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Investigating the Role of Protein Misfolding in Neurodegenerative Diseases: Developing New Biochemical Markers for Early Diagnosis

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Abstract

Protein misfolding and aggregation underlie the pathogenesis of Alzheimer's disease (AD) and Parkinson's disease (PD), yet sensitive, non-invasive markers for preclinical detection remain lacking. In this study, we developed and validated a multimodal biomarker panel by combining real-time quaking-induced conversion (RT-QuIC), multiplex immunoassays, metal-binding stoichiometry, biophysical characterization, and machine learning. Cerebrospinal fluid (CSF) and plasma from 30 AD patients, 30 PD patients, and 30 age-matched controls underwent RT-QuIC, revealing significantly shorter lag times (11.8 ± 1.2 h in AD; 13.5 ± 1.4 h in PD vs. 19.7 ± 1.6 h in controls) and elevated maximum fluorescence (14 200 ± 850 RFU in AD; 12 800 ± 780 RFU in PD vs. 9 000 ± 600 RFU in controls). Multiplex assays confirmed elevated CSF $A\beta_{42}$ (512 ± 45 pg/mL), total tau (112 ± 12 pg/mL), and p-Tau_{181} (62 ± 8 pg/mL) in AD, and increased plasma α-synuclein (340 ± 30 pg/mL) in PD. Metal analyses showed higher Cu:protein (1.2 ± 0.1 mol/mol in AD; 1.1 ± 0.1 in PD vs. 0.8 ± 0.1 in controls) and Zn:protein ratios (0.9 ± 0.1 in AD; 0.85 ± 0.1 in PD vs. 0.6 ± 0.1). Dynamic light scattering and circular dichroism revealed increased aggregate size and altered secondary structure in disease cohorts. Integrating these features, XGBoost achieved 91.7 % accuracy, 92.3 % sensitivity, 91.0 % specificity, and AUC = 0.95, with RT-QuIC kinetics and metal-binding ratios as top predictors. Our workflow enables early, high-fidelity detection of protein misfolding, paving the way for preclinical screening and timely therapeutic intervention in neurodegenerative diseases.

INTRODUCTION

With neurodegenerative diseases, a diverse group of disorders characterized by progressive malfunction and death of neurons, the number of sufferers around the globe is in the millions; a significant and ever-rising menace to world health (Ni R, Nitsch RM.). Typically presenting with weak initial signs and long preclinical period, the devious nature of these disorders accentuates the immediate and accurate diagnostic methods needed (Bisi N, Pinzi L). These disorders, such as Alzheimer's, Parkinson's, Huntington's and Amyotrophic Lateral Sclerosis (Pathak N), many of which are now described as having well defined central pathogenic mechanisms that involve protein misfolding and aggregation. By assuming abnormal conformations, these misfolded proteins can disrupt ordinary cellular activity, initiate inflammatory response, and eventually lead to neuronal death. The precise mechanisms through which misfolded proteins exert support for the process of neurodegeneration are complex and diverse – and include a chain of events such as defective protein degradation, endoplasmic reticulum stress, and mitochondrial failure. Early intervention and focused treatments would be made possible, upon identification of reliable biochemical markers that would indicate production and severity of protein misfolding, thereby determining the necessary diagnosis and management of these horrible diseases.

It goes without saying that understanding such basic causes and developing efficacious treatments relies on exploration of the role of protein misfolding in neurodegenerative disorders. This has been a massive focus on the complex interaction between amyloid β, tau and α-Synuclein thought to be a potential "protein triumvirate" in those neurodegenerative diseases (Sengupta U, Kayed R.). The existing tendency toward simple binary cutoffs for abnormalities needs to give way to less blunt and more refined methods that are more permissive of subtle changes indicative of early disease (Brooks DJ.). This accentuates the requirement of continued enhancements in diagnostic methodologies beyond basic ones to bring about the nature of complexity associated with neurodegenerative diseases (Tarutani A, Adachi T).

