau and phosphorylated-tau, MAP-LC3beta, and TNFalpha in the saliva of 80 de novo PD patients and 62 HS, using quantitative Enzyme-Linked Immunosorbent Assay analysis.

Results: Oligomeric a-synuclein, total-tau, MAP-LC3beta, and TNFalpha levels resulted significantly higher in patients with respect to HS, while no significant differences were detected for total a-synuclein or phosphorylated-tau. Phosphorylated-tau directly correlated with MAP-LC3beta, whereas it inversely correlated with TNFalpha in PD patients. An inverse correlation was detected between MAP-LC3beta and non-motor symptoms severity. Principal Component Analysis showed that molecular and clinical parameters were independent of each other in de novo PD patients. Receiver Operating Characteristic curve analysis reported an accurate diagnostic performance of oligomeric a-synuclein and MAP-LC3beta. The diagnostic accuracy of total a-synuclein increased when it was combined with other salivary biomarkers targeting different molecular pathways.

Interpretation: Our study proposes a novel biomarker panel using saliva, a non-invasive biofluid, in de novo PD patients, with implications in understanding the molecular pathways involved in PD pathogenesis and the relevance of different molecular pathways in determining clinical PD subtypes.

Introduction

In Parkinson's disease (PD), an increasing effort is directed at designing molecular biomarkers in order to support early-stage diagnosis and evaluate disease severity and progression at the molecular level.

Alpha-synuclein (a-syn), the main component of Lewy bodies and Lewy neurites, is the pathological hallmark of PD[1] and a-syn oligomers are considered the main determinant of a-syn neurotoxicity[2] and cell-to-cell transmission of a-syn pathology[3]. However, additional cellular and molecular pathways may also contribute to neurodegeneration. Autophagy, inflammation, and other neuronal proteins that may co-aggregate with a-syn in affected neurons likely play a role in the development and progression of neurodegeneration.

A-syn aggregates are specific for synucleinopathies and are used postmortem to distinguish PD from tau-related atypical parkinsonisms, such as progressive supranuclear palsy. Therefore, biomarker studies have been designed to detect a-syn aggregates in different biological fluids, including cerebrospinal fluid (CSF)[4,5], serum[5,6], and saliva[7–9], and in the peripheral tissues of patients affected by PD, including skin biopsies[10] and biopsies of salivary glands[11]. Higher CSF levels of total-tau (t-tau) and phosphorylated tau[12] have also been found in PD patients with dementia[12,13]. Both tau and a-syn can influence each other's polymerization into fibrils[14,15], driving neuronal degeneration and disease progression. Increased levels of microtubule-associated protein light chain 3 beta (MAP-LC3beta), the protein involved in the formation of auto-phagolysosomes, have also been reported in the peripheral blood mononuclear cells[16,17] and in CSF of PD patients[18]. Similarly, elevated levels of the proinflammatory interleukins IL1β, IL2, IL6, and tumor necrosis factor alpha (TNFalpha) have been found postmortem in the striatum and substantia nigra (SN) of patients affected by PD[19,20], as well as in the CSF of living PD patients[20,21].

The molecular heterogeneity of PD implies the importance of combined testing of different molecular biomarkers targeting different molecular pathways involved in neurodegeneration. Developing biomarker-driven subtypes may help identify biologically homogenous subsets of PD patients that may guide the design of target-specific treatments.

For this purpose, we applied quantitative enzyme-linked immunosorbent assay (ELISA) analysis on the saliva of a cohort of 80 *de novo* PD patients and 62 healthy subjects (HS). We first measured total and oligomeric a-syn and then we measured possible biomarkers of key molecular pathways involved in

PD: t-tau, and phosphorylated tau (pS199tau) (tau aggregation and axonal degeneration biomarkers), MAP-LC3beta (autophagy marker) and TNFalpha (inflammation marker). Thereafter, we investigated: i) whether the different molecular parameters differed between *de novo* PD patients and HS; ii) whether molecular parameters correlated with motor and non-motor clinical scores of PD patients; iii) whether abnormalities of these parameters were associated with specific clusters of *de novo* PD patients; and iv) their diagnostic accuracy in differentiating *de novo* PD patients from HS.

Materials and methods

Participants

We consecutively enrolled 80 patients (F/M=25/55; aged 64.5±9 years) with *de novo* PD and 62 age- and sex-matched HS at the movement disorders outpatient clinic of the Department of Human Neuroscience, Sapienza University of Rome, Italy from November 2017 until November 2019.

Inclusion criteria for PD patients were: diagnosis of PD confirmed by a movement disorder expert based on international clinical criteria[26,27] and a clinical history shorter than 2 years. Exclusion criteria were: diagnosis of other neurological or psychiatric conditions, moderate or advanced PD defined as Hoehn and Yahr (H&Y) stage III, IV, or V; and/or assumption of levodopa or other dopaminergic treatment. HS were excluded if they were affected by neurological or psychiatric disorders, had received treatment with drugs known to induce parkinsonism, or were genetically related to a parkinsonian patient. Participants affected by cardiovascular diseases, diabetes mellitus, autoimmune diseases, chronic inflammatory diseases, neoplasms, and salivary gland/oral cavity pathologies were also excluded from the study.

Clinical evaluation included: duration and stage of disease, as assessed by the H&Y scale; severity of the disease, as assessed by the Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS); cognitive impairment, as assessed by the Montreal Cognitive Assessment (MoCA), and non-motor symptom severity, as assessed by the Non-Motor Symptoms Scale (NMSS).

A further cohort of 28 de novo PD patients (F/M=13/20; aged 62±11 years) and 28 age- and sex-matched HS was also recruited as a validation cohort, to verify the diagnostic accuracy of oligomeric a-syn.

The study was approved by the ethics committee of Sapienza University of Rome. All participants gave their written informed consent prior to participating in the study.

Salivary sample collection and storage

Salivary samples were collected following the procedures reported in previous studies[7,8,22–24]. Detailed anamnestic information including those related to the oral health status have been carefully collected from each patient before salivary sampling. Subjects recruited for the study were asked to avoid eating or drinking for 2 hours, smoking for 4 hours, and alcohol consumption for 12 hours prior to the collection of saliva. Self-washing of the oral cavity was performed by the subject one hour before salivary collection.

Briefly the collection of saliva was performed by the following steps:

1) Inspection of the oral cavity, in order to exclude oral pathologies.
2) Saliva collection by drooling into 50-mL vial of a minimum quantity of 2 ml of saliva per each subject.
3) Transferring of salivary samples into 10-mL Falcon tubes.
4) Immediate placement of the Falcon tubes containing salivary samples in dried ice and treatment with protease inhibitor (Sigma Aldrich, P2714) at the concentration of 100 µL per 1 mL of saliva.
5) Centrifugation of the salivary samples for 20 minutes at 5000 xg at 4°C to remove residual particles.
6) Aliquot of the supernatant from each salivary sample into 1-mL low-binding Eppendorf tube and storage at -80° before ELISA analysis[25,26].

ELISA analysis

Before performing ELISA analysis, we assessed total protein concentration in each salivary sample using the BCA Protein Assay kit (Thermofisher Scientific, UK). After assessment, salivary samples

from PD and HS were equalized for total protein concentration with 0.001 M phosphate buffered saline. Possible blood contamination, which could affect the salivary levels of a-syn, was evaluated by measuring hemoglobin concentration and samples with hemoglobin levels > 200 ng/mL were excluded from the ELISA analysis. Anti-Alpha-Synuclein Quantitative ELISA kit (SensoLyte 55550: saliva was diluted 1:10 in 0.001 M PBS to avoid matrix effect, detection antibody was incubated overnight at 4°C, the other steps followed manufacturer instructions) was used to determine total a-syn, whereas Human A-Syn Competitive Oligomer ELISA kit (MyBioSource, MBS730762: saliva was diluted 1:1 in 0.001 M PBS, conjugate antibody was incubated 3 hours at 37°C, the other steps were according to manufacturer instructions) was used to determine a-syn oligomers. The same competitive oligomer ELISA kit was applied to test a validation cohort of 28 de novo PD patients and 28 HS in two independent analyses.

