Erythrocytes as biomarkers for dementia: Analysis of protein content and Alpha synuclein

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Abstract:

***Background*:** Discovering biomarkers for dementia is a pivotal step towards successful early diagnosis and treatment. Although plasma biomarkers have been explored, no consensus has been reached. Alpha-synuclein (AS), a 14kDa synaptic protein associated with several neurodegenerative diseases, exists natively within erythrocytes (ERC). This protein is characteristic of Lewy Body diseases, in which it aggregates into toxic Lewy bodies. As erythrocytes are implicated in dementia, they are a potential target for future biomarkers.

Aims: The aims of this study were to assess AS levels within ERC and whether AS can be used as a peripheral biomarker to differentiate between dementia and aged matched healthy control subjects.

Methods: A total of 114 samples (60 ageing controls, 36 Alzheimer's disease, 12 vascular dementia and 6 Dementia with Lewy Bodies subjects) were analysed. We used: Bradford assay to measure protein concentration, indirect ELISA to detect levels of AS and Immunoblotting to identify AS composition.

***Results*:** AS oligomers were present in dementia blood samples, whereas in controls, AS was largely monomeric. There was a significant increase in AS levels in DLB whole bloods ($p=0.005$), with a sensitivity and specificity of 100.0% and 93.9%. Protein concentrations in pH 5.7 ERC fractions were significantly increased in dementia patients compared to controls (17.58 vs. 40.33 μg/ml; $p=<0.005$). In the VaD group, the protein concentration in the pH5.7 ERC fraction had sensitivity and specificity of 91.7% and 62.1%.

Conclusions: Protein concentration and AS levels have a potential for development of a novel diagnostic dementia blood test.

Key words: Alpha synuclein, erythrocytes, blood, dementia, biomarker, Alzheimer's disease, Vascular dementia, Dementia with Lewy Body.

Introduction

Alpha-synuclein (AS) has emerged as one of the major neurobiological players in Parkinson's Disease (PD) pathogenesis, with its' aggregation in the form of misfolding, oligomerization and fibrillization extensively studied in PD and other synucleinopathies (reviewed in Wales et al, 2013, Lee et al, 2014). However, AS is also a widely distributed synaptic protein in the human brain, and found in various non-neuronal tissues, including muscle, liver, testis, blood vessels), and blood cells and derivates [e.g. plasma, serum, platelets, lymphocytes and erythrocytes (ERC)] (Nakai et al, 2007, Mukaetova-Ladinska et al, 2012), and skin fibroblasts (Hoepken et al, 2008). In contrast to the negligible amount of beta and gamma synuclein (Nakai et al, 2007), the ERC contain the highest amount of AS detected in the periphery (Barbour et al, 2008, De Palma et al, 2012). This makes ERC a good substrate to determine the properties of this synaptic protein in both ageing and distinct forms of dementia, with a potential to monitor these changes with the progression of dementia.

In our previous biochemical and neuropathological studies, we have shown that AS undergoes similar changes like other synaptic proteins, including synaptophysin, SNAP-25 and syntaxin, with a transient up-regulation in the pre-clinical stages of dementia (Mukaetova-Ladinska et al, 2000a), and is incorporated into proteolysis stable intraneuronal deposits in the intermediate stages of Alzheimer's disease (AD) pathology (Mukaetova-Ladinska et al, 2000b) prior the generalised synaptic loss occurring in end stages dementia (Mukaetova-Ladinska et al, 2000a). These findings were recently confirmed in our AS study using platelets and plasma (Mukaetova-Ladinska et al, 2012), where the increase in platelet AS was associated with impairment in attention, calculation, abstract thinking, depression and apathy, all associated with the early changes in the dementia clinical phenotype.

Since ERC contain about 95% of the AS found in the periphery (Barbour et al, 2008), the question arises whether these changes we have observed in platelets and in the brain tissue can be repeated using the AS derived from ERC. Argyriou et al (2012) found increased dimerization of ERC AS in people with Gaucher disease, a rare autosomal recessive disorder characterised by a deficiency of the lysosomal enzyme cerebrosidase, and parkinsonism. Argyriou et al (2012) also suggested that the increased AS dimer to monomer ratio in ERC may be a potential biomarker for Parkinson's disease (PD) risk. These findings have been now confirmed in sporadic and genetic forms harbouring the GBA mutations, but not the SNCA (synuclein alpha gene) mutations in PD subjects (Papagiannakis et al, 2018). In addition, AS posttranslational modification also appear to be distinctly affected in the PD red blood cells (Vicente Miranda et al, 2018). Two most recent study expanded on these findings, reporting the alpha-synuclein to ERC protein ratio to discriminate well between the parkinsonian clinical syndromes (i.e. PD and multiple system atrophy; MSA) from normal ageing (Wang et al, 2015, Liu et al, 2018). These studies clearly indicate that ERC derived AS can not only be measured but may also have a clinical utility for diagnosis of PD and related clinical syndromes.