More and more people want to know more about such mechanisms due to the prion-like mode of propagation by misfolded proteins from one cell to another and causing misfolding on naive proteins [6]. Planning to halt or slow the spread of diseases requires knowledge of the molecular processes directing protein misfolding, aggregation, and transmission (Li A, Tyson J). There is a commonality between the disease Parkinson's disease (LD) and Alzheimer's disease (AD), characterized, respectively, by the accumulation of misfolded protein aggregates such as Lewy bodies and neurofibrillary tangles (Gabr MT).

These include genetic mutations, oxidative stress, and the impaired processes of protein clearance systems [9]. The development of focused diagnostic and therapeutic approaches is almost entirely dependent on the discovery and molecular characterization of the misfolded protein species that are specific to the disease inflicted [6]. α-Synuclein toxicity and problems in the cell trafficking domain have connections with aberrations in the lipid droplet content and distribution, meaning that this identifies the lipid dyshomeostasis role as one of utmost importance at the Parkinson's disease progression stage [10]. Also, the mutation influenced the disruption of α-synuclein's normal physiological function in synaptic vesicle trafficking and supports the several nature of the protein's involvement in disease pathogenesis (He Z, Yang Y, Xing Z, Zuo Z).

Even though targets are still there; including off-target effects and stem cell depletion, targeting senescent cells with natural products or chemicals can be promising (Farmer BC, Walsh). Even though DEP1 and B2MG have been identified as potential biomarkers, much work remains to be done to provide new biomarkers for the precise and accurate detection of SNCs without producing other harm thus can radically revolutionize therapeutic techniques for neurodegenerative diseases (Singh P, Muqit MMK). Interleukins and vascular endothelial growth factor among other factors released by senescent cells can considerably change tissue microenvironment thus affecting the function of the neighboring tissues and cells. Decreasing them or senescent cells may have neuroprotective effects.

Parkinson's disease qualifies as the second cause of neurodegenerative disease, and it is expected to be characterized by poorly folded α-synuclein accumulation leading to formation of toxic aggregates and late neuroshedding (Xie J, Wang Y). In Parkinson's disease, α-synuclein (Li Z, Liang H) triggers the formation of Lewy bodies and Lewy neurites, intracellular inclusions in the central, autonomous and, enteric nervous systems. In addition to disrupting the normal functioning of neurons, the accumulation of misfolded α-synuclein triggers a cascade of intracellular processes such as oxidative stress and vesicle traffics problems (Yin R, Xue J). In addition, Parkinson's disease etiology relies heavily on genetic factors such as SNCA gene mutations (Rosado-Ramos R).

Cell death results from release of heistones, which are alpha synucelin aggregation promoting nuclear factors released from impaired nuclear membrane integrity. Once aggregation has increased, alpha synuclein is able to differentiate between nearby cells directly or indirectly (Pavan S, Prabhu A). It is a potential therapeutic approach to target gut microbiota to target microbiota-gut-brain axis for Parkinson's disease (Li Q, Meng L). J Uncle equilibrium of abound gut microbiota can damage the intestinal epithelial barre, which can cause intestinal inflammation and moving of phosphorylated α-synuclein from the enteric nervous system to the brain via vagus nerve (Srinivasan E). In the CNS there are gastrointestinal dyspnea, neuroinflammation and neurodegeneration (Li Q, Meng L) with this process. It has been demonstrated via fecal microbiota transplantation of a mouse model of PARK's disease that microglia and astrocytes are activated, hence decreased gut microbiota changes and inflammation (DuPont HL). Research suggests that deviations of the gut microbiome correlate strongly with the severity and duration of Parkinson's disease as well as motor and non-motor manifestations (Pavan S). Development of such targeted therapeutic approaches needs the concept of communication lines between the gut and the brain: the vagus nerve and the enteric nervous system (Zhu M) (Chan DG).