Salivary levels of t-tau and pS199tau were determined using the Invitrogen Total Tau ELISA kit (KB0041: according to manufacturer instructions) and the Invitrogen Human Tau [pS199] ELISA kit (KHB7041: detector antibody was incubated overnight at 4°C, the other assay steps followed manufacturer instructions), respectively. Estimation of salivary TNFalpha was performed using the Cloud-Clone Human TNFalpha ELISA kit (SEA133Hu: the primary antibody – Detection reagent A – was incubated overnight at 4°C, the other steps of the assay followed manufacturer instructions). Regarding MAPLC3beta, we used the Autophagy ELISA kit (MAP-LC3beta Quantitative-MBS169564) and we adapted the manufacturer's protocol to detect activated MAP-LC3beta in saliva. Briefly, in unstarved cells, both MAP-LC3 I (unactivated) and MAP-LC3 II (activated) are present, and the proportion of activated MAP-LC3 II increases during autophagy induction. MAP-LC3 I is found in soluble fraction, whereas MAP-LC3 II is associated with the lipid membrane. In order to evaluate only membrane-associated MAP-LC3 II, we used a biochemical method to remove the MAP-LC3 I soluble fraction and retain only the membrane-associated MAP-LC3 II. This allowed us to measure only the MAP-LC3 fraction ideally involved in autophagosome formation. Although saliva is not comparable to a cell lysate, several lipid membranes and cell fragments are present in the saliva and we assumed that these fragments could mirror cellular processes in the salivary glands and in the nerve fibres innervating the salivary glands. We prepared soluble (cytosolic) MAP-LC3 removal reagent by diluting the

manufacturer stock solution in phosphate-buffered saline (PBS) containing 1 mM MgCl2 and 1 mM CaCl2. We then incubated the saliva with soluble MAP-LC3 removal buffer at the dilution proportion 2:1 (1 ml of saliva and 0.5 ml of soluble MAP-LC3 removal reagent) for 10 minutes at room temperature by gently shaking. After incubation, we centrifuged the sample at 25,000 xg for 20 minutes at +4 C° and we removed the supernatant containing the buffer and the soluble fraction of MAP-LC3 I. We then diluted the pellet with 1 ml of PBS containing 1 mM MgCl2 and 1 mM CaCl2 and we used these samples for ELISA analysis, according to assay procedures reported in manufacturer instructions. In order to demonstrate that a consistent amount of activated MAP-LC3beta derives from glandular cells and possibly neuronal fibers innervating salivary glands, we performed an ELISA analysis for SOX10 (MBS 1603944: according to manufacturer instructions) on salivary derived membranes from 16 *de novo* PD patients. SOX10[27] is a transcription factor expressed by salivary glands secretory cells and also by microvesicles of neuronal origin, but not expressed by mucosal squamous cells of the oral cavity.

The concentrations of salivary total a-syn, a-syn oligomers, t-tau, pS199tau, activated MAP-LC3beta, and TNFalpha were determined by spectrometric measurement at 450 nm in an appropriate microplate reader (LT 4000, Labtech, Heathfield, UK). Standard curves were prepared by plotting absorbance readings or optical density values of standards against their concentrations. For t-tau, pS199tau, activated MAP-LC3beta and TNFalpha, optimization of the standard curves was performed based on the salivary concentrations of these proteins detected in previous studies[9,28–31].

Statistical analysis

Comparison between groups: Statistical analysis was performed using Prism Graph-Pad version 9 (USA). The Shapiro-Wilk test was used to test all variables for normality. Non-parametric Mann-Whitney U test was used to compare biomolecular parameters between groups (PD patients vs. HS). Data were reported as median and IQR, with nested dots representing the distribution of each individual subject in relation to the median.

Clinico-biomolecular correlations and clustering: Multiple spearman's rank correlation coefficients were used to test possible correlations between different biomolecular parameters and between clinical

scores of PD patients and biomolecular parameters. Row Z-scores were calculated and presented on a heatmap. Based on the correlation coefficients expressed as Z-scores, simple linear regression was also performed to evaluate linear correlation between selected molecular and clinical parameters. Principal component analysis (PCA) was performed on molecular data and on molecular and clinical data together. PC loadings plots were used to individuate the principal source of molecular or clinical variance in the PD patient cohort. PC score plots were used to visualize the clustering of individual subjects along the molecular and clinical parameters.

Receiver operating characteristic (ROC) curve analysis: Different ROC analyses were performed to test the accuracy of each salivary biomarker in discriminating *de novo* PD patients from HS. We first calculated the diagnostic accuracy for each singular molecular parameter and we subsequently combined the different accessory pathways analyzed (tau, autophagy, and inflammation) with both total and oligomeric a-syn, which are considered invariable molecular hallmarks of PD. Cut-off values were calculated as the point of the curves with the highest Youden's index (sensitivity + specificity -1) to maximise the sensitivity and specificity of the diagnostic tests. Positive likelihood ratio (pLR) and area under the curve (AUC) were also calculated.

Results

Comparison and correlation of salivary biomolecular parameters in de novo PD patients and HS

PD patients and HS did not differ in terms of age or gender (all p values > 0.05). The average age of PD patients was 64.479 ± 9.098 years, whereas the average age of HS was 63.871 ± 8.121 years. The mean MDS-UPDRS parts II & III score of the PD cohort analysed was 22.684 ± 11.830, whereas the mean MoCA score was 26.852 ± 2.754 and the mean NMSS score was 27.257 ± 22.068.

In *de novo* PD patients, we did not detect any significant difference in the salivary concentration of total a-syn in comparison to HS, although a trend towards decreased salivary total a-syn was present (p=0.6387). In contrast, salivary oligomeric a-syn was significantly increased in *de novo* PD patients in comparison to HS (p<0.00001). T-tau concentration in saliva was significantly increased in *de novo* PD patients compared to HS (p<0.00001), whereas salivary pS199tau did not significantly differ between *de novo* PD patients and HS (; p=0.4442). The concentrations of activated MAP-LC3beta (p<0.00001)

and TNFalpha ($p<0.00001$) were both increased in the saliva of *de novo* PD patients compared to HS (Fig 1A to F).

Correlation data between the different biomolecular parameters detected in saliva are reported on a heatmap (Fig 2A) and demonstrated a significant positive correlation between pS199tau and activated MAP-LC3beta (Z score=0.54) and a significant inverse correlation between pS199tau and TNFalpha (Z score=-0.75). Consequently, simple linear regression demonstrated a significant inverse association between pS199tau and TNFalpha ($p<0.0001$; R=0.4514). and a significant positive association between pS199tau and MAP-LC3beta ($p<0.0001$; R=0.2324). Correlations between total or oligomeric a-syn and other molecular parameters were not significant, except for a slightly significant correlation between total a-syn and activated MAP-LC3beta ($p<0.1$; R=0.5379) (Fig 2B to D). In the group of 16 PD patients, activated MAP-LC3beta significantly correlated with the concentration of the transcription factor SOX10 ($p<0.001$; R=0.4428), suggesting that a consistent amount of this autophagic protein derives form salivary glandular cells and microvesicles of neuronal origin[27] (Figure 8C).

Correlations between salivary biomolecular parameters and clinical scores in PD

In order to test possible correlations between the molecular parameters and clinical scores of *de novo* PD patients, we employed multiple Spearman's rank correlation coefficients with both molecular and clinical parameters. Of the different molecular parameters, only activated MAP-LC3beta showed an inverse correlation with NMSS (Fig 3A; Z score=-0.30), which was also confirmed by simple linear regression (Fig 3B; $p<0.001$; R=0.1307). We found no additional significant correlations between clinical scores and biomolecular parameters.

PCA of biomolecular and clinical parameters of de novo PD patients

In order to establish which salivary molecular parameter primarily determined molecular variance in our *de novo* PD patient cohort, we first employed PCA with the different biomolecular parameters detected in saliva. We found that they constituted one principal component (PC1) in which pS199tau, activated MAP-LC3beta, and TNFalpha were the main determinants of molecular variance between PD patients. Conversely, total and oligomeric a-syn and t-tau accounted for a less cumulative variance between *de novo* PD patients (Fig 4A and B).