In this study, we describe a protocol for extracting ERC-AS that can be used to measure AS in ERC. We report the presence of AS polymers in erythrocytes and whole bloods in subjects with dementia, and propose the clinical relevance of AS content in bloods

Material and methods

Participants' demographic and clinical data

The study was conducted on blood samples obtained from a total number of 114 subjects and included: 60 ageing control participants and 54 people with dementia [AD, n=36), Vascular dementia (VaD), n=12 and Dementia with Lewy Body (DLB), n=6]. We obtained demographic (gender and age) and extensive clinical data for all subjects enrolled in the study. Thus, both control and dementia subjects underwent cognitive assessment using the CAMCOG (Cambridge Cognitive Examination) and MMSE (Mini Mental State Examination), whereas dementia patients had additional assessments for their behaviour, which included NPI (Neuropsychiatric Inventory) and Cornell (Depression Scale) (Mukaetova-Ladinska et al, 2012) (Table 1).

The analysed groups differed in respect to age, gender and cognitive status. The VaD and DLB participants were older than control and AD subjects ($p=0.004$) with more males in the AD group ($p=0.010$). Similarly, the VaD and DLB subjects had more pronounced cognitive impairment compared to control and AD subjects ($p<0.005$). In contrast, the dementia participants had equal extent of behavioural and mood problems, as measured with the NPI and Cornell depression rating scale, respectively (table 1).

Blood Samples

Blood samples were obtained following the clinical assessments and they were further processed to obtain 6 blood fractions from each blood sample: whole bloods (WB), ERC and ERC fraction, obtained at pH 5.7 as product of the platelet protocol, as described previously (Mukaetova-Ladinska et al, 2012), as well as their respective heat stable fractions.

Whole Bloods: Whole blood samples were collected from the patient and 1ml sample was immediately frozen and stored at -8°C.

Untreated ERC Fractions: 1ml of whole blood sample was subjected to centrifugation for 20min at 13,000rpm at room temperature. The supernatant consisting of plasma was then removed leaving behind the ERC fraction which was then stored at -8°C.

pH 5.7 ERC Fractions**:** The pH 5.7 ERC fraction was collected as a by-product of our platelet protocol (Mukaetova-Ladinska et al, 2012). Briefly, the whole blood samples were diluted in citric buffer (pH 5.7) by using a 1:5 ratio and centrifuged at 1000 rpm for 20 minutes at 18°C. The pellet, containing ERCs, was collected and frozen at -8°C.

Heat Stable ERC fractions: To obtain the heat stable fractions, whole blood and ERC samples were heated at 200°C for 5 minutes and centrifuged at 13,000 rpm for 15 minutes. The supernatant, consisting of the heat stable proteins, including native AS, was then collected and stored at -8°C, whilst the pellet, containing precipitated protein, was discarded.

Protein Concentration

The protein concentration for each analysed blood fraction was established using the Bradford Assay. A standard curve was produced using standard concentrations of bovine albumin serum (BSA), ranging from 0 μg/ml to 25 μg/ml. Untreated whole blood and ERC samples were diluted 1:1000 whereas the heat stable samples were diluted 1:150. The Coomassie protein assay solution, (Coomassie Plus- The Better Bradford protein assay, Thermo Scientific, Loughborough, UK), was then added to each of the wells and incubated on a shaker at room temperature for 10 minutes. OMEGA software was used to determine the protein concentrations at 595.

SDS-PAGE and Immunoblotting

Loading samples were prepared by mixing ERC preparations (40microgram/ml, determined using the Coomassie Plus-The Better Bradford protein assay, Thermo Scientific, Loughborough, UK, as per manufacturer's instructions) with 2x SDS loading buffer [0.125M

Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M dithiothreitol (DTT), 0.02% bromophenol blue] to give the final protein concentration of 20microgram/ml and boiled for 5mins at 95°C. SDS-PAGE was carried out using 17% acrylamide gels (OmniPAGE Mini Wide electroblotting module, SLS, UK), containing electrophoresis buffer (0.025M Tris, 0.192 M glycine, 0.1% w/v SDS] and ran at 100V for 1 hour at room temperature (RT). Gels were then immediately electroblotted using the Trans-Blot® Cell system (Bio-Rad), onto nitrocellulose membrane (pore size 0.45μm, Thermo Scientific Pierce, UK) while immersed in transfer buffer [0.4M glycine, 0.025M Tris, 5% w/v isopropanol] at a constant current of 350mA and 16°C for 2 hours.

Membranes were first blocked in blocking buffer consisting of 5% BSA in Tris-Buffered Saline and Tween-20 [TBST, 20mM Tris HCl pH 7.5, 150mM NaCl, 0.1% (w/v) Tween 20] for 1.5 hours at RT, then incubated with a primary antibody against the C-terminal end of alpha-synuclein {pAb α-synuclein [(C-20)-R; SANTA CRUZ Biotechnology, INC] diluted 1:100 in blocking buffer. The incubation with the primary antibody was carried overnight at 4°C.