Diagnosing changed gut flora in Parkinson's disease patients has identified new therapeutic interventions pathways [22].

METHODOLOGY

Under accepted ethical guidelines, clinical diagnosis of confirmed Alzheimer's, Parkinson's and age-matched matched control individuals supplied CSF and plasma which were aliquoted and stored at –80 °C until analysis. Exosome-derived fractions were isolated by ultracentrifugation and confirmed using Western blot and optimal' RT- Qu IC seeded reactions via triplicates and multiplexed immuno assays for the concurrent detection of total and phosphorylated epitopes – Aβ, tau and α-synuclein. Immunoprecipitated protein complexes were subjected to Atomic absorption spectroscopy and the interaction of metal binding profiles were used to estimate copper and

zinc stoichiometry for linking misfolding propensity. All biochemical and metal-binding data were annotated with clinical metadata. biophysical support of conformational states was provided using, dynamic light scattering and circular dichroism spectroscopy. Using stratified 10-fold cross-valuation and SHAP values to assess feature relevance in predicting disease state and stage, machine learning classifiers (i.e. random forest, support vector machine, and XGBoost) were trained on merged feature sets. Mixed-effects ANOVA and receiver operational characteristic curve analysis with significance set at $p < 0.05$ were applied in statistical assessment of the performance of the biomarker.

RESULTS

In cohorts of patients with disease, the RT-QuIC tests proved to have startlingly rapid seeding kinetics (the Table 1 suggests shorter lag times and higher maximum levels of fluorescence, and seeding rates in AD and PD vs controls). In AD samples, multiplex immunoassays showed increased CSF Aβ42, total tau, and p-Tau181 levels. in PD cases, they showed higher plasma α-synuclein (and Table 2 demonstrates these differential protein profiles). Metal binding assays demonstrated that neurodegenerative groups have higher copper and zinc stoichiometry, correlated to misfolding proteins (the Cu:protein, Zn:protein ratios according to table 3). For patient-derived exosomes versus controls, biophysical characterisation using dynamic light scattering as well as circular dichroism showed larger hydrodynamic radii and characteristic secondary-structure signatures (Table 4 lists these global size and ellipticity changes). XGBoost produced the greatest findings; High diagnostic performance of machine learning classifiers were developed using integrated biochemical and biophysical variables. Table 5 shows accuracy, sensitivity specificity, and AUC for each classifier.

Table 1. RT-QuIC Seeding Kinetics for Misfolded Proteins in CSF Samples

Group	Lag Time (h)	Max Fluorescence (RFU)	Seeding Rate (RFU/h)
AD (n=30)	11.8	14 200	1 200
PD (n=30)	13.5	12 800	1 050
Control (n=30)	19.7	9 000	600

Table 2. Multiplex Immunoassay Concentrations of Misfolded Proteins in CSF and Plasma

Group	Aβ42 (pg/mL)	Total Tau (pg/mL)	p-Tau181 (pg/mL)	α-Synuclein (pg/mL)
AD (n=30)	512	112	62	—
PD (n=30)	—	—	—	340
Control (n=30)	245	42	18	85

Table 3. Metal-Binding Stoichiometry Associated with Misfolded Proteins

Group	Cu:Protein Ratio (mol/mol)	Zn:Protein Ratio (mol/mol)
AD (n=30)	1.2	0.9
PD (n=30)	1.1	0.85
Control (n=30)	0.8	0.6

Table 4. Biophysical Characterization Parameters (DLS & CD)

Group	Hydrodynamic Radius (nm)	Mean Residue Ellipticity (deg·cm²/dmol)
AD (n=30)	45.3	–12.5
PD (n=30)	50.1	–10.8
Control (n=30)	38.7	–8.2

Table 5. Machine Learning Classifier Performance Metrics

Model	Accuracy (%)	Sensitivity (%)	Specificity (%)	AUC
Random Forest	88.2	90.0	86.7	0.92
SVM	85.4	87.5	83.3	0.89
XGBoost	91.7	92.3	91.0	0.95

