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Potential Diagnostic Value of Red Blood Cells α-Synuclein Heteroaggregates in Alzheimer's Disease

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Abstract

A plethora of complex misfolded protein combinations have been found in Alzheimer disease (AD) brains besides the classical pathological hallmarks. Recently, α-synuclein (α-syn) and its heterocomplexes with amyloid-β (Aβ) and tau have been suggested to be involved in the pathophysiological processes of neurodegenerative diseases. These pathological features are not limited to the brain, but can be also found in peripheral fluids. In this respect, red blood cells (RBCs) have been suggested as a good model to investigate the biochemical alterations of neurodegeneration. Our aim is to find whether RBC concentrations of α-syn and its heterocomplexes (i.e., α-syn/Aβ and α-syn/tau) were different in AD patients compared with healthy controls (HC). The levels of homo- and heteroaggregates of α-syn, Aβ and tau, were analyzed in a cohort of AD patients at early stage either with dementia or prodromal symptoms ($N = 39$) and age-matched healthy controls ($N = 39$). All AD patients received a biomarker-based diagnosis (low cerebrospinal fluid levels of Aβ peptide combined with high cerebrospinal fluid concentrations of total tau and/or phospho-tau proteins; alternatively, a positivity to cerebral amyloid-PET scan). Our results showed lower concentrations of α-syn and its heterocomplexes (i.e., α-syn/Aβ and α-syn/tau) in RBCs of AD patients with respect to HC. RBC α-syn/Aβ as well as RBC α-syn/tau heterodimers discriminated AD participants from HC with fair accuracy, whereas RBC α-syn concentrations differentiated poorly the two groups. Although additional investigations are required, these data suggest α-syn heteroaggregates in RBCs as potential tool in the diagnostic work-up of early AD diagnosis.

Keywords Alzheimer's disease · α-Synuclein · α-Synuclein heterocomplexes · β-Amyloid · Tau · Red blood cells

Abbreviations

AD	Alzheimer's disease
Aβ	β-amyloid$_{1–42}$
LB	Lewy bodies
MCI	mild cognitive impairment
ND	neurodegenerative disease
PD	Parkinson's disease
RBCs	red blood cells
α-syn	α-synuclein

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Introduction

Alzheimer's disease (AD) is traditionally considered a neurodegenerative disease (ND) belonging to the group of proteinopathies, and characterized by cerebral deposits of amyloid-β (Aβ) peptide and of abnormally phosphorylated tau protein in form of amyloid plaques and neurofibrillary tangles, respectively [1]. However, several studies are confirming that a plethora of complex misfolded protein combinations can be found in the AD brains, besides the classical pathological hallmarks, i.e., the extracellular Aβ aggregates and the hyperphosphorylated tau protein deposits [2–8]. In

particular, recent studies have showed cerebral α-synuclein (α-syn) depositions in about 30% of AD cases [8, 9], and a higher α-syn concentration has been found in the cerebrospinal fluid (CSF) of AD patients compared to healthy controls (HC) [10–13] and in asymptomatic subjects at risk of AD [14], although so far, findings have been controversial concerning this aspect. α-Syn protein, that is the principal component of Lewy bodies (LB) in the synucleinopathies, such as Parkinson's disease [15–17], binds to presynaptic proteins potentially involved in the neurotransmitter release and protection of axonal terminals, suggesting its potential use as a proxy of synaptic disruption also in traditionally retained non-synucleinopathic NDs [18, 19], such as AD. Eventually, α-syn could be actively and early involved in the pathophysiological processes of the preclinical AD phases [14] and may promote the fibrillization of Aβ peptides and tau proteins suggesting a crucial role for α-syn toxicity in neurodegenerative mechanisms [19]. Several studies reported that α-syn is highly expressed in red blood cells (RBCs), too [20]. Additionally, RBCs may represent an interesting peripheral model of neurodegeneration since it is likely they are involved in the accumulation and clearance of the misfolded proteins [13, 21–23]. α-Syn has been demonstrated to physically interact with other proteins of neurodegeneration such as tau proteins [24, 25] and Aβ peptides [25, 26], producing hybrid oligomers (heteroaggregates) in RBCs of healthy subjects [27] and Parkinson's disease (PD) individuals [27].

Our objective is to assess the potential diagnostic accuracy of the levels of RBC α-syn and of heteroaggregates of α-syn with tau and Aβ in discriminating AD individuals showing both the typical hippocampal phenotype and the positivity of core AD pathophysiological biomarkers (i.e., low CSF Aβ peptides concentrations and high tau or pospho-tau (p-tau) protein levels or, alternatively, high cortical amyloid-PET uptake) [6] from cognitively HC.

Material and Methods

A total of 78 consecutive participants were examined. Clinical and biological data from these 78 individuals (Alzheimer disease dementia (ADD) patients = 28, prodromal AD or mild cognitive impairment due to AD subjects = 11, and healthy controls (HC) = 39) were collected in our Centre for Cognitive Disorders of the Neurology Clinic at the University of Pisa. Patients and HC were matched for age and sex. We initially enrolled 87 patients then we excluded from further analysis six clinically demented and three MCI subjects since during the diagnostic work-up, they did not show the CSF or amyloid PET hallmarks of AD pathophysiology. All participants underwent a clinical examination and an extensive cognitive evaluation with psychometric tests, including the Mini Mental State Examination (MMSE). All enrolled subjects had a clinical diagnosis of amnestic mild cognitive impairment (MCI) [28] or mild AD (hippocampal type) dementia [6] with a MMSE ≥ 20. They all performed a magnetic resonance imaging (MRI) scan to rule out secondary causes of dementia/cognitive impairment, and in particular, we excluded mixed cognitive impairment participants with a Fazekas modified scale score [29] (mFss) ≥ 2. All patients underwent either a diagnostic lumbar puncture (cutoff points of 600 pg/mL for Aβ, 275 pg/mL for tau, and 60 pg/mL for p-tau) [30], or a cerebral Aβ ^{18}F-florbetapir PET scan, or both of them. All patients included had received both clinical- and biomarker-based diagnosis of prodromal AD (or mild cognitive impairment due to AD) or ADD hippocampal type, according to the IWG-2 and NIAA criteria [6, 28].