The following day, membranes were washed thrice with TBST, each time for 10 minutes, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, anti-rabbit-HRP P0448, DAKO Cytomation, Gostrup, Denmark) at RT for 1.5 hours diluted in TBST. The washing procedure was then repeated as above before products were visualised using a chemiluminescent kit (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The intensity of alpha synuclein products was visualised with a digital imager (ImageQuant LAS 4000, GE Healthcare, Buckinghamshire, UK), and the exposure was standardised to 30 seconds.

ELISA alpha-synuclein immunoassay

The level of ERC alpha-synuclein was measured with an indirect ELISA immunoassays, using the same commercial immunoprobe against the C-terminal end of alpha-synuclein {pAb α-synuclein [(C-20)-R; SANTA CRUZ Biotechnology, INC] as per the SDS-PAGE, but used in 1:5000 dilution (Mukaetova-Ladinska et al, 2012). Briefly, triplicates of double dilutions of the antigen (ERC; 13.5 microgram/ml); 1:20 dilution for the untreated blood samples and 1:100 dilution for the heat stable samples, which were diluted in coating buffer (carbonate-bicarbonate) over 12 wells were coated over night at 4ºC using carbonate-bicarbonate buffer, and washed in 0.05% Tween, blocked with 1% dried skimmed milk and incubated for 1h at 37ºC. Plates were washed and incubated for 1h at 37ºC with the primary antibody diluted in 0.05% Tween in phosphate buffer solution (PBS, pH 6.8). Following another wash in 0.05% Tween in PBS, plates were incubated with the secondary antibody conjugated to horseradish peroxidase (HRP) and incubated for 1h at 37ºC. Colorimetric analysis of reaction with 3,3',5,5'- trimethylbenzidine (TMB; Sigma) was performed, and reaction quenched after 10min with 2N H_2SO_4. Plates were read with Vmax plate-reader (Molecular Devices, Sunnyvale, California), and assay curves plotted using SOFTmaxPro (Version 4.7.1, molecular Devices, Sunnyvale, California). All values were expressed as relative arbitrary unites of immunoreactivity similar to previous reports (Mukaetova-Ladinska et al, 2012).

Statistical Analysis

The data were analysed using SPSS v.21. Normality of data was established with the Kolmogorov-Smirnov Test. Since data were non-parametrically distributed we used non parametric analysis (Kruskal-Wallis test) to establish differences between dementia and control subjects, and mean rank values are presented. Sensitivity and specificity of protein

concentration and AS measures was established with Receiver Operating Characteristics (ROC) curve, whereas the relationship between clinical measures of cognitive and behavioural changes and biochemical measures with regression analysis, Statistical significance was set at $p \leq 0.05$.

Results:

AS detection in ERC and bloods

AS was detected in all analysed groups at 14 kDa, predominantly in the monomeric form. The 14kDa band was strongly represented in all heat stable (Figure 1; lanes 1-3) compared to the untreated samples (Figure 1; lanes 4-6). In AD patients, less immunoreactive AS positive bands were also seen at approximately 28 kDa, which were not observed in the controls. Another band present in all fractions of AD samples was noted at 42 kDa, which was absent in the controls except from the untreated ERC fraction. There was a further band at approximately 56 kDa, which appeared in untreated ERC samples of both AD and control subjects (Figure 1).

DLB and VaD subjects exhibited similar results to AD. Firstly, bands at 14 kDa were observed in all samples of both DLB and VaD, where a greater intensity of the bands was seen in the heat stable preparations (figure 1). Bands at approximately 28 kDa and 42 kDa were also noted in DLB and VaD cases. However, they were absent in pH5.7 ERC preparations (lanes 2 and 2'; Figure 1). The ~56 kDa were bands observed in all untreated but not in the heat stable preparations. Unlike AD and control subjects, these were seen in all three fractions of VaD samples (lanes 4, 5 and 6) and WB and ERC preparations in DLB (Figure 1).

Protein Concentration Analysis

The differences in protein concentration were largely seen within the ERC and pH 5.7 ERC fractions where most increases were evident in VaD and DLB dementia subjects. In untreated WB samples, the protein concentrations are similar for all cases irrespectively of their cognitive status (Table 2). However, in the ERC untreated fractions, the VaD group had significantly elevated protein concentration compared to their control, AD and DLB counterparts ($p< 0.001$). The heat stable ERC fractions had a similar level of protein concentration between control and AD subjects. In the pH 5.7 fractions there was an increase in protein concentrations across the three sub-types of dementia, compared to control subjects ($p < 0.001$), markedly so in the VaD samples which were increased by a factor of 2.5. The protein concentrations of the heat stable pH 5.7 ERC were similar in all analysed groups (Table 2). The protein concentration of 14.1 μg/ml in the pH 5.7 untreated samples showed a significant sensitivity of 91.7% and a specificity of 62.1% to discriminate VaD patients from control subjects ($p = <0.001$) (Figure 2a).

