Use of whole blood for analysis of disease-associated biomarkers

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Abstract:

Analyses for diagnosis and monitoring of pathological conditions often rely on blood samples, partly due to relative ease of collection. However, many interfering substances largely preclude use of whole blood itself, necessitating separation of plasma or serum. We present a feasibility study demonstrating potential use of fresh or frozen whole blood to detect soluble biomarkers using an ELISA-based method. Good correlation between levels of soluble CD25 in plasma and whole blood of healthy individuals or Alzheimer's patients was established. These results provide a basis for development of a novel biosensor approach for disease-associated biomarker detection in whole blood.

Keywords:

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Introduction:

Blood is a commonly used biological specimen for diagnosis and monitoring of disease processes, however, the vast majority of analyses performed on blood are conducted only on the plasma or serum fraction, thus requiring separation steps, which can require specialist equipment and be time-consuming, increasing cost. Most routine laboratory analyses utilise either plasma or serum [1], as do many tests for specific pathologies such as malignancy-associated biomarkers [2].

Whole Blood (WB) is a more attractive option for rapid point-of-care (POC) testing, completely removing preparatory steps. However, WB is a highly complex sample, with many potentially interfering factors. Available biosensing systems utilising WB are very limited in comparison to sample types such as plasma, therefore presenting a rapidly developing and potential new approach for biosensing technologies. Only a few examples of POC tests utilise WB, mostly for analysis of erythrocyte-related parameters such as haemoglobin [3]. One commonly used WB biosensor is blood glucose monitoring, where POC tests routinely require just a finger-prick of blood, and show excellent correlation between plasma and WB glucose levels [4].

Additionally, whilst some POC tests such as cardiac troponin detection can be performed on WB these are generally less sensitive than laboratory methods (typically performed on serum), with studies indicating need for confirmation using standard methods and therefore mainly useful for exclusion purposes [5].

'Lab-on-a-chip' technologies have recently begun to show promise by incorporating steps to separate plasma or serum fractions prior to analysis [6]. There remains a need to develop novel technology for POC testing utilising WB, with potential application in settings such as emergency departments, GP surgeries and areas with limited healthcare facilities. Additionally, ability to use WB frozen without preparatory or protective stages would be beneficial for batch-testing.

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We describe a method for analysis of a disease-associated biomarker using frozen WB. CD25 was selected as a representative biomarker for initial method development, as it is found to be increased on activated MNC in Alzheimer's disease (AD) against age-matched cohorts (our unpublished data) and [7]. Currently, despite extensive research into AD biomarkers there is still no robust blood-based diagnostic biosensor available. Most of the available methods involve invasive sampling such as cerebrospinal fluid (CSF) [8], hence there is increasing need to develop methods for blood-based analysis of potential biomarkers for AD. Whilst CD25 was utilised for this feasibility study, it is important to highlight that this approach could be used for any soluble blood-based biomarker and thus may have wide future applications.

Aim:

To compare the use of fresh or frozen WB as an alternative to plasma for analysis of disease-associated biomarkers, as a possible basis for development of biosensing systems using WB.

Methods:

Reagents were from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

Blood Samples:

Two sets of samples were utilised: (i) fresh and frozen WB samples from healthy volunteers and (ii) frozen WB and plasma samples from AD and healthy individuals.

WB samples were obtained from 13 healthy consenting volunteers (age range 25-51 years) at the University of the West of England, Bristol with Faculty Research Ethics Committee approval.

WB and plasma samples were obtained from the OPTIMA Biobank (John Radcliffe Hospital, Oxford) with prior donor consent and National Research Ethics Committee approval (10 samples from patients with a conclusive AD diagnosis post-mortem and 10 from age-matched healthy controls).

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Isolation and stimulation of MNC from healthy volunteer samples:

Mononuclear cells (MNC) were isolated by density centrifugation, by layering WB onto an equal volume of lymphoprep (Axis-Shield, Cambridgeshire, UK) and centrifuging at 600xg for 30mins. The buffy coat was harvested, washed, and MNC seeded in RPMI (Lonza, Suffolk, UK) supplemented with 10% foetal calf serum (Lonza), 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin, in 24-well culture plates at $1x10^6$ cells/ml. MNC were stimulated with 5µg/ml phytohemagglutinin (PHA) over 3 days.