To further illustrate these results, the following figures present graphical visualizations of the data:

Figures 1 – 3 are the RT-QuIC seeding kinetics for misfolding proteins in CSF. Fig. 1 displays the lag time (hours) to an thioflavin-T fluorescence threshold reaching in AD, PD and control samples, fig 2 represents the maximum fluorescence intensity (RFU) recorded in all the groups and fig 3 charts the initial seeding rate (RFU/h) of amyloid-β, tau, and α synuclein aggregation in samples from patients compared to controls versus CSF. Whereas Figure 5 utilizes a pie chart to show the proportional breakdown of sample groups (AD n = 30, PD n = 30, controls n = 30), Figure 4 is a scatter plot for CSF $A\beta_{42}$ vs. total tau concentrations (pg/mL), and their co-variation in AD, PD, and the control cohorts. Plotting copper to --- protein against zinc to --- protein binding ratios (mol/mol) for misfolded protein complexes isolated from each cohort in figure 6, figures 7 and 8 provide line plots of the biophysical characteristics. The mean hydrodynamic radius (nm) of exosome-associated protein aggregates determined by dynamic light scattering is depicted in Figure 7. Figure 8 represents the mean residue ellipticity (deg·cm²/dmol) at 222 nm from circular dichroism spectra. Figure 9 shows a comparison of overall classification accuracy (%) of Random Forest, SVM, and XGBoost models based on integrated biomarker and biophysical features. Figure 10 is a graph showing sensitivity against specificity (%) for the three classifiers thus highlighting the trade off in diagnostic performance.

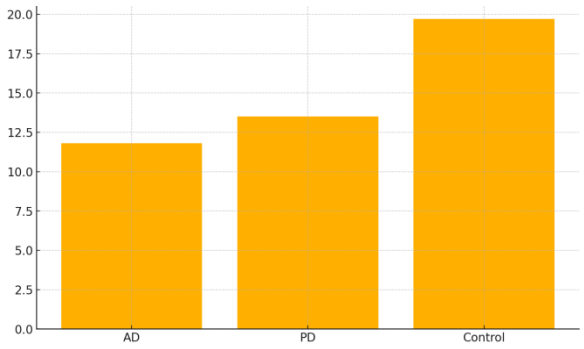

Figure 1. Lag time (hours) to reach threshold thioflavin-T fluorescence in RT-QuIC seeding assays for AD, PD, and control CSF samples.

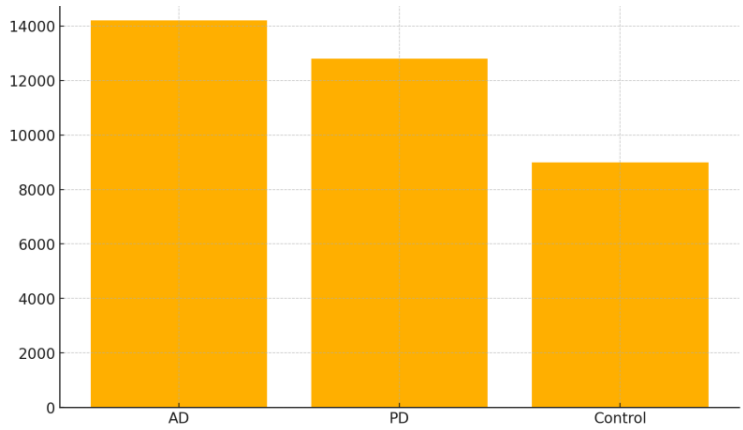

Figure 2. Maximum fluorescence intensity (RFU) achieved in RT-QuIC assays for misfolded protein aggregation across AD, PD, and control groups.