DLB subjects had significantly lower protein levels in their heat stable preparations derived from whole bloods compared to the control, AD and VaD subjects ($p= 0.02$) (Table 2). However, the ROC curve analysis showed low sensitivity and specificity (Figure 2b). We also performed correlation analysis to determine the relationship between the protein concentrations detected in the untreated and heat-treated blood samples. It is of note that there is an inverse relationship between the heat stable and the untreated samples in the pH 5.7 samples (r -0.353, $p=0.01$). This occurs in the dementia subjects but not in controls, showing that increase in protein concentration in the dementia group was not caused by a heat stable protein. This rules out the possibility that the increase in proteins was due to the increase in AS, a heat stable synaptic protein.

Analysis of AS levels in bloods fractions

The untreated whole blood samples had a significant increase in AS content in the DLB subjects compared to controls, AD and VaD participants ($p = 0.024$). This increase in AS in the DLB subjects showed a high sensitivity (100%) and specificity (93.9%) to discriminate DLB patients from controls, with a RV of 192.4. (Figure 2b)

In the whole blood heat stable samples, the VaD subjects had significantly elevated AS levels ($p=0.038$), and similar, but not statistically significant increase was also seen in the DLB subjects (Table 3).

ROC curve analysis for the VaD group in the whole blood heat stable samples showed a sensitivity and specificity of 75% and 57.4%. ($p=0.038$; figure 2c). Similar high levels of sensitivity and specificity were obtained for the DLB subjects when whole blood heat stable fractions were analysed, but this did not reach statistical significance when compared to control subjects (figure 2b).

Protein Concentration and AS content Analysis

Analysis between the protein concentrations and the AS content data was carried out to identify relationships between the two and, in particular, if AS could be responsible for any changes occurring in the protein concentration.

The heat stable protein concentrations and heat stable AS levels displayed two significant correlations. The first was seen in the ERC heat stable fractions, (for controls and all dementia subtypes), which exhibited a positive correlation with a coefficient value of 0.349 between the two sample groups therefore indicating that as the protein concentration increased there was also an increase in the AS content. ($p=0.040$) The second significant correlation of 0.296 was observed in the pH 5.7 ERC fractions again suggesting that as the protein concentration increases the AS content increases. ($p=0.037$) Collectively, findings

from this analysis showed that the AS levels were elevated in the ERC fractions when protein concentration increased (Table 4).

The untreated protein concentrations and the untreated AS levels showed different results to the heat stable correlations. The only significant correlation displayed for the untreated samples is regarding the pH 5.7 fractions, where there was a negative correlation, with a value of -0.286 ($p=0.047$). This observation indicated that as the protein concentrations of the pH 5.7 untreated samples increased, the AS content decreased. The pH 5.7 untreated fraction was noted to have the larger increase in dementia patients compared to controls. Therefore, this further demonstrates that AS was not responsible for this increase.

Clinical relevance of AS blood content

Regression analysis was used to explore the correlation between the levels of AS detected in the blood samples and the clinical data of the dementia patients and control subjects (Table 5). This analysis showed that for the whole blood samples there was a positive correlation between age and AS level in all the cases (control and dementia patients; $p=0.007$), suggesting that AS increases with age. However, this finding was only found in controls, and there was no correlation when the dementia group was analysed separately. Interestingly, the opposing relationship was noted between age and pH 5.7 ERC for the whole and the control group.

The cognitive assessment analysis (CAMCOG and MMSE) highlighted a negative relationship between the heat stable AS and the cognitive measures in the dementia group, but not in control subjects. This was especially evident in the ERC heat stable fraction. In addition, the global behavioural changes, as measured by NPI and Cornell test, were positively related to the heat stable AS in both the ERC and pH 5.7 ERC fractions (Table 5).

Discussion

The main findings from this study confirm that blood samples and ERC, in specific, could be reliable biomarkers for dementia. Thus, elevated protein concentration has high sensitivity and specificity to discriminate VaD subjects from normal ageing and other forms of dementia, such as AD and DLB. This significant increase in protein concentration was confined to the ERC fraction processed at pH 5.7 but was not evident in whole bloods or the ERC fraction alone. Furthermore, we demonstrate that this increase is not due to the upregulation of AS or other heat stable proteins. In addition to this, AS levels in the whole bloods are highly increased in DLB subjects, with high sensitivity and specificity for the disease, whilst the ERC AS measures do not appear to differ between control and dementia subjects. The reported findings for the DLB subjects, however, need to be interpreted with caution, since they derive from a small group of patients.