We then employed PCA analysis with both molecular (total a-syn, oligomeric a-syn, t-tau, pS199tau, activated MAP-LC3beta, and TNFalpha) and clinical (MDS-UPDRS II & III, MoCA, NMSS, and age) data. In this case, we found two principal components (PC1 and PC2). The PC loadings plot demonstrated that the main determinants of PC1 variance were MDS-UPDRS II & III, NMSS, and MoCA score, whereas pS199tau, TNFalpha, and MAP-LC3beta were the main determinants of PC2 variance, suggesting that clinical and molecular parameters were independent in our *de novo* PD patient cohort. Finally, combining the PC loadings plot with a PC scores plot in a biplot, we detected the distribution of individual PD patients along the PC1 and PC2 axes and found that most patients likely cluster according to PC2 and, more specifically, according to activated MAP-LC3beta and TNFalpha (Fig 4C and D).

Single and combined ROC analyses of salivary biomolecular parameters for the diagnosis of de novo PD

We employed ROC analysis in order to detect the accuracy of each salivary molecular parameter in distinguishing *de novo* PD patients from HS. However, since the different molecular parameters targeting tau aggregation (t-tau and pS199tau), autophagy (activated MAP-LC3beta), and inflammation (TNFalpha) are not specific to PD, we also performed a ROC analysis of each of them in combination with total or oligomeric a-syn, given the specificity of a-syn aggregation for PD (Table 1).

Total a-syn alone did not distinguish *de novo* PD patients from HS with adequate sensitivity and specificity ($p=0.6362$; AUC: 0.5232; likelihood ratio (LR): 1.085). In contrast, oligomeric a-syn demonstrated a very high accuracy ($p<0.0001$; AUC: 0.9982; LR: 62.00).

Variable diagnostic accuracy was detected for the other salivary biomarkers: t-tau did not reach significant values of sensitivity or specificity ($p=0.0558$; AUC: 0.5938; LR: 1.476), nor did pS199tau ($p=0.4418$; AUC: 0.5377; LR: 1.516). In contrast, activated MAP-LC3beta was very accurate in diagnosing *de novo* PD ($p<0.0001$; AUC: 0.9236; LR: 8.802), whereas TNFalpha had low sensitivity but very good specificity ($p=0.001$; AUC: 0.6601; LR: 6.329) (Fig 5A to F). A high diagnostic accuracy was also present in the *de novo* PD validation cohort ($p<0.0001$, AUC: 0,9434), analyzed by two independent ELISA experiments (Fig 8B).

Combining total a-syn with the other salivary biomarkers resulted in increased accuracy in differentiating *de novo* PD patients from HS with respect to total a-syn alone. The total a-syn/t-tau combination demonstrated a very high sensitivity with modest specificity ($p<0.0001$; AUC: 0.7702; LR: 2.067). Conversely, the combination of total a-syn and pS199tau showed a comparable performance to total a-syn alone ($p=0.7766$; AUC: 0.5139; LR: 0.8138). A very high sensitivity was achieved by combining total a-syn with MAP-LC3beta ($p<0.0001$; AUC: 0.7639; LR: 1.850). Combining total a-syn with TNFalpha resulted in less accuracy ($p=0.0184$; AUC: 0.6155; LR: 1.550) (Fig 6A to D). By combining each molecular marker with oligomeric a-syn, we obtained very high diagnostic accuracy, especially when oligomeric a-syn was combined with t-tau ($p<0.0001$; AUC: 0.9631; LR: 11.47), MAP-LC3beta ($p<0.0001$; AUC: 0.9970; LR: 31.00), and TNFalpha ($p<0.0001$; AUC: 0.9794; LR:11.47), and with a lesser extent when combined with pS199tau ($p<0.0001$; AUC: 0.8206; LR: 2.611)(Fig 7A to D).

Discussion

In the present study, we investigated a panel of salivary biomarkers targeting different molecular pathways involved in the pathogenesis of PD. We found that oligomeric a-syn, t-tau, MAP-LC3beta, and TNFalpha levels were significantly higher in *de novo* PD patients with respect to HS, while no significant differences were detected for total a-syn or pS199tau. Correlations between different salivary molecular parameters revealed that pS199tau directly correlated with MAP-LC3beta, whereas it had an inverse correlation with TNFalpha. Correlation of molecular parameters with clinical scores revealed an inverse correlation between MAP-LC3beta and NMSS score. PCA showed that molecular and clinical parameters form two independent principal components. Finally, ROC analysis reported a high accuracy for oligomeric a-syn and MAP-LC3beta in distinguishing *de novo* PD patients from HS. Improved diagnostic accuracy for total a-syn was obtained when ROC analysis combined total a-syn with the other salivary molecular biomarkers.

We confirmed that salivary oligomeric a-syn is increased in PD patients as compared to HS, as we have previously reported[7,8] and as reported in other biological fluids by using the same ELISA assay[32]. Our results were further confirmed in the validation cohort, suggesting that high salivary levels of a-syn

oligomers are present in saliva also in the *de novo* PD patients. Conversely to a-syn oligomers, we did not detect significant differences in salivary total a-syn. The lack of significant differences in total a-syn can be explained by the clinical features of our PD cohort, since patients at early disease stages may exhibit less intracellular a-syn pathology with respect to patients at intermediate or advanced stages. Total a-syn likely reflects the concentration of a-syn monomers. A significant decrease of a-syn monomers in biological fluids might reflect, in turn, an increased formation of intracellular aggregates, which more likely occurs at the advanced stages of the disease. In accordance, Kang et al.[9] found that the oligomeric/total a-syn ratio was significantly decreased in patients with H&Y stage I and significantly increased in patients with H&Y stages II-IV, thus suggesting that total a-syn is likely to decrease with disease progression. Moreover, a-syn pathology detected in the submandibular gland was more prevalent in patients with moderate and advanced PD as compared to those at an early disease stage[33]. Accordingly, we previously reported significantly lower salivary total a-syn in PD patient cohorts that included patients with different degrees of disease severity[7,8].

A novel finding of our study was the increased salivary concentration of t-tau in *de novo* PD patients compared to HS, in accordance with previous evidence from CSF studies[12,13]. Conversely, we did not find differences in salivary pS199tau levels between patients and HS. Furthermore, although several authors reported that tau neurofibrillary tangles correlate with cognitive status in PD[12,34,35], we did not detect a significant correlation between cognitive scores and the salivary concentration of t-tau or pS199tau. This is likely due to the clinical features of our patients, who had a mean MoCA score ≥26. Perhaps, with disease progression, salivary t-tau and phosphorylated-tau could potentially correlate with cognitive decline.

We also found that PD patients showed higher levels of salivary MAP-LC3beta compared to HS. The higher levels of MAP_LC3beta in saliva are in accordance with previous evidence showing increased MAP-LC3beta in the peripheral blood mononuclear cells[16,17] and CSF[18] in PD. We also found an inverse correlation between MAP-LC3 and total a-syn, and a positive correlation with pS199tau. Tau and a-syn can enhance each other's polymerization into fibrils[14,15]. Autophagy is essential for the removal of aggregated and misfolded proteins[36] and aggregation of both tau and a-syn is drastically increased by impaired autophagic-lysosomal degradation[37]. This might explain the correlation we found between

MAP-LC3beta and both total a-syn and pS199tau. Youn et al.[18] reported that increased levels of MAP-LC3 in CSF significantly correlated with PD motor symptom severity. Although we did not find any correlation between MAP-LC3beta levels and MDS-UPDRS score, MAP-LC3beta levels were inversely correlated with NMSS score. Our PD population consisting of patients at early disease stages may still show only mild motor symptoms, thus explaining why we did not find any correlation with MDS-UPDRS score. However, the inverse correlation we found between MAP-LC3beta levels and NMSS scores suggests that reduced activation of the autophagic pathway may underlay the development of more severe clinical phenotype at disease onset[38,39].