Flow cytometry analysis:

MNC ($1x10^5$) were stained with CD25 primary antibody (Caltag-Medsystems) for 30mins at 4°C, followed by addition of anti-mouse FITC-conjugated secondary antibody for 30mins at 4°C. Analysis used an Accuri C6 flow cytometer, measuring median fluorescence intensity (MFI) relative to a secondary antibody only background control. Cells were gated to exclude debris and dead cells, with 10,000 gated events analysed.

CD25 ELISA

Expression of MNC cellular CD25 (cCD25) has been reported to be proportional to soluble CD25 (sCD25) due to proteolytic cleavage of transmembrane CD25 [9]. This correlation was confirmed in our laboratory using 6 healthy blood donors. cCD25 expression of PHA-stimulated MNC was analysed using flow cytometry as previously described. Soluble CD25 (sCD25) levels in plasma from the same samples was analysed using a sCD25 (IL2Rα) human ELISA kit (Abnova, Taiwan) according to the manufacturers' instructions. Briefly, 50µl biotinylated anti-CD25 antibody was added to the pre-coated microtitre plate. Samples were diluted 1/2 and 1/5, 100µl added, and incubated for 1h (all incubations at room temperature). After 5 washes, avidin conjugate was added (100µl), mixed and incubated for 1h. After washing, substrate solution (100µl) was added for 15mins, before adding stop solution (100µl) to the substrate solution already in the well and Optical Density measured at 450nm within 30mins, using a microplate reader (Anthos Labtec, Cambridge, UK). Standards of

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known CD25 concentrations (doubling dilutions from 2000pg/ml-62.5pg/ml) were also analysed, allowing CD25 concentration estimation within blood samples.

Plasma samples from AD patients and age-matched controls were analysed for sCD25 expression using the same sCD25 ELISA kit, in duplicate, at two different dilutions. Frozen WB samples from the same individuals were also obtained, sonicated for 10mins, and then similarly analysed directly for sCD25 without any preparatory steps.

Statistical Analysis

All data are presented as mean ± SE unless stated otherwise. Statistical analyses utilised paired t-tests, with $p < 0.05$ considered significant.

Results:

Initially fresh blood samples from young healthy donors were used to confirm highly significant upregulation of cCD25 expression following PHA stimulation as a validation step only (Figure 1A and B) ($p < 0.001$).