This is the first report to recognise the role of protein concentration in relation to dementia (VaD) diagnosis. Although increases in individual proteins has been reported in bloods, this has been restricted to certain plasma proteins, identified either in proteomic studies (Liu et al, 2015) or in studies exploring dementia-related blood proteins, e.g. inflammatory markers (α1-antichymotrypsin, interleukin 6, and C-reactive protein) or AS (Engelhart et al, 2004, Kasuga et al, 2012). It appears that the by-product of the platelet preparation in pH 5.7 contributes to significant increase in the protein concentration in this fraction that does not occur when ERC are prepared as a result of one step centrifugation. This finding invites us to explore into possible proteins which could lead us to future potential biomarkers, specifically for VaD as the largest increase is seen here. ERCs contain several proteins that are also found in the brain; one of them is ferritin. This protein is found in blood and contains a ferric ion. Previous studies have shown that iron regulation in AD and PD can often be disturbed where levels of iron increase and cause disruptions to proteins such as AS (Eassa et al, 2009). Increased iron, is also found in subcortical ischaemic vascular dementia and its elevation

corresponds to the cognitive impairment (Liu et al, 2015). Ferritin releases the ferric ion which can then interact with AS and causes mis-folding leading to aggregation and formation of Lewy Bodies (Liu et al, 2015) and AS synaptic loss. Furthermore, the AS

coding gene possesses an iron regulatory element (IRE) which requires binding of iron to regulate it's expression. The amyloid precursor protein (APP) also possesses an IRE which relates ferritin to both DLB and AD. Since iron is highly involved in various dementia pathologies, it needs to be explored further as a potential cause of elevated protein concentration (Cahill et al, 2009). A previous study reported a positive relationship between the severity of AD and aluminium ferritin (Cahill et al, 2009).

Other candidates contributing to protein increases seen in VaD are the heat shock proteins. Due to their involvement in refolding misfolded proteins and thereby preventing aggregation, they have been reported to be upregulated in diseases such as dementia (Engelhart et al, 2004). Proteomic studies are now needed to expand on this research to identify blood proteins which increase as a result of VaD pathology. This will help to understand the real nature of the overall increase in blood protein concentration in VaD and thus aid the development of diagnostic biomarkers for the disease.

The increase in protein concentration in VaD subjects at pH 5.7 argues there is a specific phenomenon associated with this dementia subtype. If so, this may be closely associated with the increased blood viscosity leading to vascular events in these patients. Indeed, acidosis impairs blood flow properties and previous studies have described a possible decrease in pH caused by oxidative stress, which occurs in dementia (Reinhart et al, 2002, Mulkey et al, 2004). This may lead to a sequential precipitation of a specific protein seen in the pH 5.7 fractions. The inverse correlation between the heat stable and untreated pH 5.7 protein concentrations rules out the heat stable AS to contribute to this. Understanding this phenomenon is now a crucial step in elucidating the pathophysiological mechanisms underlying VaD.

AS has been previously explored as a peripheral, blood biomarker for dementia. However, most of the studies have concentrated on plasma and in Parkinson's disease (PD), with

somewhat inconclusive results (Kasuga et al, 2012). Thus, an observed increase, decrease and unchanged AS plasma levels have all been reported in PD. However, increased plasma AS oligomers reflecting the Lewy body pathology, appear to be a more consistent finding in the bloods of PD subjects (El-Agnaf, et al, 2006). In all dementia blood samples that we analysed, we detected presence of oligomers and polymers, both in the whole blood and the ERC fractions. However, the ELISA method we used was able to address the total AS content in the blood samples and not AS polymers. Furthermore, the increase in AS in the DLB subjects was detected in the whole bloods, and not in ERC fractions. This suggests that the AS increase in the DLB group is a result of AS elevation either in plasma or other blood cellular components, such as platelets. However, a recent study also demonstrated an increase in total AS in sedentary people (Iofrida et al, 2017), raising an interesting hypothesis that this ERC AS increase may be confined to people with Lewy body diseases, i.e. PD/PDD and DLB, known to have significant motor impairment (Fritz et al, 2016). These findings now need to be explored further to determine both the exact blood fraction where the AS increase occurs, as well as determine the putative lifestyle factors that may additional influence the latter. This may well enhance the specificity of the current total AS blood measure to differentiate DLB from other dementia subtypes. However, we must point out both plasma (Gorostidi et al, 2012) and ERC (Abd-Elhadi et al, 2015) AS levels do not have a predictive value in the diagnosis of Parkinson's disease and this has to be further investigated for DLB subjects.

In contrast to Barbour et al, 2008, we found an increase of AS in the heat stable blood preparations. Furthermore, we provide further quantitative evidence for the increase in the heat stable AS throughout all analysed fractions of blood (Figure 1), using a much greater sample size of 114 compared to 4 young adult samples used in Barbour et al study (2008). It is of interest to point out that AS tetramers (56 kDa) but not smaller oligomers also occur in control subjects in the whole blood, but not heat stable fractions. Previous studies have shown that AS may exist natively as a tetramer which therefore explains the presence of the tetramer but no other oligomer, within the control subjects (Bartels et al, 2011). Both the qualitative (Western blot) and quantitative (ELISA) data of increased AS levels in the heat stable blood preparations are a further confirmation that AS in bloods undergoes self-polymerisation, with both oligomers, tetramers and higher molecular weight polymers present.