RBC Collection

Whole blood was collected from AD patients and healthy volunteers into a tube containing EDTA as an anticoagulant. RBCs were separated from plasma by a centrifugation at 200×*g* at 4 °C for 10 min [27]; then, RBCs were centrifuged at 1000×*g* for 10 min, washed three times with PBS, and the RBC pellet was frozen at − 20 °C until use. The amount of proteins was determined by the Bradford method. For immunoenzymatic assays, RBCs were suspended in 2 mM SDS to a final concentration of 40 mg of total proteins in 100 μL.

Immunoassay Methods for Total α-Syn

Total α-syn was evaluated in RBCs following literature's protocols [27]. Briefly, wells were pre-coated overnight at 4 °C using a full-length antibody to α-syn (sc-10717, Santa Cruz Biotechnology), and bovine serum albumin (BSA) was used to block non-specific sites for 1 h at 37 °C [27]. RBCs (0.2 mg/100 μL) were added to the wells for 2 h at 25 °C. Purified recombinant protein standards of α-syn were analyzed in parallel with human samples to generate a standard curve. Samples were then probed with a mouse monoclonal antibody to α-syn (sc-12767, Santa Cruz Biotechnology) and subsequently with an anti-mouse HRP antibody. The wells were washed three times with phosphate-buffered saline containing 0.01% Tween 20 (PBS-T), and incubated with the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Thermo Scientific). Absorbance values were read at 450 nm.

Preparation of Aged Solutions of α-Syn and of the α-Syn Biotinylated Antibody

Recombinant α-syn was incubated in parafilm-sealed tubes at 37 °C for 4 days in an Eppendorf Thermomixer with constant mixing (1000 rpm), as reported previously [27, 31]. To prepare the α-syn biotinylated antibody, Sulfo-NHSLC-Biotin (Pierce, Rockford, IL, USA) (200 mg) was reacted with the 211 mouse monoclonal antibody (mAb) (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) [32]. The mixture was desalted on Bio-Spin-6 columns (Bio-Rad, UK) to eliminate excess uncoupled biotin.

Detection of Oligomeric α-Syn

Oligomeric α-syn levels in RBCs were quantified with an immunoenzymatic assay, as previously described [27, 31]. Wells were pre-coated overnight at room temperature with the mouse monoclonal α-syn 211 antibody (sc-12767, Santa Cruz Biotechnology). RBCs (0.04 mg/100 μL) were added to each well, after the blocking of non-specific sites with 1% BSA, and incubated at 25 °C for 2 h. α-Syn oligomers were identified with the α-syn biotinylated antibody, which binds amino acid residues 121–125 of human α-syn. Streptavidin horseradish peroxidase conjugate antibody (1:1000, GE Healthcare) was used for antigen detection. After several washing with PBS-T, 100 μL/well of the substrate TMB was added, as reported above.

Detection of Total Beta-Amyloid

Aβ levels in RBC samples were assessed using an immunoenzymatic assay, as described previously [27, 32]. A specific antibody to Aβ (ab10148, Abcam) was used to pre-coat the wells overnight at 4 °C. After extensive washing with PBS-T, non-specific sites were blocked with 1% BSA. RBCs (0.2 mg/100 mL) were loaded to each well and incubated at 25 °C for 1 h. After several washing with PBS-T, samples were probed using a specific antibody to Aβ (sc-28365, Santa Cruz Biotechnology). The standard curve was obtained using recombinant human Aβ solutions at eight different concentrations [27, 32].

Detection of Total Tau

Tau levels in RBCs were detected with the use of an immunoenzymatic assay, as described previously [27]. The plate was pre-coated overnight at 4 °C with a specific antibody to tau (sc-32274, Santa Cruz Biotechnology), and after washing, non-specific sites were blocked with 1% BSA. RBCs (0.5 mg/100 mL) were added to each well and incubated at 25 °C for 1 h. After extensive washing with PBS-T, samples were detected using a specific tau antibody (sc-5587, Santa Cruz Biotechnology). The standard curve was created using recombinant human tau solutions at eight different concentrations [27].

Immunoassay Detection of α-Syn/β-Amyloid Heterocomplexes

The quantification of α-syn/Aβ heterocomplexes was assessed using a "homemade" method employing a "sandwich" immunoenzymatic assay [27, 33, 34], as follows. Standards of α-syn/Aβ at eight different concentrations were prepared, following capturing on wells pre-treated with a specific antibody to Aβ (ab10148, Abcam) [27] in poli-L-ornithine/$NaHCO_3$, pH 9.6. After washing twice with PBS-T, RBCs (40 mg/sample in 2 mM SDS) were added to each well and incubated at 25 °C for 2 h. Non-specific sites were blocked with the addition of 1 % BSA for 30 min at 37 °C. To evaluate α-syn bound to Aβ, samples were probed with a specific antibody to α-syn (sc-12767, Santa Cruz Biotechnology) for 2 h at 37 °C, and next, with the appropriate HRP-conjugated antibody. After 1.5 h, and after two washes with PBS-T, the substrate TMB was added to each well. Absorbance was measured at 450 nm. Relative concentrations of α-syn/Aβ complexes were measured according to the standard curve created in each microplate. The assays of blood were all carried out in duplicate. Blood samples from patients and healthy subjects were analyzed together in batch runs. For some subjects, various assays were performed on diluted RBCs from a single subject to confirm that low or high concentrations were in the linear range of the assay. All measurements were repeated twice, and the average value was determined [27].