Due to proteolytic cleavage of transmembrane CD25, sCD25 reportedly correlates with cCD25 (R^2 0.929) [9], and therefore, would be a viable approach to analyse plasma or WB rather than isolation of MNC to analyse cCD25. This correlation was also confirmed in our laboratory (Figure 1C).
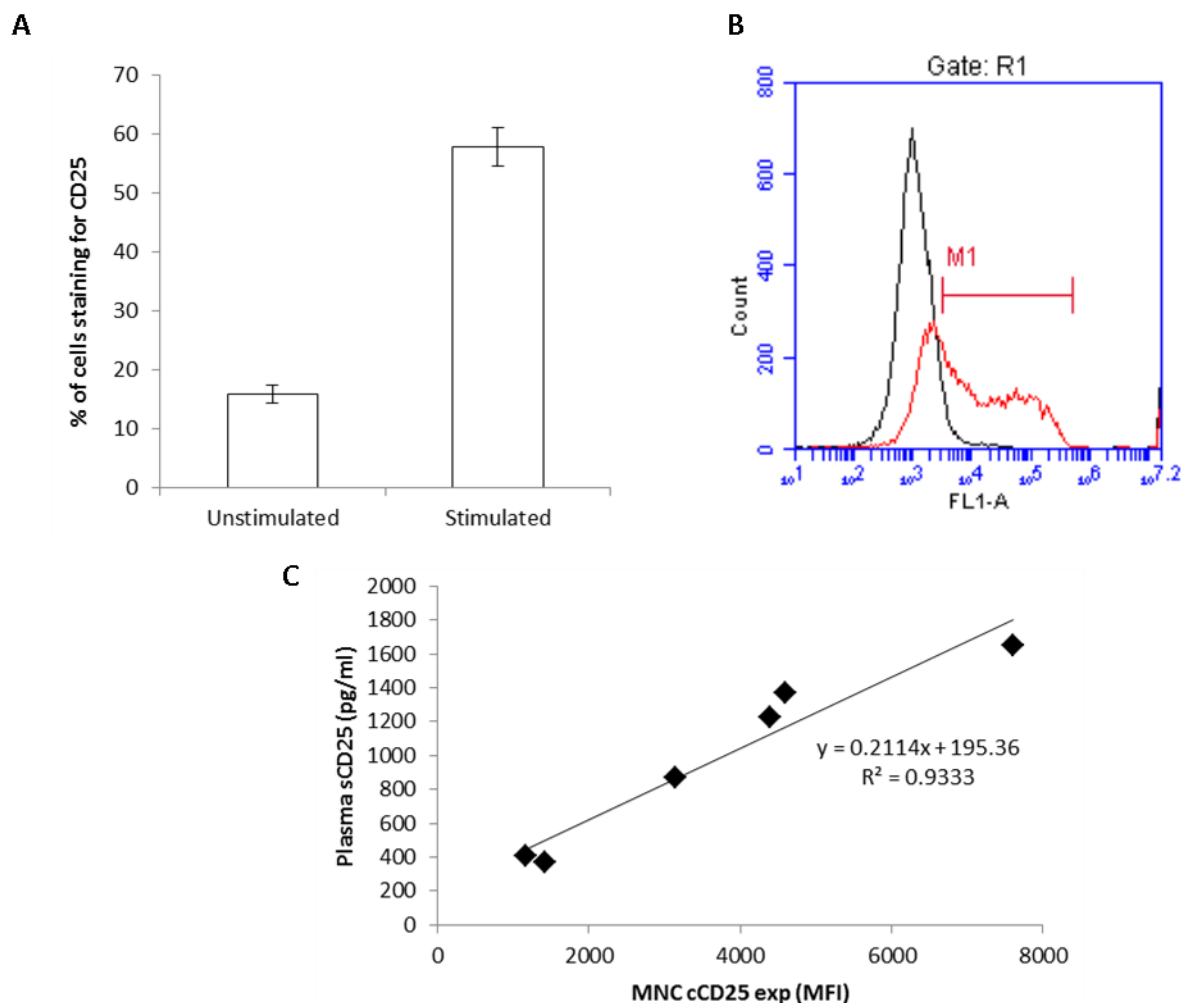
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Figure 1 - (A) MNC CD25 expression following 3day PHA stimulation. Mean±SE of cells staining positively for CD25 presented, from 12 healthy individuals. (B) Representative flow cytometry histogram plot of one sample, showing upregulation of CD25 expression (FL-1) after stimulation. Unstimulated cells (black), stimulated cells (grey). (C) Correlation between cCD25 on stimulated MNC by flow cytometry analysis in comparison to sCD25 in plasma measured by ELISA, in healthy blood donors (n=6). MFI = median fluorescence intensity, used to indicate number of antigen sites per cell.

Following confirmation of this correlation, sCD25 was subsequently measured directly in frozen

samples from AD patients and age-matched controls, where analysis of cCD25 was impractical.

Initially sCD25 in plasma was analysed in both cohorts, with detectable levels in all individuals (AD

patients range 331-2472pg/ml, controls 423-2197pg/ml). Analysis of sCD25 in WB from the same

individuals was also performed, with detectable levels in all samples (AD patients range 550-

2194pg/ml, controls 573-2243pg/ml).