The clinical analysis highlighted further the distinct contribution of monomeric (heat stable) and total AS content in blood samples. In contrast to the total AS content that appears to be able to discriminate between DLB and other forms of dementia and control subjects, the monomeric (heat stable) AS seems to be more closely related to the cognitive and behavioural profiles in subjects with dementia. More importantly, the increase in both protein concentration and AS in VaD and AD may be also driving the observed clinical relevance in our dementia cohort. In addition, a previous correlative clinico-biochemical study described a transient upregulation of AS in the temporal and frontal lobes of people with AD in mild to moderate stages (Mukaetova-Ladinska et al, 2000a). The AD group in the current study was mildly cognitively impaired, as demonstrated via the MMSE and CAMCOG scores. It is intriguing to speculate that blood AS measures may also be mirroring the central neuropathological process seen in AD.

Our study is not devoid of limitations. Firstly, the sample sizes for different dementia sub-types (e.g. DLB) were relatively small compared to AD and VaD. This meant that the results

for the DLB cases needed to be analysed with caution as they may not represent the majority of DLB cases. The pharmacological heterogeneity of the analysed samples was not subjected to further analysis, and thus there is a possibility that medication may influence some of the results. In addition, heat stable samples could not be prepared from all ERC fractions largely due to the limited amount of available samples, with some samples showing signs of coagulation. Other studies have also reported limitations of their ERC work caused by the contamination of plasma within the separate fractions (Barbour et al, 2008). Since plasma also contains AS this harbour the potential of disrupting AS data, though we took great precautions to eliminate plasma especially from the ERC samples.

In conclusion, we report novel clinical relevance of both blood protein concentration and AS content. One of our most significant findings was the potential use of whole bloods for dementia diagnosis, specifically DLB by monitoring AS levels. The replication in a larger sample size, will enable the use of archived blood material from previously conducted longitudinal studies on ageing and dementia to compare findings with neuropathological diagnosis.

In addition to this, we were able to detect an elevation in the pH 5.7 treated ERC, specifically for VaD. We know from our work that the latter is not due to AS elevation. Overall the results from the current study have provided, an extensive clinical insight about the ERC derived AS and how it can facilitate the development of novel biomarkers for dementia.

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Variable	Control	AD	VaD	DLB	F	p
Age/years	72.07±11.45	77.34±5.62	80.9±4.86	81.75±4.99	4.712	0.004
Gender (M/F)	15/45	21/15	7/5	4/2	13.920	<0.005
CAMCOG	95.31±6.00	70.31±19.33	55.40±18.00	48.75±28.34	42.675	<0.005
MMSE	27.89±2.026	21.29±5.829	15.90±5.174	12.00±8.29	40.210	<0.005
NPI	NA	16.40±14.74	23.38±14.19	26.25±18.93	1.302	0.282
Cornell	NA	6.06±4.54	5.75±2.71	2.50±1.92	1.311	0.280

Table 1: Demographic and clinical data of the subjects. Numbers represent mean ±SD. Abbreviations: AD, Alzheimer disease, VaD, vascular dementia; DLB, dementia with Lewy body; CAMCOCG, MMSE, Mini Mental State Examination; NPI, Neuropsychiatric Inventory; Cornell, Cornell Scale for Depression in Dementia; NA, data not available; M, male; F, female.

Variables	Control	AD	VaD	DLB	P
WB					
Untreated	63.45	42.29	66.77	87.50	0.002
Heat Stable	58.20	56.96	73.31	38.67	0.191
ERC					
Untreated	52.61	37.50	81.00	78.00	<0.001
Heat Stable	18.79	18.18	NA	NA	0.862
pH 5.7 ERC					
Untreated	17.58	33.75	40.33	27.83	<0.001
Heat Stable	28.54	21.75	21.25	34.50	0.224

Table 2: Protein concentrations in blood samples. Heat stable samples were not available for VaD and DLB ERC fractions due to both a limited amount of ERC available and samples having a higher tendency to coagulate. All values expressed in μg/ml. *Abbreviations:* ERC, erythrocytes; NA, not available; WB, whole blood.

	Control	AD	VaD	DLB	p
WB					
Untreated	49.73	50.72	56.05	97.00	0.024
Heat Stable	48.71	50.06	72.31	60.50	0.180
ERC					
Untreated	50.44	39.56	55.73	45.63	0.221
Heat Stable	21.00	14.84	NA	NA	0.075
pH 5.7 ERC					
Untreated	28.20	25.81	20.71	16.25	0.277
Heat Stable	26.42	24.44	23.32	27.20	0.927

Table 3 –AS levels in blood fractions. Kruskal-Wallis analysis. Values refer to relative values. Abbreviations: ERC, erythrocytes; WB, whole bloods.

Parameters	**Statistical measures**	**PC RBC HS**	**PC WB HS**	**PC pH 5.7 HS**
RBC HS	ρ Sig.	0.349 0.040	-0.195 0.261	NA
WB HS	ρ Sig.	-0.086 0.620	-0.032 0.749	0.066 0.684
pH 5.7 HS	ρ Sig.	NA	0.169 0.240	0.296 0.037

Table 4 – Summary of correlations between the heat stable protein concentrations (PC) and the heat stable AS content. The data refer to all analysed samples grouped together.