Immunoassay Detection of α-Syn/Tau Heterocomplexes

The quantification of α-syn/tau heterocomplexes was performed using a "homemade" method similar to the previously reported (see the precedent paragraph) [27]. Standard α-syn/tau was prepared by incubating 1 mg of each protein, diluted in 2 mM SDS in parafilm-sealed tubes at 37 °C for 1 h in an Eppendorf Thermomixer with constant mixing (500 rpm). The plate was pre-coated overnight at room temperature with a specific antibody to α-syn (sc-514908, Santa Cruz Biotechnology) in poli-L-ornithine/NaHCO3, pH 9.6. After two washes with PBS-T, RBCs (80 mg/sample in 2 mM SDS) were loaded to each well and incubated at 25 °C for 2 h. The wells were washed, and 1% BSA was used to block non-specific sites for 30 min at 37 °C. To determine α-syn bound to tau, samples were probed for 2 h at 37 °C with a specific antibody to tau (sc-5587, Santa Cruz Biotechnology) and subsequently with the appropriate HRP-conjugated antibody. After 1.5 h, the wells were washed twice with PBS-T before the addition of 100 μL/well of TMB. Absorbance was read at 450 nm. Relative concentration of α-syn/tau complexes was measured according to the standard curve obtained in each microplate.

Statistical Analysis

Prodromal AD and mild ADD subjects were merged and considered as a single diagnostic category named AD group [6]. Associations between sex and diagnostic group were assessed by a chi-square test with continuity correction. The differences

of the quantitative variable values between AD and HC groups were assessed through a nonparametric Mann-Whitney test after Shapiro-Wilk test for normality.

Then, the diagnostic potential of each biomarker was examined, calculating the area under the receiver operating characteristic curve (AUROC) and its associated confidence intervals (CIs). The Standards for Reporting Diagnostic Accuracy Studies (STARD) criteria for the reporting of diagnostic test accuracy studies (available at http://www.equator-network.org/reporting-guidelines/stard/) were followed. The discriminatory performance of each biomarker to correctly allocate individuals to diagnostic group was classified as follows: excellent (AUROC 0.90–1.00), good (AUROC 0.80–0.89), fair (AUROC 0.70–0.79), poor (AUROC 0.60–0.69), and fail/no discriminatory capacity (AUROC 0.50–0.59) [35]. Spearman's rank correlation coefficients were calculated to compare each RBC biomarker concentrations with those of the CSF Aβ, p-tau, and tau proteins. All statistical analyses were performed in the SPSS statistical environment version 18. Two-tailed p values, 0.05, were considered statistically significant.

Results

RBC Biomarkers Concentrations According to Clinical Diagnosis

Table 1 summarizes the concentrations of RBC Aβ peptide, α-syn, tau protein concentrations, and the RBC α-syn/Aβ and α-syn/tau heterodimers levels in the two groups. RBC α-syn, α-syn/Aβ, and α-syn/tau heterodimers concentrations were significantly lower in AD patients than HC ($p = 0.036$, 0.001, < 0.001, respectively). No other significant intergroup differences were found (Fig. 1).

Accuracy of RBC Biomarkers for Different Clinical Diagnosis

Figure 2 AUROCs summarize the accuracy of RBC biomarkers in distinguishing HC from AD. Particularly, the performance of α-syn/Aβ and α-syn/tau heterodimers in discriminating AD participants from HC was fair (AUROCs = 0.76, 0.72, respectively), whereas α-syn poorly differentiated the two groups (AUROC = 0.63). RBC Aβ and tau levels were unable to discriminate HC from AD

Correlations of RBC Biomarkers in the Whole Study Cohort

The correlation matrix among all biomarkers in the entire study population *(N = 78)* was reported in Table 2. The correlations between CSF core AD biomarkers (Aβ, p-tau, and tau) concentrations and the other biomarkers values have been showed in a subset of AD patients *(N = 32)* as well as the CSF α-syn levels and those of the other biomarkers in a further subgroup of AD patients *(N = 10)*. RBC α-syn strongly correlated with RBC α-syn/tau and with RBC tau ($\rho_s = 0.623$ and 0.567 $p < 0.001$, respectively). RBC α-syn/tau moderately correlated with RBC tau ($\rho_s = 0.031$ $p = 0.003$). RBC Aβ moderately correlated with RBC tau and RBC α-syn ($\rho_s =$ 0.329 and 0.031 $p = 0.003$, respectively). Moreover, RBC Aβ and RBC α-syn/Aβ heterocomplex moderately correlated with CSF Aβ ($\rho_s = 0.435$ and 0.368, respectively; $p = 0.015$ and 0.042) in a subset of 32 AD patients.

Discussion

Our results showed that α-syn concentrations, α-syn/Aβ as well as α-syn/tau heterodimers concentrations are significantly lower in RBCs of AD patients than in RBC of HC (Fig. 1).