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Importantly, very good correlation was seen between sCD25 in plasma and WB (AD patients R^2 0.78, controls R^2 0.80), indicating potential use of WB for detection of soluble blood biomarkers as an alternative to plasma (Figure 2).
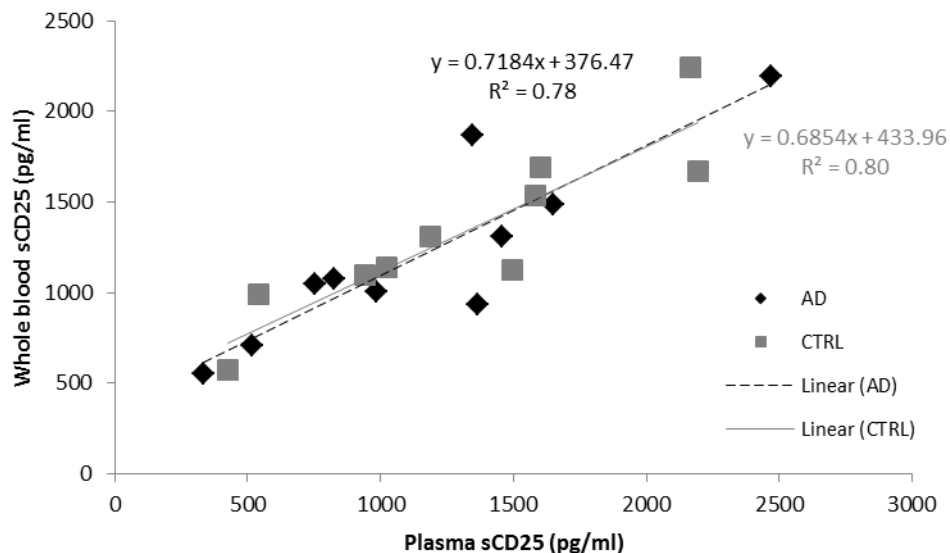

Figure 2 – Correlation between sCD25 in whole blood and plasma in AD patients and healthy age-matched controls (n=10 per group).

Generally, lower values for sCD25 were detected in plasma (p>0.05), with mean values of 1170±188pg/ml and 1316±182pg/ml measured in AD and age-matched controls respectively. In comparison, WB levels were 1217±153pg/ml and 1336±139pg/ml in AD and controls respectively, representing an increase of 4% in AD patients compared to plasma levels, and a difference of only 1% in healthy controls between sample types.

The sCD25 ELISA method proved very sensitive, with standards detected as low as 62.5pg/ml; and a broad concentration range measurable in frozen samples (331pg/ml to 2472pg/ml).

Discussion:

Consistent detection of sCD25 in all samples indicates that ELISA measurement of this marker is a robust approach for measuring soluble blood biomarkers, even within a highly complex sample.

Good correlation between sCD25 levels in plasma and WB provides proof of concept for rapid

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detection of markers in WB without preparatory steps. WB is relatively easy to obtain (potentially requiring only a finger-prick) and less invasive than samples such as CSF currently used in AD diagnosis. Additionally, good correlation between WB and plasma was seen in previously frozen samples, highlighting potential use of samples frozen without preparation.

The results presented illustrate the potential for this method in detecting soluble markers; however CD25 did not show a significant difference between AD and healthy cohorts. Previous work identified a difference between control and AD MNC only following activation (unpublished results) [7] but not in unstimulated cells, thus in agreement with the current study. It is also acknowledged that the stage at which the samples are taken may affect differential expression levels in AD and control samples as differences are reported to be enhanced at later stages of the disease [7]. Whilst the marker used for development purposes in this study does not currently provide evidence of usefulness as a biomarker for AD, the approach used for the interrogation of biomarkers generally in WB is potentially widely applicable.

The ELISA-based approach showed good detection limits, but requires considerable hands-on time, substantial cost, and is less amenable to high-throughput testing. Bio-sensing technology, for example an electrochemical approach such as that used by diabetics for glucose monitoring using WB [10, 11] could offer a rapid, low cost alternative. An electrochemical method incorporating an antibody immobilised onto a screen-printed carbon electrode has been developed by our group for detection of picomolar concentrations of estradiol [12] and could be adapted for measurement of soluble markers in WB. There is potential to develop a multiplex system, enabling simultaneous detection of a panel of biomarkers. A recent study by O'Bryant *et al.,* employed this approach to interrogate altered serum proteins as a potential AD screening tool [13], and exhibited excellent results at distinguishing an inflammatory phenotype. Similar approaches utilising WB would remove preparatory steps, with potential cost and time benefits, and enhanced POC suitability.

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Summary:

In conclusion, the immunoassay antibody-based method reported showed strong positive correlation between CD25 levels in plasma and WB, demonstrating feasibility for analysis of disease-associated biomarkers in WB. This has potential for further development towards a screen-printed electrochemical biosensor approach for fresh and frozen WB, with advantages including improved sensitivity, low-cost and applicability to POC testing. Future work will include evaluation of WB for suitability to detect other soluble blood-based biomarkers, which may have wide application for a range of pathologies.

Acknowledgements:

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