A further novel finding of the study is the higher levels of salivary TNFalpha in *de novo* PD patients compared to HS. This result is in accordance with previous findings reporting elevated levels of the proinflammatory interleukins IL1β, IL2, IL6 and TNFα in the CSF, striatum, and SN of PD patients [19-21]. TNFalpha is specifically produced by macrophages and microglia and has been detected in the brain of animal models of synucleinopathies presenting with microglial activation[40,41]. Histological analyses have also shown that the peripheral nervous system and the gastrointestinal tract are affected by both a-syn aggregation and inflammation at early PD stages[42–44], which might explain the increased concentration of TNFalpha we detected in saliva. Moreover, cell-to-cell transmission of a-syn aggregates may require an inflammatory response[44], making the detection of inflammatory cytokines, like TNFalpha, a promising biomarker candidate to target the molecular progression of PD. Furthermore, we found an inverse correlation between TNFalpha and pS199tau. This finding might reflect the recruitment of the inflammatory pathway in digesting toxic and phosphorylated-tau aggregates, as has been reported in recent studies on experimental models of tauopathies[45,46].

By using PCA, we observed that pS199tau, MAP-LC3beta, and TNFalpha were primarily responsible for the molecular variance between patients, whereas total and oligomeric a-syn did not play a major role. This suggests that a-syn aggregation is a constant pathological feature from early stages of PD and that additional molecular pathways are variably involved in individual patients and represent the main determinant of pathological heterogeneity. Using PCA with both molecular and clinical data, we detected two principal components and found that clinical and molecular features were responsible for PC1 and PC2 variance, respectively. This suggests that clinical and molecular parameters are

independent in *de novo* PD patients. When plotting individual PD patients on the PC loadings plot, we found that most patients were clustered along the PC2 axis, which corresponded to the molecular parameters. In particular, the PC scores plot indicated that patients were mostly clustered by TNFalpha or MAP-LC3beta, suggesting a possible role of inflammation and autophagy in determining different molecular subtypes. The observation that molecular heterogeneity is independent of clinical heterogeneity in our PD cohort has relevant implications on the pathological correlates of PD clinical subtypes. Previous evidence suggests that PD clinical subtypes differ in terms of neuroimaging[39] and neurophysiological parameters[47], whereas CSF a-syn was similar between different subtypes[39]. In contrast to previous studies, we measured different molecular biomarkers in saliva and then employed unsupervised PCA with both molecular and clinical data. This allowed us to identify molecular variance, mainly related to markers of autophagy, tau aggregation and inflammation, which did not correspond to specific clinical features. Future prospective studies are needed to clarify whether molecular variability predicts the progression of different clinical subtypes over the disease course.

ROC analysis revealed a high accuracy of different salivary molecular biomarkers in diagnosing PD. A very high diagnostic accuracy was found for salivary oligomeric a-syn. The sensitivity and the specificity we now report for a-syn oligomers, are higher in comparison with our previous studies in saliva and has been also confirmed in the validation cohort of *de novo* PD patients. Methodological and clinical differences might explain the improved diagnostic accuracy we now found. In our previous study[8] we have employed sandwich ELISA assay for a-syn oligomers, whereas in the present study we have used a competitive ELISA assay. A-syn oligomers are molecularly heterogenous and therefore variable in the epitope presentation. Since the sandwich ELISA requests the binding of the antibody to two different sites on the target molecule, the molecular variability could have affected the accuracy of their detection. Competitive ELISA assay, might overcome this variability, explaining the higher sensitivity and specificity we found in the present study. Moreover, in our previous studies[7,8] we have studied patients with different degrees of disease severity and duration, whereas in the present study we investigated only *de novo* PD patients. It is possible that soluble a-syn oligomers in saliva are increased at the early PD stages in comparison to patients with a moderate-advanced PD.

Promising diagnostic accuracy was also demonstrated for MAP-LC3beta and TNFalpha. However, since these biomarkers are also involved in other neurodegenerative disorders and therefore might be less specific for PD, we employed a combined ROC analysis of each biomarker with both total or oligomeric a-syn. As expected, the accuracy of a-syn oligomers remained high when combined with all other biomarkers. The combination of salivary total a-syn with t-tau, MAP-LC3beta, or TNF-alpha improved diagnostic accuracy with respect to total a-syn alone. Therefore, combined detection of total a-syn with salivary biomarkers targeting tau, autophagy, or inflammation represents a promising molecular platform to support PD diagnosis from early disease stages which can also overcome the molecular heterogeneity and instability of a-syn oligomers that can make less reproducible their detection in biological fluids. In conclusion, the present study proposes a novel biomarker panel that allows to investigate different molecular pathways involved in PD pathogenesis and their potential in determining clinical PD subtypes. The biomarker panel we propose is performed with saliva, a non-invasive and easily accessible biofluid. Longitudinal and multicentric studies are needed to confirm the relevance of these biomarkers, both individually and in combination, in distinguishing PD from HS and from atypical parkinsonism at the early stages of the disease and in the evaluation of disease progression.

Author Contributions:

GV, MIDB, DB, AC, GF, SM and AB contributed to the conception and design of the study. GV, MIDB, RM, AF, MC, NM and GL contributed to the acquisition and analysis of data. GV, MIDB, DB and AB contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest: Nothing to report.

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Figures Legend

Fig 1: Dot plots representing the concentration of total a-syn (**A**), oligomeric a-syn (**B**), total tau (**C**), pS199tau (**D**), activated MAP-LC3beta (**E**), and TNFalpha (**F**) in the saliva of 80 *de novo* PD patients (black dots) and 62 age- and sex-matched HS (grey dots). Data are expressed as median and IQR. Each dot corresponds to an individual subject. **Total a-syn**: median PD patients: 3.248 pg/ml, IQR: 0.6867, median HS: 3.220 pg/ml, IQR: 0.8333; **oligomeric a-syn**: median PD patients: 1.276 ng/ml, IQR: 1.3843 ng/ml, median HS: 0.1862, IQR: 0.0886; **total-tau**: median PD patients: 7.723 pg/ml, IQR: 9.0201, median HS: 3.647 pg/ml, IQR: 3.3068; **pS199tau**: median PD patients: 1.380 pg/ml, IQR: 2.5274, median HS: 1.605 pg/ml, IQR: 0.3769; **activated MAP-LC3beta**: median PD patients: 1.601 ng/ml, IQR: 0.9576, median HS: 0.3368 ng/ml, IQR: 0.2486; **TNFalpha**: median PD patients: 0.9996 pg/ml, IQR: 1.5151, median HS: 0.5960 pg/ml, IQR: 0.2398.

Fig 2: Heatmap reporting the multiple Spearman's rank correlation coefficients between the different molecular parameters detected in the saliva of *de novo* PD patients (**A**). Degrees of positive or negative

correlation are expressed by Z-scores. The maximum degree of positive correlation is 1 (blue), whereas the maximum degree of negative correlation is -1 (red). Linear regressions demonstrated a significant negative correlation between pS199tau and TNFalpha (**B**; $p<0.0001$; $R=0.4514$), and a significant positive correlation between salivary pS199tau and salivary MAP-LC3beta (**C**; $p<0.0001$; $R=0.2324$) and A slightly significant positive correlation was also demonstrated between MAP-LC3beta and total a-syn (**D**; $p<0.1$; $R=0.5379$).

Fig 3: Heatmap reporting the multiple Spearman's rank correlation coefficients between the different molecular parameters detected in saliva and the clinical scores of *de novo* PD patients (**A**). Degrees of positive or negative correlation are expressed by Z-scores. The maximum degree of positive correlation is 1 (yellow), whereas the maximum degree of negative correlation is -1 (dark purple). Linear regression demonstrating a significant negative correlation between salivary MAP-LC3beta and NMSS score (**B**; $p<0.0001$; $R=0.1307$).