Table 1 Demographic data and biomarker levels of the population

	HC	AD	p value
Sex, n (F/M)	39 (17/22)	39 (19/20)	0.650
Age at sample collection (years)	68.0 (60.0–72.0)	69.2 (63.3–72.7)	0.577
RBC Aβ	10.32 (6.79–14.41)	9.34 (4.65–24.15)	0.924
RBC tau	4.984 (1.908–8.475)	3.154 (1.549–6.881)	0.173
RBC α-syn	20.26 (3.63–52.14)	10.68 (4.12–18.09)	*0.036*
RBC o-α-syn	10.41 (6.94–13.00)	8.48 (2.95–13.91)	0.164
RBC α-syn-Aβ	2.730 (1.603–6.658)	1.473 (0.675–2.299)	*0.001*
RBC α-syn-tau	1.800 (0.632–2.611)	0.762 (0.475–1.062)	*< 0.001*

AD, Alzheimer disease; *HC*, healthy controls; *F*, female; *M*, male; *RBC*, red blood cell; *α-syn-Aβ*, α-synuclein/β-amyloid peptide(1–42) heterocomplexes; *α-syn-tau*, α-synuclein/tau protein heterocomplexes; *α-syn*, total α-synuclein; *o-α-syn*, oligomeric α-synuclein; *Aβ*, β-amyloid peptide(1–42); *tau*, total tau protein

Data are presented as median values (25th–75th quartiles)

p values < 0.05 were considered statistically significant (in italics)

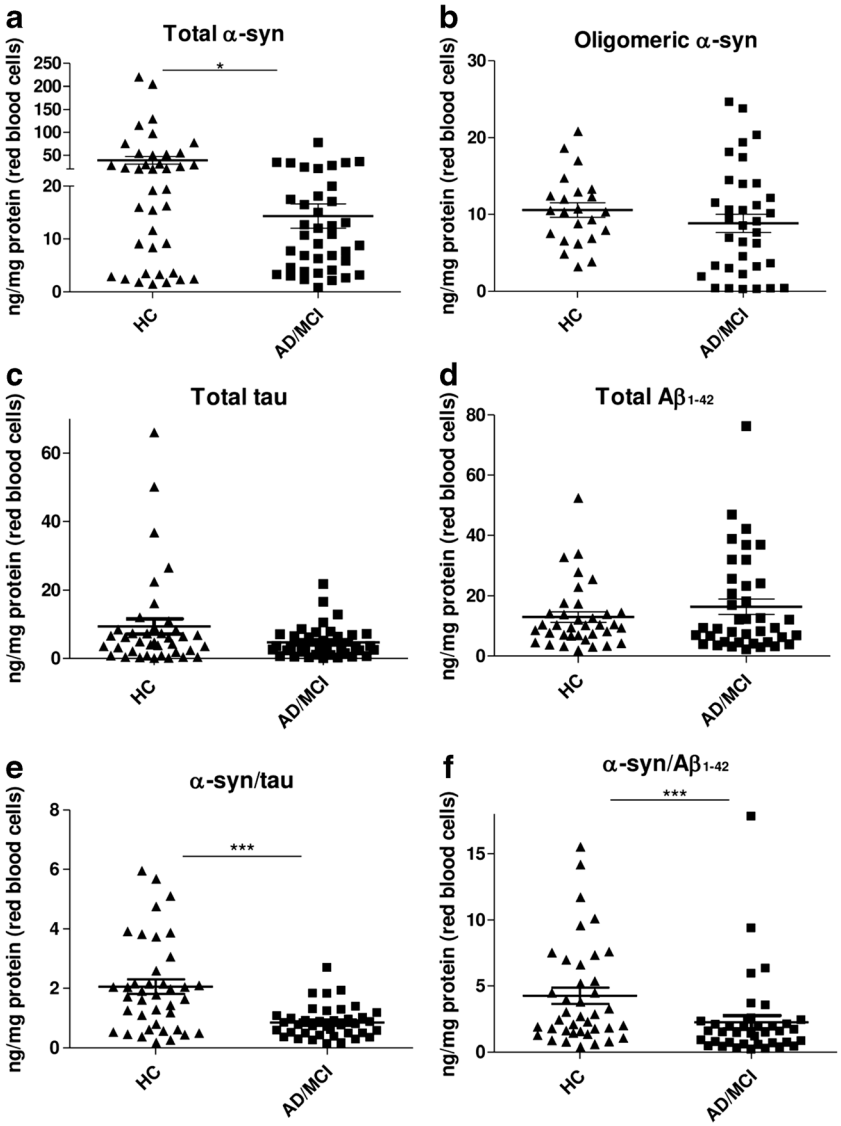

Fig. 1 Immunoassay determinations in RBCs of AD patients and HC. **a**–**e** RBC levels of total (**a**) and oligomeric (**b**) α-syn, total tau (**c**), $A\beta_{1–42}$ (**d**), α-syn-tau (**e**), α-syn-$A\beta_{1–42}$ (**f**) in the cohort of AD patients, and HC was quantified by specific immunoenzymatic assays, as described in the Methods section. The data are mean ± SD. Differences between the two groups were evaluated by a non-parametric analysis (Mann-Whitney): $*p < 0.05$, $***p < 0.001$ versus HC

RBC α-syn/Aβ as well as RBC α-syn/tau heterodimers discriminated AD participants from HC with fair accuracy whereas RBC α-syn concentrations differentiated poorly the two groups (Fig. 2).

Recent findings have suggested that each ND is not related to the brain deposition of a misfolded specific protein, but rather to a mixed pattern of misfolded proteins [36, 37]. Concomitantly, with their presence in the CNS, these altered proteins can be found also in peripheral compartments, possibly configuring a systemic disease [38]. In particular, RBCs seem to be prominently involved among peripheral cells, based on recent findings demonstrating an accumulation of misfolded proteins in these cells with a potential relationship with NDs [13, 21, 22, 27, 39, 40].