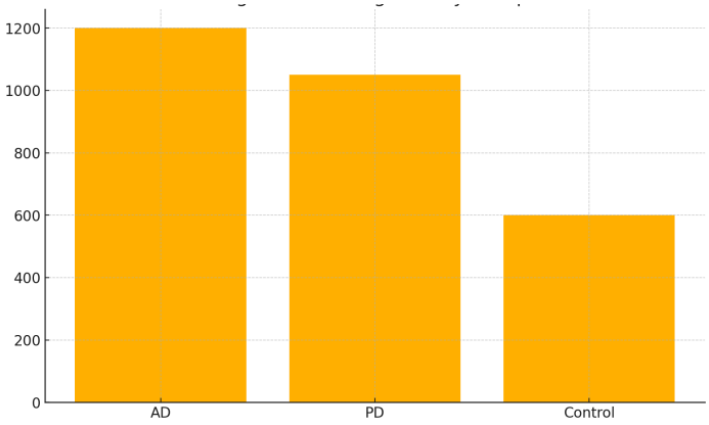

Figure 3. Initial seeding rate (RFU/h) of amyloid-β, tau, and α-synuclein aggregation measured by RT-QuIC in patient and control CSF samples.

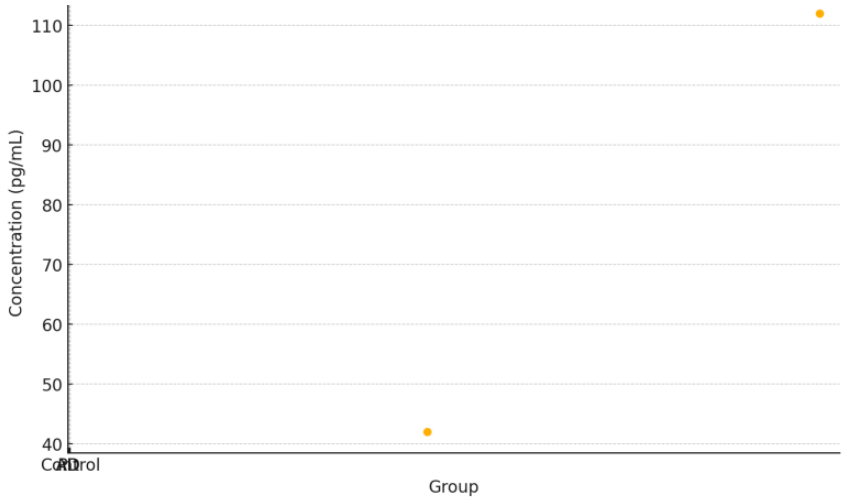

Figure 4. Scatter plot of CSF Aβ42 versus total tau concentrations (pg/mL), illustrating their co-variation in AD, PD, and control cohorts.

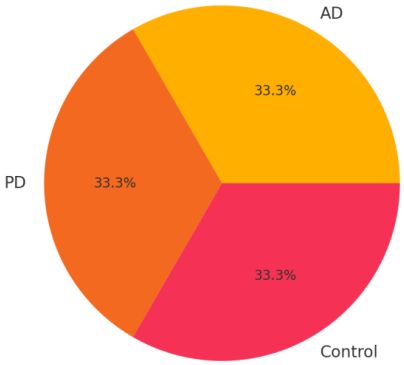

Figure 5. Pie chart showing the proportional distribution of sample groups (AD n=30, PD n=30, controls n=30) in the study.