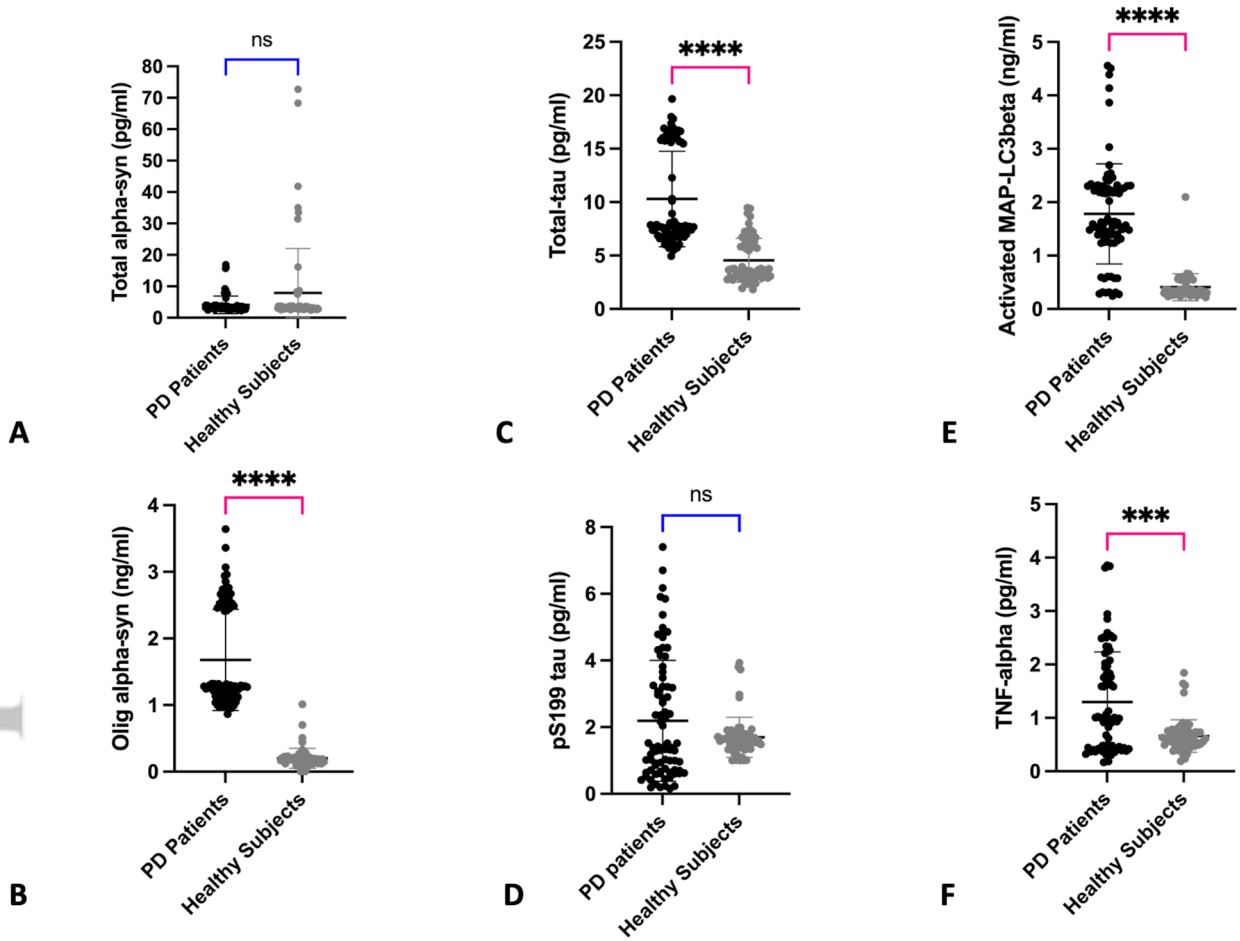
Fig 4: The PCA loadings plot for salivary molecular parameters in PD patients demonstrates that PC1 variance is mostly attributed to salivary concentrations of pS199tau, MAP-LC3beta, and TNFalpha (**A**). Linear bars show that components 4, 5, and 6, respectively corresponding to pS199tau, MAP-LC3beta, and TNFalpha, are the main sources of cumulative variance in the PD patient cohort. Dotted lines represent individual variance for each component (**B**). After performing PCA with both clinical score and molecular parameters, the PC loadings plot (**C**) showed that PC1 variance was almost equally attributable to MDS-UPDRS II & III, NMSS, and MoCA scores, whereas the molecular parameters pS199tau, TNFalpha, and MAP-LC3beta were mostly involved in PC2 variance, although TNFalpha also slightly contributed to PC1 variance. The biplot (**D**) combines the PC loadings plot with the PC scores plot, showing the distribution of individual patients in relation to PC1 and PC2. Most patients are likely to cluster according to PC2, with the main determinants of clustering being TNFalpha, pS199tau, and MAP-LC3 beta.

Fig 5: ROC analysis of salivary levels of total a-syn (**A**; AUC: 0.5232), oligomeric a-syn (**B**; AUC: 0.9982), total tau (**C**; AUC: 0.5938), pS199tau (**D**; AUC: 0.5377), MAP-LC3 beta (**E**; AUC: 0.9236), and TNFalpha (**F**; AUC: 0.6601) in differentiating *de novo* PD patients from HS.

Fig 6: Combined ROC analysis with salivary levels of total a-syn and total tau (**A**; AUC: 0.7702; $p<0.0001$), pS199tau (**B**; AUC: 0.5139; $p=0.7766$), MAP-LC3 beta (**C**; AUC: 0.7639; $p<0.0001$) and TNFalpha (**D**; AUC: 0.6155; $p<0.05$) in differentiating *de novo* PD patients from HS.

Fig 7: Combined ROC analysis with salivary levels of oligomeric a-syn and salivary levels of total tau (**A**; AUC: 0.9631; $p<0.0001$), pS199tau (**B**; AUC: 0.8179; $p<0.0001$), MAP-LC3beta (**C**; AUC: 0.9970; $p<0.0001$), and TNFalpha (**D**; AUC: 0.9794; $p<0.05$) in differentiating *de novo* PD patients from HS.

Fig 8: (**A**) Dot plots representing the concentration of oligomeric a-syn in the validation cohort of 28 *de novo* PD patients and 28 HS. Data are expressed as median and IQR. Each dot corresponds to an individual patient. Median HS: 0.2258 ng/ml, IQR: 0.0774; median PD patients: 1.410 ng/ml, IQR: 1.6886. (**B**) ROC analysis of salivary levels of oligomeric a-syn in the validation cohort (AUC: 0.9434; $p<0.05$). (C) Linear regression demonstrating a significant positive correlation between salivary MAP-LC3beta and salivary SOX10 ($p<0.0049$; $R=0.4428$).