Herein, a cohort of AD patients with a biomarker-based diagnosis [6] was recruited to measure the RBC concentrations of α-syn and its heterocomplexes with tau and Aβ, to test their potential discriminatory accuracy and to demonstrate, in a subset sample, the putative correlation with the levels of the same proteins in CSF.

In the present study, comparable concentrations of Aβ were found in RBCs of HC and AD patients. In contrast, a recent paper, in which Aβ presence and aggregation in fibrils were assessed in RBCs through thioflavin T (ThT) staining, has reported that the percentage of Aβ binding-positive RBCs is higher in AD compared to HC [41]. However, in this study, the subjects had been diagnosed by purely clinical criteria only [42], which could not be sufficient to allow establishing a correct profile of the disease [1, 2, 6]. Furthermore, the quantification of Aβ ThT staining had been performed in RBCs only, without considering the corresponding levels in CSF (a key parameter to confirm the diagnosis of AD), not allowing a correlation between central and peripheral compartments. Finally, these discrepancies in the biochemical measurements may be attributed to the different methods employed to detect Aβ by products (i.e., ThT staining versus immunoenzymatic assay), which may mark different

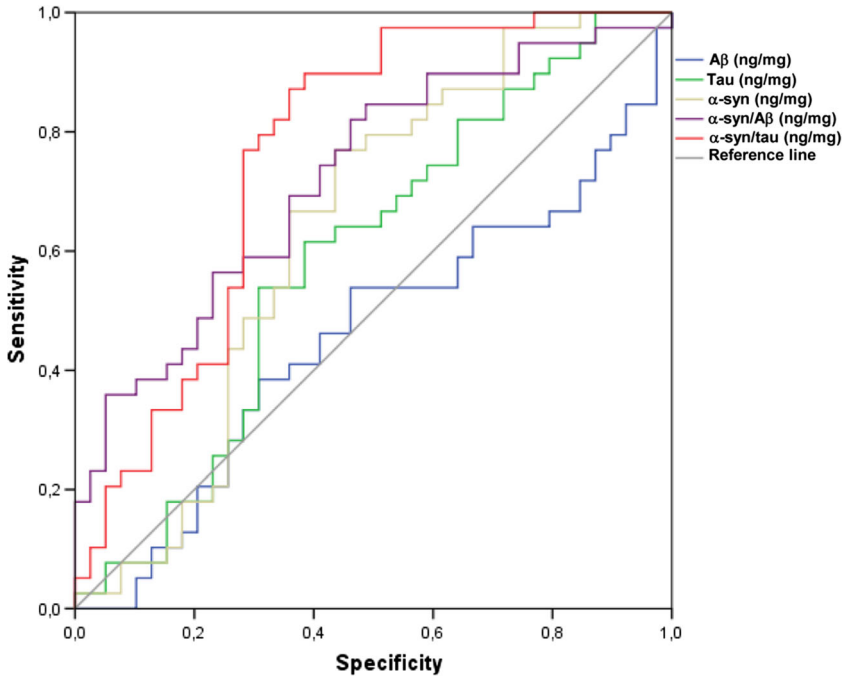

Fig. 2 ROC curves to evaluate the utility of RBC biomarkers concentrations in discriminating AD patients from HC. α-Syn-tau heterodimers AUROC = 0.76 (95% CI 0.65–0.87, $p < 0.001$); α-syn-$A\beta_{1–42}$ heterodimers AUROC = 0.72 (95% CI 0.61–0.84, $p = 0.001$); AUROC α-syn = 0.64 (95% CI 0.51–0.77, $p = 0.036$); AUROC tau = 0.59 (95% CI 0.46–0.72, $p < 0.173$); $A\beta_{1–42}$ AUROC = 0.46 (95% CI 0.33–0.59, $p < 0.50$)

states of protein aggregation. In this regard, we suggest that the total amount of Aβ peptides might be unchanged in RBCs of AD, but the degree of fibrils enhanced in pathological conditions [43, 44].

Interestingly, Aβ concentrations in RBCs correlated with CSF Aβ in a subset of 32 AD patients, suggesting that RBCs protein modifications may reflect central pathological alterations. In contrast, plasma Aβ concentrations have not shown neither significant differences between AD and HC, nor correlation with the levels of the same protein in CSF [45].

Similar to Aβ, the total amount of tau protein in RBCs was similar in AD and HC. Several recent studies on neurodegeneration-related diseases, including AD, showed higher tau levels in plasma of patients [46–49]. To our knowledge, very few studies have evaluated the RBC tau protein concentrations in NDs [27]. Further evaluations are needed to assess the validity of RBC tau as a biomarker of AD.

Interestingly, total α-syn levels in RBCs significantly decreased in our AD cohort. Nevertheless, ROC analysis demonstrated that total α-syn protein poorly differentiated the two groups (AUROC = 0.63). Consistently, with our finding in AD subjects, significant lower total α-syn concentrations have been also reported in human RBCs from PD patients [13, 20, 50] compared with age-matched HC. In this regard, these results suggest that the peripheral total α-syn, which is accumulated especially in RBCs [20], is possibly lower in general in NDs, but not specifically.