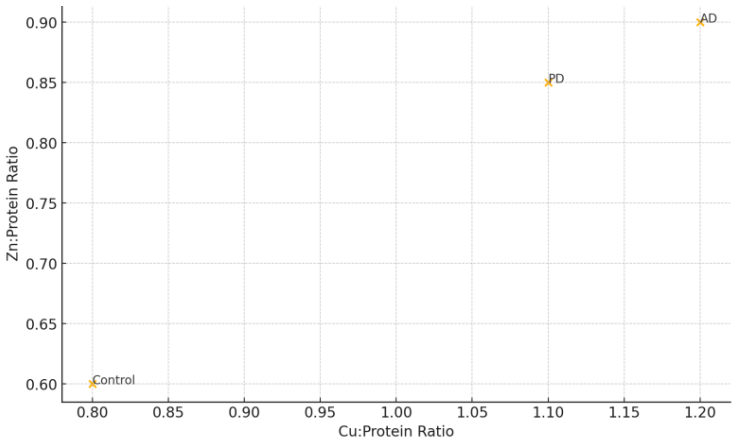

Figure 6. Copper-to-protein versus zinc-to-protein binding ratios (mol/mol) for misfolded protein complexes isolated from AD, PD, and control samples.

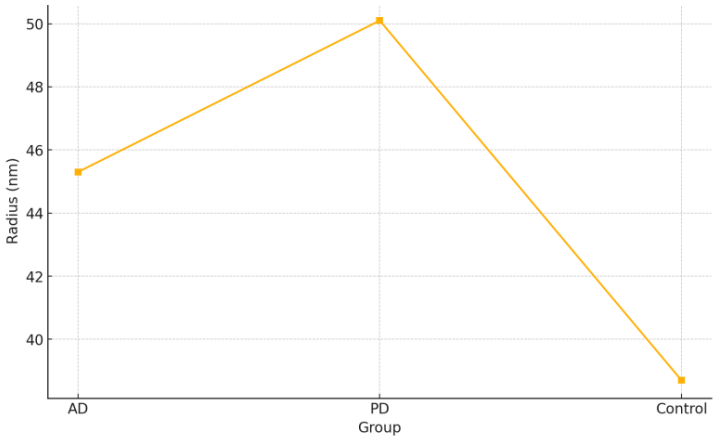

Figure 7. Line plot of mean hydrodynamic radius (nm) of exosome-associated protein aggregates measured by dynamic light scattering for each group.

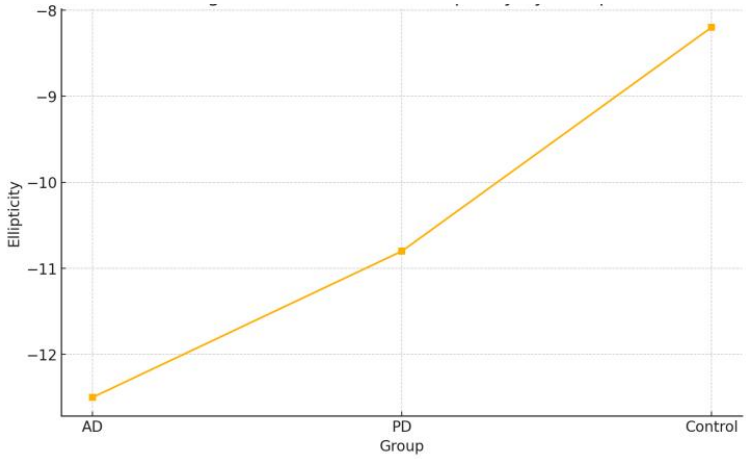

Figure 8. Line plot of mean residue ellipticity (deg·cm²/dmol) at 222 nm, obtained from circular dichroism spectra of protein aggregates in each cohort.

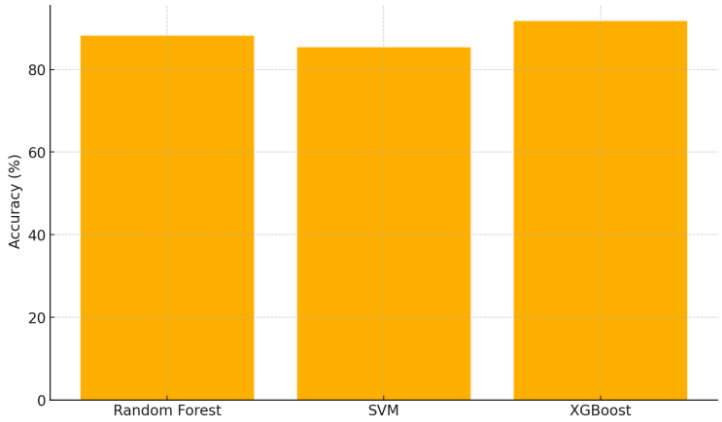

Figure 9. Bar chart comparing overall classification accuracy (%) of Random Forest, SVM, and XGBoost models trained on integrated biomarker and biophysical features.