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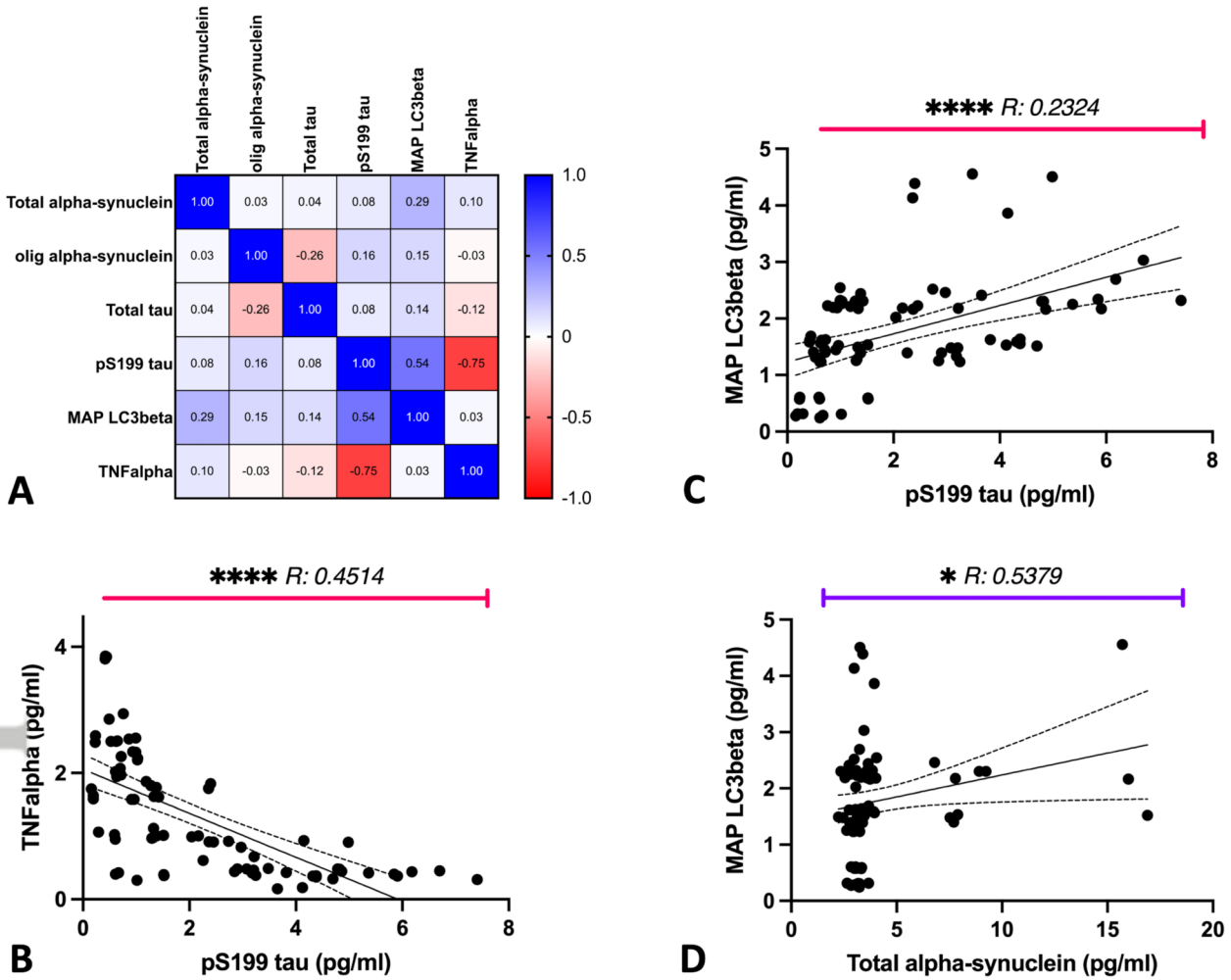

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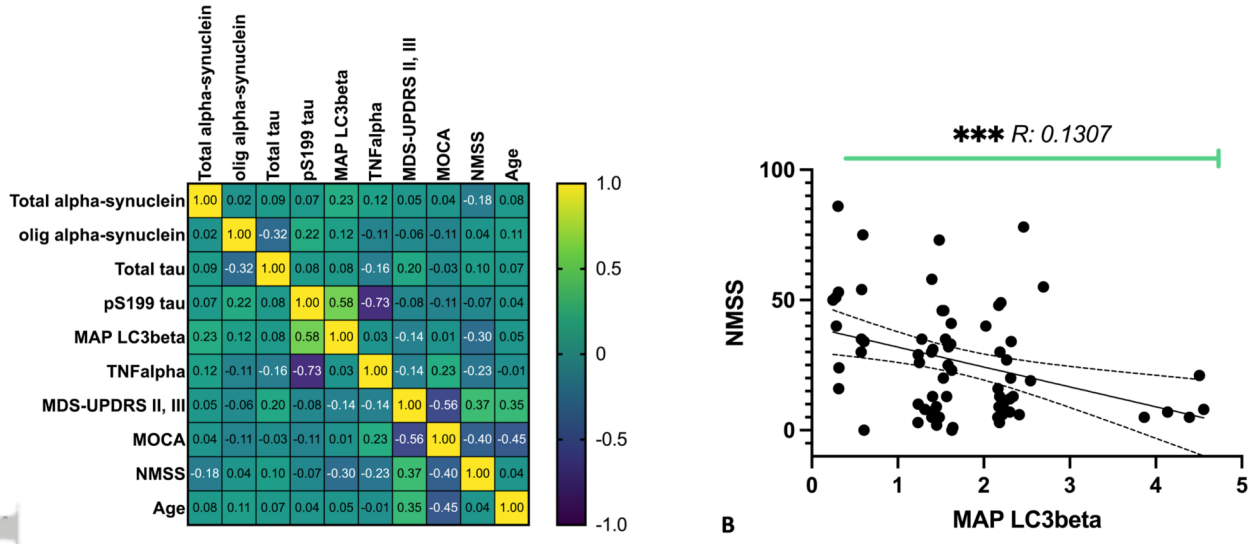

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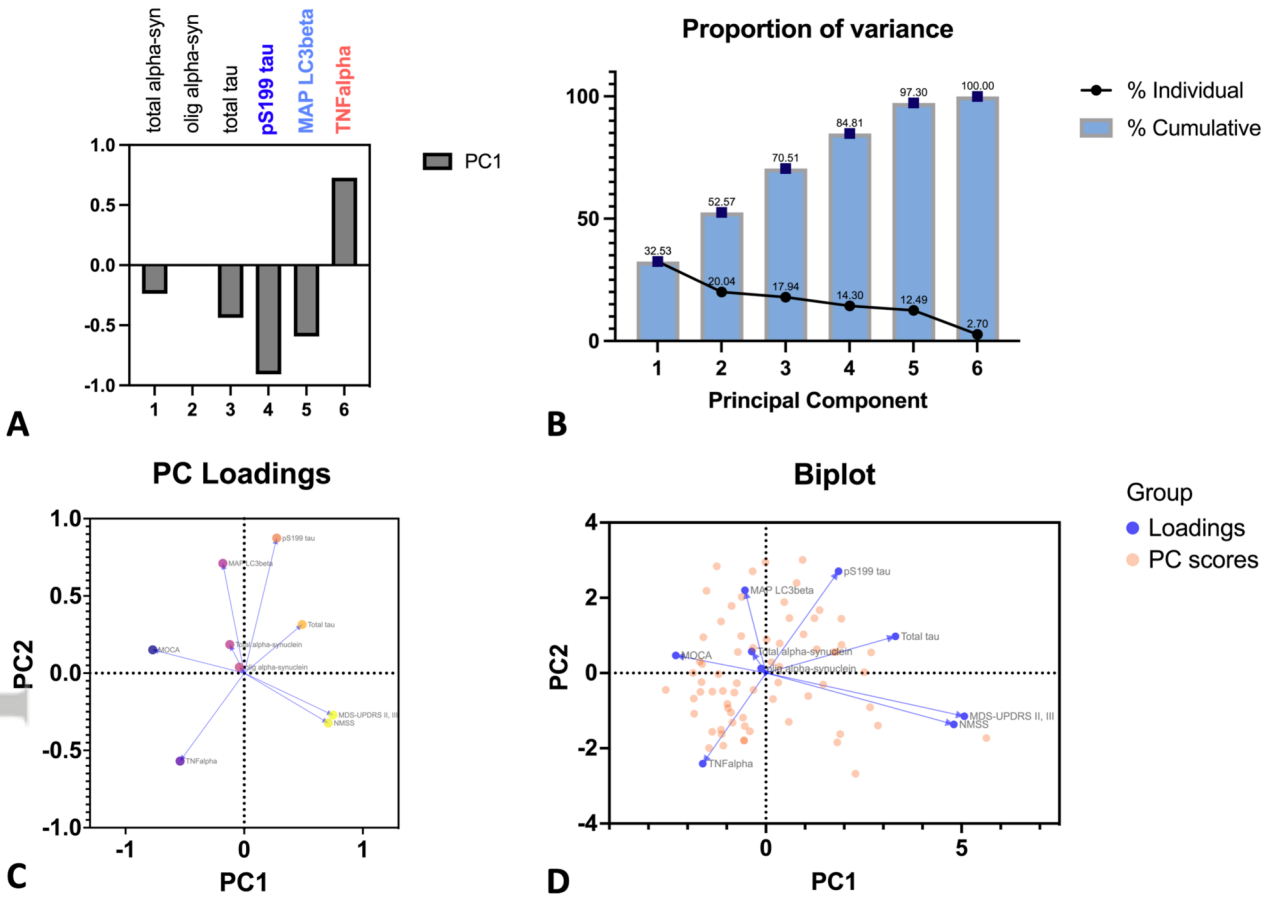