In addition to homoaggregates of the aforementioned proteins, heterocomplexes of α-syn with tau and Aβ have been proven to occur both in cellular models and in patients' brains

Table 2 Correlations between the analyzed biomarkers in the selected cohort

	RBC tau	RBC α-syn	RBC α-syn-Aβ	RBC α-syn-tau	CSF p-tau	CSF tau	CSF α-syn	CSF Aβ
RBC Aβ	0.329**	0.331**	−0.084	0.673	0.076	− 0.017	0.188	0.435**
RBC tau		0.567***	0.104	0.331**	0.085	− 0.018	−0.030	−0.009
RBC α-syn			−0.120	0.623***	0.176	0.191	0.127	0.050
RBC α-syn-Aβ				0.137	0.133	0.207	−0.600	0.368*
RBC α-syn-tau					0.288	0.151	−0.152	−0.175
CSF p-tau *(Pts = 32)*						0.682***	−0.361	0.033
CSF tau *(Pts = 32)*							−0.333	0.232
CSF α-syn *(Pts = 10)*								0.283

AD, Alzheimer disease; *HC*, healthy controls; *F*, female; *M*, male; *RBC*, red blood cells; *α-syn-Aβ*, α-synuclein/β-amyloid peptide(1–42) heterocomplexes; *α-syn-tau*, α-synuclein/tau protein heterocomplexes; *α-syn*, total α-synuclein; *o-α-syn*, oligomeric α-synuclein; *Aβ*, β-amyloid peptide(1–42); *tau*, total tau protein. Data are presented as median values (25th–75th quartiles)

p values < 0.05 were considered statistically significant

[24–26, 37, 50, 51]. On this basis, α-syn heterocomplexes were measured in our cohort through a "homemade" immunoenzymatic assays. Notably, α-syn has been demonstrated to form heterocomplexes with both Aβ and tau proteins in brain tissues and in RBCs of senescence-accelerated mice, similar with previous data reported in human samples [27, 37, 50, 52]. Moreover, several evidences have reported a very likely contribute of α-syn combining with other misfolded proteins, such as tau, thus enhancing the neurodegenerative effects [36, 37].

Herein, both α-syn/Aβ and α-syn/tau concentrations in RBCs were significantly lower in AD patients than HC. Moreover, both heterodimers were fairly performing in discriminating AD participants from HC (AUROCs = 0.76, 0.72, respectively). In fact, α-syn/Aβ heterocomplexes correlated with CSF Aβ in a subset of our AD cohort (Table 2). Further, RBC α-syn heterocomplexes correlated with the Aβ and tau concentrations (Table 2). Thus, these data suggest that α-syn heterocomplexes in blood may reflect neurodegenerative mechanisms within the central nervous system.

It is worth noting that we recently have reported that RBC α-syn/Aβ heterocomplexes in PD patients were higher than in controls and correlated with the disease's progression [50]. Thus, α-syn/Aβ accumulation in RBCs seems to have different trend in PD versus AD.

Our study has some caveats. First, the relatively small sample number and the lack of a clinical follow-up. Then, we did not include atypical AD phenotype. Moreover, we omitted in our analysis a further group of patients that received a non-AD cognitive impairment diagnosis. However, all our patients received a gold standard biomarker-based diagnosis of AD and matched for age and sex with the control group.

In summary, we suggest that the RBC concentrations of α-syn heterocomplexes may be lower in the early AD stages, showing a fair discriminating accuracy compared with controls. Moreover, we guess that since RBCs are rich of peripheral α-syn proteins and are easily accessible with a simple blood sample, they could become, in the near future, an interesting peripheral model of neurodegenerative mechanisms.

Authors' Contributions FB, SD, MLT, GT, UB, and CM were involved with the conception, design, and interpretation of data. FB, SD, RP, LG, DP, ALG, LP, and ED performed the experiments. FB, SD, RP, LG, DP, MLT, and UB were involved with data analysis. FB and LG collected the clinical material. CM, MLT, GS, GT, and UB provided general overall supervision of the study, and acquired funding. All authors contributed to the drafting and critical revision of the manuscript and have given final approval of the version to be published.

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Compliance with Ethical Standards

Study procedures were approved by our local board and were in accordance with the provisions of the Declaration of Helsinki. All participants or their representatives gave written informed consent for the use of their clinical data for research purposes.

Conflict of Interest The authors declare that they have no conflict of interest.

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References

1. Scheltens P, Blennow K, Breteler MM, de Strooper B, Frisoni GB, Salloway S, Van der Flier WM (2016) Alzheimer's disease. Lancet 388:505–517
2. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Kawas CH, Klunk WE, Koroshetz WJ et al (2011) The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:263–269
3. Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C et al (2012) National Institute on Aging-Alzheimer's association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement 8:1–13
4. Kovacs GG, Milenkovic I, Wöhrer A, Höftberger R, Gelpi E, Haberler C, Höftberger S, Reiner-Concin A et al (2013) Non-Alzheimer neurodegenerative pathologies and their combinations are more frequent than commonly believed in the elderly brain: a community-based autopsy series. Acta Neuropathol 126:365–384
5. Warren JD, Rohrer JD, Schott JM, Fox NC, Hardy J, Rossor MN (2013) Molecular nexopathies: a new paradigm of neurodegenerative disease. Trends Neurosci 36:561–569
6. Dubois B, Feldman HH, Jacova C, Hampel H, Molinuevo JL, Blennow K, DeKosky ST, Gauthier S et al (2014) Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria. Lancet Neurol 13:614–629
7. Kovacs GG (2016) Molecular pathological classification of neurodegenerative diseases: turning towards precision medicine. Int J Mol Sci 17. https://doi.org/10.3390/ijms1702089
8. Rabinovici GD, Carrillo MC, Forman M, DeSanti S, Miller DS, Kozauer N, Petersen RC, Randolph C et al (2017) Multiple comorbid neuropathologies in the setting of Alzheimer's disease neuropathology and implications for drug development. Alzheimers Dement 3:83–91
9. Tofaris GK, Buckley NJ (2018) Convergent molecular defects underpin diverse neurodegenerative diseases. J Neurol Neurosurg Psychiatry 89:962–969
10. Mattsson N, Insel P, Tosun D, Zhang J, Jack CR Jr, Galasko D, Weiner M (2013) Effects of baseline CSF α-synuclein on regional brain atrophy rates in healthy elders, mild cognitive impairment and Alzheimer's disease. PLoS One. https://doi.org/10.1371/journal.pone.0085443
11. Hansson O, Hall S, Ohrfelt A, Zetterberg H, Blennow K, Minthon L, Nagga K, Londos E et al (2014) Levels of cerebrospinal fluid α-synuclein oligomers are increased in Parkinson's disease with dementia and dementia with Lewy bodies compared to Alzheimer's disease. Alzheimers Res Ther 6:25
12. Slaets S, Vanmechelen E, Le Bastard N, Decraemer H, Vandijck M, Martin J-J, De Deyn PP, Engelborghs S (2014) Increased CSF α-synuclein levels in Alzheimer's disease: correlation with tau levels. Alzheimers Dement 10:S290–S298
13. Wang Z-Y, Han Z-M, Liu Q-F, Tang W, Ye K, Yao Y-Y (2015) Use of CSF α-synuclein in the differential diagnosis between