Variables	AGE	CAMCOG	MMSE	NPI	Cornell
WB	**0.304**	-0.020	-0.035	-	-
	0.005	0.845	0.728	-	-
C	**0.265**	-0.05	-0.005	-	-
	0.007	0.724	0.971	-	-
D	-0.055	-0.056	-0.109	0.193	0.100
	0.711	0.710	0.465	0.199	0.508
WB HS	-0.101	-0.149	**-0.193**	-	-
	0.320	0.142	**0.056**	-	-
C	-0.135	0.215	0.107	-	-
	0.440	0.121	0.446	-	-
D	0.086	-0.131	-0.196	0.088	0.075
	0.569	0.390	0.196	0.565	0.606
ERC	0.044	0.057	0.019	-	-
	0.683	0.601-	0.862	-	-
C	.0440	**0.271**	0.233	-	-
	0.683	**0.082**	0.137	-	-
D	0.044	-0.120	-0.123	-0.174	0.060
	0.773	0.444	0.431	0.265	0.703
ERC HS	-0.135	**0.340**	0.251	-	-
	0.440	**0.046**	0.147-	-	-
C	-0.135	0.268	0.365	-	-
	0.440	0.282	0.136	-	-
D	-0.053	**-0.538**	-0.499	-0.174	**0.452**
	0.839	**0.026**	**0.041**	0.265	**0.069**
pH 5.7 ERC	**-0.455**	0.182	0.179	-	-
	0.003	0.262	0.270-	-	-
C	**-0.64**	0.012	0.062	-	-
	0.003	0.961	0.794	-	-
D	0.085	-0.143	-0.113	0.330	0.038
	0.693	0.559	0.646	0.195	0.881
pH 5.7 ERC HS	0.013	-0.095	-0.179	-	-
	0.935	0.561	0.268	-	-
C	-0.065	-0.104	-0.098	-	-
	0.786	0.662	0.608	-	-
D	-0.165	0.036	0.057	**0.655**	**0.437**
	0.431	0.887	0.821	**0.003**	**0.070**

Table 5 – Summary of regression analysis of clinical data and AS content in different blood fractions. B value defines the regression coefficient whilst p defines the level of significance. Abbreviations: C, control subjects; D, dementia subjects.

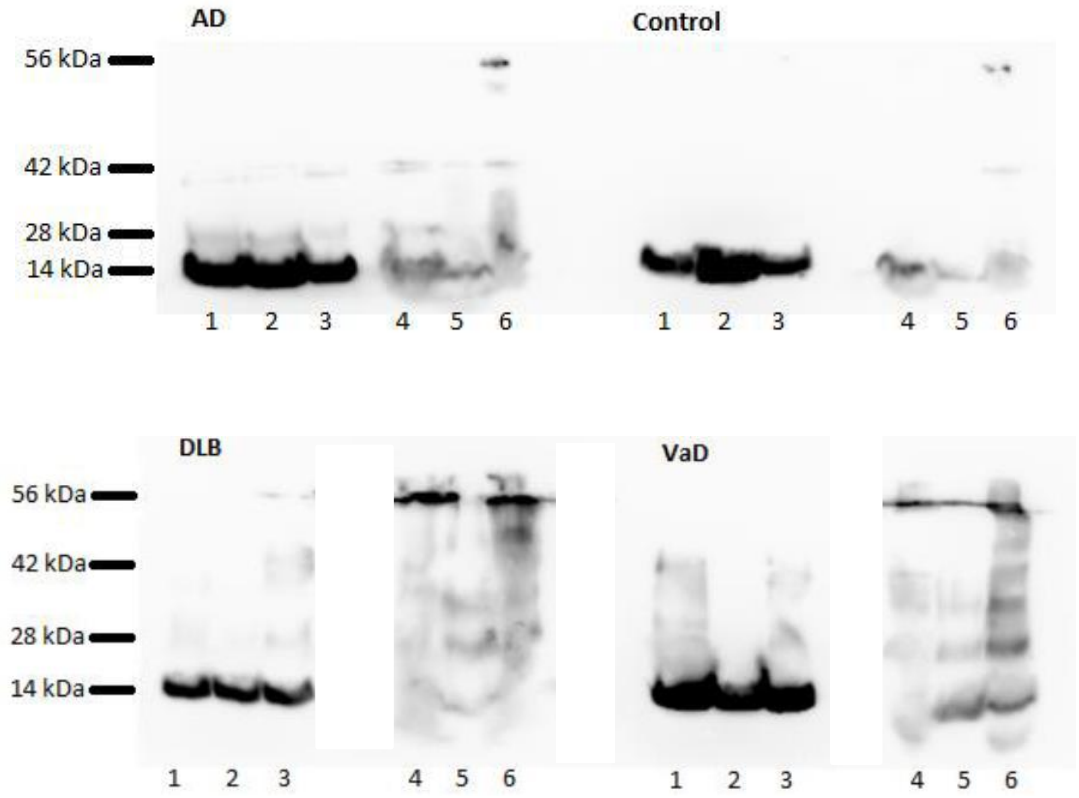

Figure 1: Alpha synuclein (AS) immunoreactivity in ERC fractions. Western blot. Lanes 1 – 3 WB, pH 5.7 ERC and ERC heat stable preparations. Lanes 4 – 6 contain untreated WB, pH 5.7 ERC and ERC fractions. Markers on the left show the approximate molecular weight of the bands. This was repeated X times with similar results?