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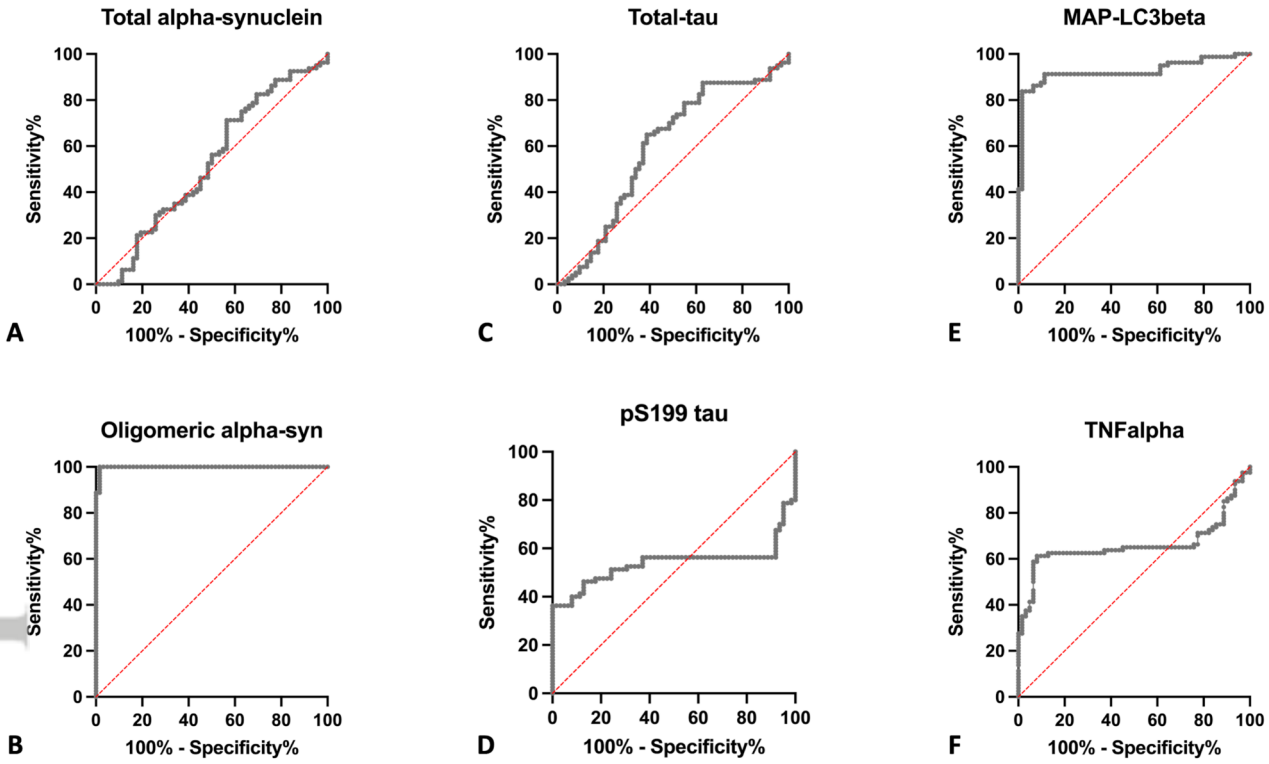

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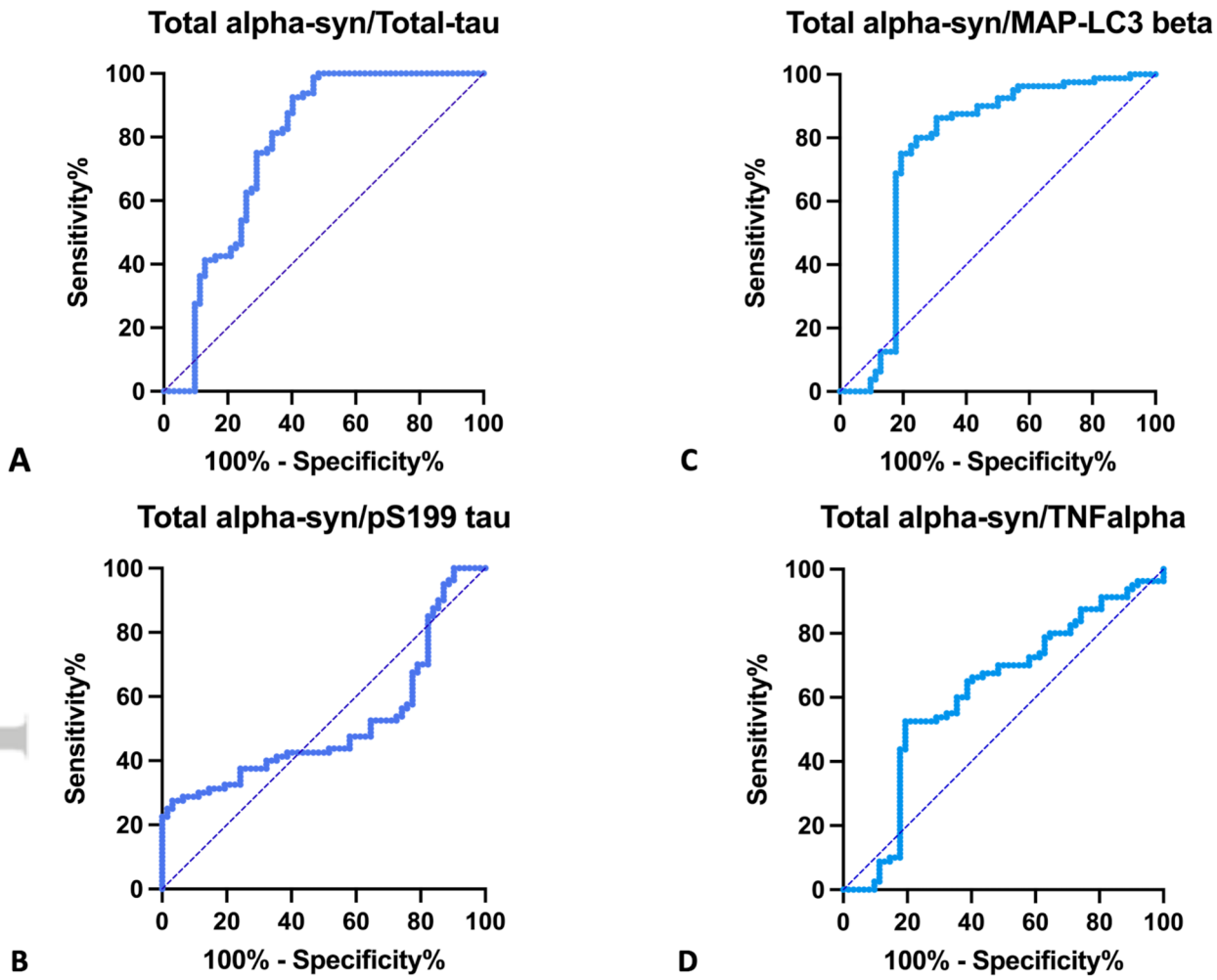