Alzheimer's disease and other neurodegenerative disorders. Int Psychogeriatr 27:1429–1438
14. Vergallo A, Bun R-S, Toschi N, Baldacci F, Zetterberg H, Blennow K, Cavedo E, Lamari F et al (2018) Association of cerebrospinal fluid α-synuclein with total and phospho-tau181 protein concentrations and brain amyloid load in cognitively normal subjective memory complainers stratified by Alzheimer's disease biomarkers. Alzheimers Dement S1552-5260:33241–33242
15. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388: 839–840
16. Dickson DW (2012) Parkinson's disease and parkinsonism: Neuropathology. Cold Spring Harb Perspect Med. https://doi.org/10.1101/cshperspect.a009258
17. Miraglia F, Betti L, Palego L, Giannaccini G (2015) Parkinson's disease and alpha-synucleinopathies: from arising pathways to therapeutic challenge. Cent Nerv Syst Agents Med Chem 15:109–116
18. Fang F, Yang W, Florio JB, Rockenstein E, Spencer B, Orain XM, Dong SX, Li H et al (2017) Synuclein impairs trafficking and signaling of BDNF in a mouse model of Parkinson's disease. Sci Rep 7:3868
19. Wong YC, Krainc D (2017) α-Synuclein toxicity in neurodegeneration: mechanism and therapeutic strategies. Nat Med 23:1–13
20. Barbour R, Kling K, Anderson JP, Banducci K, Cole T, Diep L, Fox M, Goldstein JM et al (2008) Red blood cells are the major source of alpha-synuclein in blood. Neurodegener Dis 5:55–59
21. Eisele YS, Obermüller U, Heilbronner G, Baumann F, Kaeser SA, Wolburg H, Walker LC, Staufenbiel M et al (2010) Peripherally applied Aβ-containing inoculates induce cerebral beta-amyloidosis. Science 330:980–982
22. Pandey KB, Rizvi SI (2010) Markers of oxidative stress in erythrocytes and plasma during aging in humans. Oxidative Med Cell Longev 3:2–12
23. Brubaker WD, Crane A, Johansson JU, Yen K, Garfinkel K, Mastroeni D, Asok P, Bradt B et al (2017) Peripheral complement interactions with amyloid β peptide: erythrocyte clearance mechanisms. Alzheimers Dement 13:1397–1409
24. Sengupta U, Guerrero-Muñoz MJ, Castillo-Carranza DL, Lasagna-Reeves CA, Gerson JE, Paulucci-Holthauzen AA, Krishnamurthy S, Farhed M et al (2015) Pathological interface between oligomeric alpha-synuclein and tau in synucleinopathies. Biol Psychiatry 78: 672–683
25. Andersen AD, Binzer M, Stenager E, Gramsbergen JB (2017) Cerebrospinal fluid biomarkers for Parkinson's disease - a systematic review. Acta Neurol Scand 135:34–56
26. Parnetti L, Castrioto A, Chiasserini D, Persichetti E, Tambasco N, El-Agnaf O, Calabresi P (2013) Cerebrospinal fluid biomarkers in Parkinson disease. Nat Rev Neurol 9:131–140
27. Daniele S, Pietrobono D, Fusi J, Iofrida C, Chico L, Petrozzi L, Gerfo AL, Baldacci F et al (2018) α-Synuclein aggregates with β-amyloid or tau in human red blood cells: correlation with antioxidant capability and physical exercise in human healthy subjects. Mol Neurobiol 55:2653–2675
28. Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM et al (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:270–279
29. Wahlund LO, Barkhof F, Fazekas F, Bronge L, Augustin M, Sjögren M, Wallin A, Ader H et al (2001) A new rating scale for age-related white matter changes applicable to MRI and CT. Stroke 32:1318–1322
30. Vergallo A, Carlesi C, Pagni C, Giorgi FS, Baldacci F, Petrozzi L, Ceravolo R, Tognoni G et al (2017) A single center study: Aβ42/p-Tau181 CSF ratio to discriminate AD from FTD in clinical setting. Neurol Sci 38:1791–1797
31. El-Agnaf OMA, Salem SA, Paleologou KE, Curran MD, Gibson MJ, Court JA, Schlossmacher MG, Allsop D (2006) Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. FASEB J 20:419–425
32. Pesini P, Pérez-Grijalba V, Monleón I, Boada M, Tárraga L, Martínez-Lage P, San-José I, Sarasa M (2012) Reliable measurements of the β-amyloid pool in blood could help in the early diagnosis of AD. Int J Alzheimers Dis 2012:1–10. https://doi.org/10.1155/2012/604141
33. Daniele S, Taliani S, Da Pozzo E, Giacomelli C, Costa B, Trincavelli ML, Rossi L, La Pietra V et al (2014) Apoptosis therapy in cancer: the first single-molecule co-activating p53 and the translocator protein in glioblastoma. Sci Rep 4:47–49
34. Zappelli E, Daniele S, Abbracchio MP, Martini C, Trincavelli ML (2014) A rapid and efficient immunoenzymatic assay to detect receptor protein interactions: G protein-coupled receptors. Int J Mol Sci 15:6252–6264
35. Xia J, Broadhurst DI, Wilson M, Wishart DS (2013) Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 9:280–299
36. Baldacci F, Lista S, Garaci F, Bonuccelli U, Toschi N, Hampel H (2016) Biomarker-guided classification scheme of neurodegenerative diseases. J Sport Health Sci 5:383–387
37. Giacomelli C, Daniele S, Martini C (2017) Potential biomarkers and novel pharmacological targets in protein aggregation-related neurodegenerative diseases. Biochem Pharmacol 131:1–15
38. Wang J, Gu BJ, Masters CL, Wang Y-J (2017) A systemic view of Alzheimer disease - insights from amyloid-β metabolism beyond the brain. Nat Rev Neurol 13:612–623
39. Kiko T, Nakagawa K, Satoh A, Tsuduki T, Furukawa K, Arai H, Miyazawa T (2012) Amyloid β levels in human red blood cells. PLoS One 7:e49620. https://doi.org/10.1371/journal.pone.0049620
40. Brown BM, Peiffer JJ, Taddei K, Lui JK, Laws SM, Gupta VB, Taddei T, Ward VK et al (2013) Physical activity and amyloid-β plasma and brain levels: results from the Australian imaging, biomarkers and lifestyle study of ageing. Mol Psychiatry 18:875–881
41. Lan J, Liu J, Zhao Z, Xue R, Zhang N, Zhang P, Zhao P, Zheng F et al (2015) The peripheral blood of Aβ binding RBC as a biomarker for diagnosis of Alzheimer's disease. Age Ageing 44:458–464
42. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 34:939–944
43. Hanon O, Vidal J-S, Lehmann S, Bombois S, Allinquant B, Tréluyer J-M, Gelé P, Delmaire C et al (2018) Plasma amyloid levels within the Alzheimer's process and correlations with central biomarkers. Alzheimers Dement 14:858–868
44. Verberk IMW, Slot RE, Verfaillie SCJ, Heijst H, Prins ND, van Berckel BNM, Scheltens P, Teunissen CE et al (2018) Plasma amyloid as prescreener for the earliest Alzheimer pathological changes. Ann Neurol 84:648–658. https://doi.org/10.1002/ana.25334
45. Mehta PD, Pirttilä T, Mehta SP, Sersen EA, Aisen PS, Wisniewski HM (2000) Plasma and cerebrospinal fluid levels of amyloid beta proteins 1-40 and 1-42 in Alzheimer disease. Arch Neurol 57:100–105
46. Chiu MJ, Yang SY, Chen TF, Chieh JJ, Huang TZ, Yip PK, Yang HC, Cheng TW et al (2012) New assay for old markers-plasma beta amyloid of mild cognitive impairment and Alzheimer's disease. Curr Alzheimer Res 9:1142–1148
47. Chiu MJ, Yang SY, Horng HE, Yang CC, Chen TF, Chieh JJ, Chen HH, Chen TC et al (2013) Combined plasma biomarkers for diagnosing mild cognition impairment and Alzheimer's disease. ACS Chem Neurosci 4:1530–1536