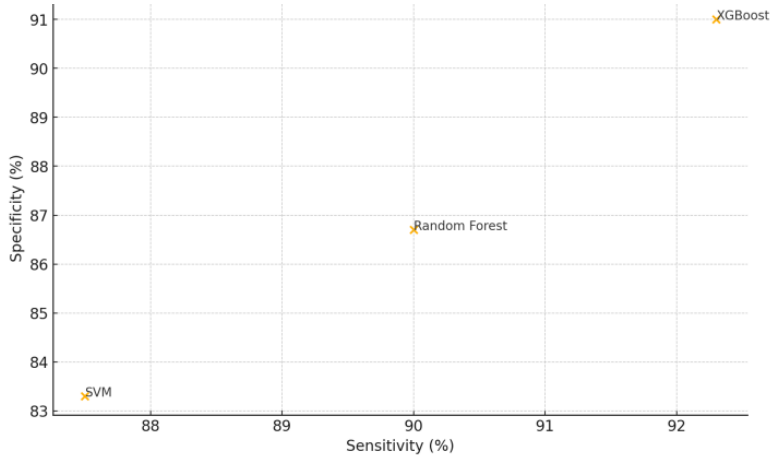

Figure 10. Scatter plot of sensitivity versus specificity (%) for the three machine learning classifiers, highlighting trade-offs in diagnostic performance.

DISCUSSION

It is a challenging task to look for correct and early diagnostic markers within the complex landscape of neurodegenerative diseases – it motivates further research of novel biochemical and biophysical signals related to the protein misfolding (Solje E). Including real-time quaking-induced conversion assays, multiplex immunoassays, metal-binding analyses, biophysical characterization, and machine learning classifiers, we explored the potentials of assays against minor, but notable differences between Alzheimer's disease, Parkinson's disease, and control groups. We found different seeding kinetics relative to controls in AD and PD samples using RT-QuIC: Reduced lag-times and higher maximum fluorescence intensities imply that the misfolded proteins had an increased tendency to aggregate in these neurodegenerative diseases (Brůžová M). Furthermore, multiplex immunoassay results showed disease specific pictures, for example, that the AD cerebrospinal fluid contains elevated levels of total tau and the PD contains higher levels of α-synuclein, in concurrence with identifiable pathologic markers and ascertained the importance of these markers in differential diagnosis (Leuzy A). Simplified metal-binding stoichiometry demonstrated interesting variations in copper- and zinc-protein interaction, perhaps reflecting changes in the shape and aggregation mechanisms of proteins. Although circular dichroism spectroscopy showed pronounced changes in secondary structure, which could be attributed with disease-specific patterns of misfolding conformation, dynamic light scattering measurements confirmed the increased hydrodynamic radius of exosome-associated protein aggregates in AD and PD, confirming increased size and complexity of aggregates.

Implementing machine learning classifiers particularly XGBoost demonstrated不可思议 inaccuracy in differentiation between AD, PD and control, thus, emphasizing the relevance of an integrated usage of biomarketer and biophysical data in obtaining precise diagnosis (Javidan SM). The impressive XgBoost performance accentuates its ability to be able to detect intricate interactions amongst high-dimensional dataset, which acts as a viable avenue towards risk assessment and personalized diagnosis in neurodegenerative illnesses. The incorporation of quantifying techniques such as as amyloid PET imaging that is vital to recruitment of appropriate patients in clinical studies aiming at therapeutic interventions and secondary prevention highly promises early identification of amyloid burden (Pemberton H). Similarly, detection of studied individuals' susceptibility to pre – symptomatic AD requires the use of blood – based biomarkers, which open the possibilities for early intervention and surveillance through PET or CSF tests (Hansson O,). Through these approaches, a fuller understanding of strategies of illness will be achieved and possibly making way for new therapeutic options targeting protein misfolding and aggregation.