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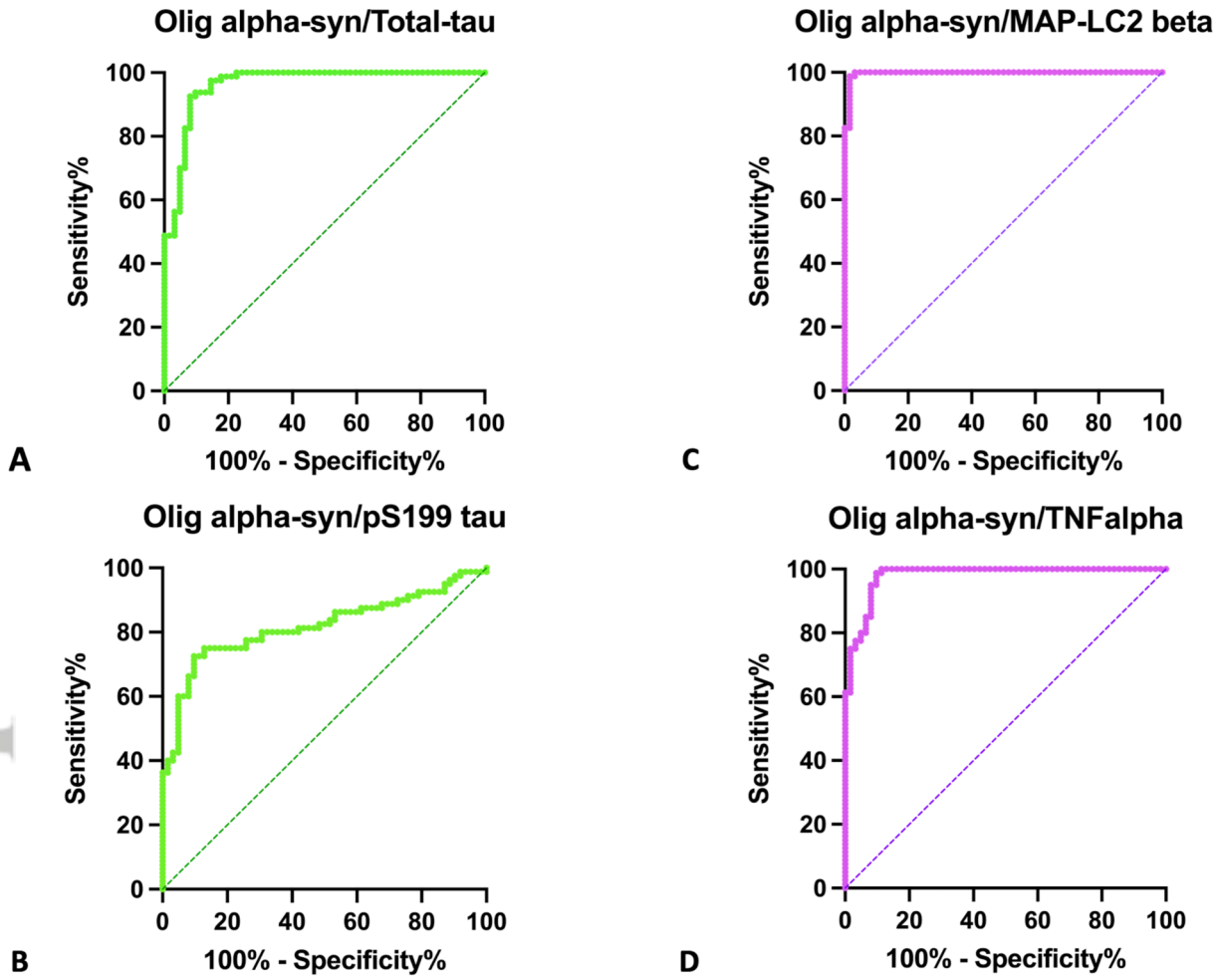

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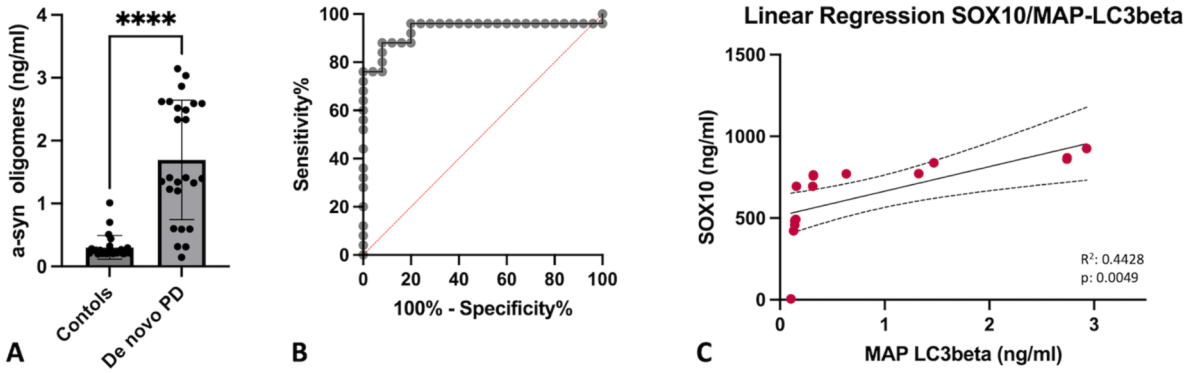

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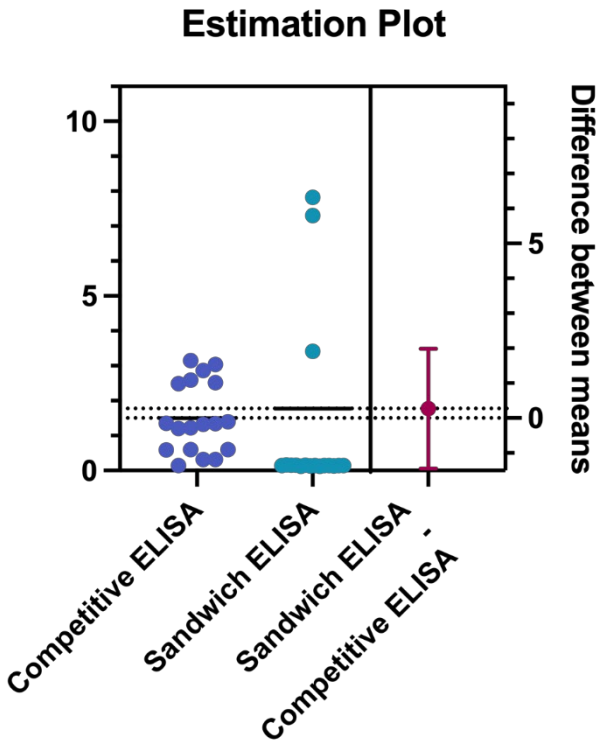

Graph 1. Estimation plot summarizing the comparison between competitive and sandwich ELISA for oligomeric a-synuclein in the saliva of 18 *de novo* PD patients, dot plots show a higher performance of competitive ELISA in detecting oligomeric a-synuclein. Linear bar demonstrate a significative variance ($p<0.0001$) in oligomeric a-synuclein detection with competitive ELISA in comparison to sandwich ELISA. *

* not for publication in the main manuscript

Table 1. ROC curve analysis results for single and combined salivary biomarkers in de novo Parkinson's disease patients.

Biomarkers	Cut-off values	Sensitivity (%)	95% CI sensitivity	Specifity (%)	95% CI specifity	Likelihood Ratio
Total a-syn	3.240 pg/ml	52.50	41.70%-63.08%	51.61	39.45%-63.59%	1.085
Olig a-syn	0.7831 ng/ml	100	95.42%-100%	98.39	91.41%-99.92%	62.00
T-tau	3.241 pg/ml	50	39.30%-50.70%	66.13	56.72%-76.66%	1.476
pS199-tau	1.530 pg/ml	56.25	45.34%-66.59%	62.90	50.46%-73.84%	1.516
MAPLC3	0.5659 pg/ml	91.25	83.02%-95.70%	88.71	78.48%-94.22%	8.802
TNF-alpha	0.8257 pg/ml	61.25	50.29%-71.18%	90.32	80.45%-95.49%	6.329
Total a-syn/t-tau		100	95.42%-100%	51.61	39.45%-63.59	2.067
Total a-syn/pS199tau		52.50	41.70%-63.08%	35.48	24.74%-47.92%	0.8138
Total a-syn/MAPLC3		92.50	84.59%-96.52%	50.00	37.92%-62.08%	1.850
Total a-syn/TNFalpha		67.50	56.64%-76.76%	56.45	44.09-68.06%	1.550
Olig a-syn/T-tau		92.50	84.59%-96.52%	91.94	82.47%-96.51%	11.47
Olig a-syn/pS199-tau		80	69.95%-87.30%	69.35	57.03%-79.42%	2.611
Oligo a-syn/MAPLC3		100	95.42%-100%	96.77	88.98%-99.43%	31.00

Oligo a-syn/TNFalpha	92.50	84.59%-96.52%	91.94	82.47%-96.51%	11.47

95% CI: 95% confidence interval