48. Tzen KY, Yang SY, Chen TF, Cheng TW, Horng HE, Wen HP, Huang YY, Shiue CY et al (2014) Plasma Aβ but not tau is related to brain PiB retention in early Alzheimer's disease. ACS Chem Neurosci 5:830–836
49. Mattsson N, Zetterberg H, Janelidze S, Insel PS, Andreasson U, Stomrud E, Palmqvist S, Baker D et al (2016) Plasma tau in Alzheimer disease. Neurology 87:1827–1835
50. Daniele S, Frosini D, Pietrobono D, Petrozzi L, Lo Gerfo A, Baldacci F, Fusi J, Giacomelli C et al (2018) α-Synuclein heterocomplexes with β-amyloid are increased in red blood cells of Parkinson's disease patients and correlate with disease severity. Front Mol Neurosci. https://doi.org/10.3389/fnmol.2018.00053
51. Iofrida C, Daniele S, Pietrobono D, Fusi J, Galetta F, Trincavelli ML, Bonuccelli U, Franzoni F et al (2017) Influence of physical exercise on β-amyloid, α-synuclein and tau accumulation: an in vitro model of oxidative stress in human red blood cells. Arch Ital Biol 155:33–42
52. Daniele S, Pietrobono D, Fusi J, Lo Gerfo A, Cerri E, Chico L, Iofrida C, Petrozzi L et al (2018) α-Synuclein aggregated with tau and β-amyloid in human platelets from healthy subjects: Correlation with Physical Exercise. Front Aging Neurosci. https://doi.org/10.3389/fnagi.2018.00017