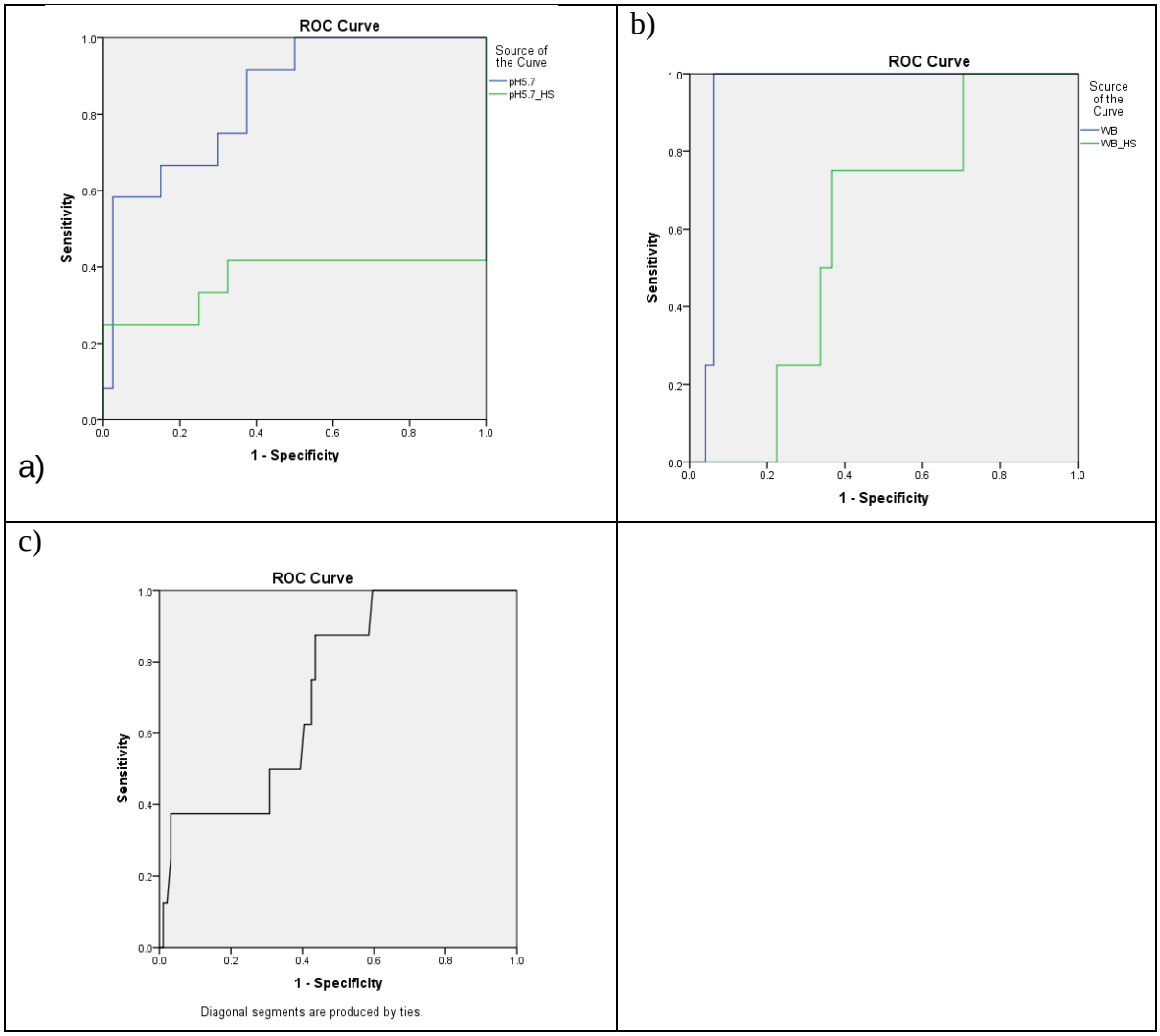

Figure 2: a) ROC (Receiver Operation Characteristics) Curve for protein concentration, showing sensitivity and specificity (91.7%, 62.1%) of pH 5.7 samples for VaD subjects in blue (p = <0.005). This was not the case for the heat stable fraction of pH 5.7 ERC (in green).

b) Graph showing the AS measures sensitivity and specificity of the whole blood (100%, 93.9%) and whole blood heat stable (87.5%, 56.4%) samples for DLB. AS content in blue and green respectively.

c) ROC Curve analysis for the AS measures in VaD WB HS fraction. Sensitivity and Specificity of 75% and 57.4% (p=0.038).