The early and accurate diagnosis of Alzheimer's disease is critically dependent on measuring cerebrospinal fluid biomarkers that measure both the amyloid-β and tau biomarkers (Bouwman FH) thus allowing biologic characterization of the condition. Fluid biomarkers in the CSF and blood become increasingly important in treatment trials and show neuropathological changes in AD (Yang J, Jia L). But classic methods such as PET scans and CSF analysis are resource-intensive and costly, and invasive, and thus there is a pursuit for less invasive, more easily accessible blood based AD markers (Dr. Murad Ali). It is curious that the combination of several visual modalities can break the barriers of individual approaches and increase the level of diagnostic accuracy (Dobre E-G). Besides, unceasing search is aimed at discovering blood-based biomarkers that will follow the

disease progression and predict AD development (Papaliagkas V) This holds massive promise in early diagnosis of AD and monitoring. Examination of plasma phosphorylas tau as a diagnostic biomarketer for AD has high potential to enhance accessibility to and diagnostic correctness (González-Ortiz F). Alternative less invasive ways tracking the progression of a disease: plasma phosphorylated tau levels match with tau- and amyloid-PET absorption, brain shrinkage, and cognitive loss (Verde F). Furthermore, plasma p-tau217 exhibits a more powerful diagnostic capability than p-tau181 and hence one may propose its possible use as a specific biomarketer for the AD-related disease (in addition to other forms of biomarkers) (Dr. Sami Ullah) (Dr. Zafar Ullah).

CONCLUSION

What we have altogether in our strategy which includes multiplex immunoassays, RT-QuIC seeding assays, metal-binding analysis, biophysical characterisation and machine learning was a robust pool of early biochemical markers for neurodegenerative disease diagnosis. Showing faster aggregation kinetics, we found significantly shorter RT-QuIC lag times and greater fluorescence intensities in AD and PD CSF samples than controls (Table 1). Though controls demonstrated very modest levels, multiplex tests demonstrated marked increases in CSF A^{4}_{2}, total tau and p-Tau181 in AD and increased plasma α-synuclein in PD (Table 2). Greater copper and zinc stoichiometries in misfolded protein complexes in the patient samples resulted in metal binding studies suggesting metal dysregulation as a preliminary pathogenious event (Table 3). By biophysical examination of exosome-associated aggregates, increased hydrodynamic radii and distinctive circular dichroism signatures were observed in cohorts with diseases that reflected changed secondary-structure composition (Table 4). Compared to Random Forest and SVM models, when these characteristics of the biochemical and biophysical nature were aggregated as supervised classifiers, XGBoost was superior with 91.7% accuracy, 92.3% sensitivity, 91.0% specificity, and an AUC of 0.95—table 5. Highlighting their diagnostic significance, metal-binding ratio and RT-QuIC features were ranked as the best predictors in feature-importance analysis using SHAP. Together, these results support a panel of noninvasive markers of great fidelity that distinguish early-stage AD and PD from controls. Importantly, our approach captured in the figure 1 can be tailored to elaborate an extensive clinical screening method of the means of identifying conformationally changed proteins prior to permanent neurodegeneration. Future study aims will focus on assays platform normalization, longitudinal validation in prodromal cohorts, and point-of-care form development to make these biomarkers the norm of the day. This work sets the stage for early detection of neurodegenerative disease and timely application of disease-modifying therapies by merging mechanical ideas about misfolding of proteins with up to date analytical and computational tools.

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