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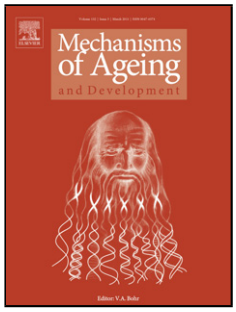

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$CD4^+$ T cell surface alpha enolase is lower in older adults

Running title: T cell surface CD4+ α-enolase in ageing

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Highlights

- α-enolase is lower on the surface of CD4+ T cell in healthy older adults.
- Cardiovascular disease patients express less CD4+ T cell α-enolase than age-matched controls.
- A hypothesis-free proteomic discovery approach has been adopted successfully to study human ageing
- Membrane protein enrichment has been developed to reduce bias towards cytosolic protein discovery.

ABSTRACT

To identify novel cell ageing markers in order to gain insight into ageing mechanisms, we adopted membrane enrichment and comparison of the $CD4^+$ T cell membrane proteome (purified by cell surface labelling using Sulfo-NHS-SS-Biotin reagent) between healthy young (n=9, 20-25y) and older (n=10; 50-70y) male adults. Following two-dimensional gel electrophoresis (2DE) to separate pooled membrane proteins in triplicates, the identity of protein spots with age-dependent differences (p<0.05 and >1.4 fold difference) was determined using liquid chromatography-mass spectrometry (LC-MS/MS). Seventeen protein spot density differences (ten increased and seven decreased in the older adult group) were observed between young and older adults. From spot intensity analysis, $CD4^+$ T cell surface α-enolase was decreased in expression by 1.5 fold in the older age group; this was verified by flow cytometry (n=22) and qPCR with significantly lower expression of cellular α-enolase mRNA and protein compared to young adult $CD4^+$ T cells (p<0.05). In an independent age-matched case-control study, lower $CD4^+$ T cell surface α-enolase expression was observed in age-matched patients with cardiovascular disease (p<0.05). An immune-modulatory role has been proposed for surface α-enolase and our findings of decreased expression suggest that deficits in surface α-enolase merit investigation in the context of immune dysfunction during ageing and vascular disease.

Keywords: enolase, $CD4^+$T cell, plasma membrane proteomics, 2D gel electrophoresis, phenotype

1. INTRODUCTION

Biological ageing is a complex process influenced by genetic and environmental factors that varies in rate from person to person; chronological age is considered to be a poor indicator of the rate of biological ageing (1). While the life spans of identical twins are significantly more similar than those of non-identical twins, genetic factors are only estimated to account for between a quarter and a third of life span (2). Environmental factors including healthy diet and increased levels of physical activity have also been linked to longer life expectancy (3,4). Biomarkers represent an attractive measure of biological ageing and potentially may improve our understanding of underlying ageing processes and age-related disease (5). Recent studies suggest that cell surface receptors may also be modified post-translationally, undergo changes in trafficking or level of expression (6-9). However, to date the cell surface proteome has not been investigated systematically for age-dependent biomarkers.

$CD4^+$ T cells are required for effective acquired immunity, and a decline in their function is thought to contribute to reduced efficacy of response to infection, vaccination, an increase in autoimmunity and cardiovascular disease typically seen with ageing (10-14). This may in part be due to the age-related decrease in naïve and increase in antigen experienced $CD4^+$ T cells (15,16). Given the observed age-related changes to $CD4^+$ T cell function and the importance of cell surface receptor expression and binding for activation, the $CD4^+$ T cell plasma membrane proteome may be an important source of biomarkers of ageing.

2DE provides a non-hypothesis driven proteomic approach for biomarker identification, which separates all protein isoforms within a sample at a given time point. The technique has been

successfully applied to simple experiments undertaken in cell lines and to more complex tissue and biological fluids for the purpose of biomarker discovery (17). In the majority of such proteomic studies, membrane proteins are often under represented due to their relatively low abundance, large size and hydrophobic properties (18). Pre-enrichment of sample with plasma membrane proteins prior to separation by 2DE, is one strategy which has been used to improve membrane protein representation for proteomic studies (19-21). In this work, we describe a membrane purification method which involves labelling of primary amine groups present on lysine residues of $CD4^+$ T cell surface membrane proteins with Sulfo-NHS-SS-Biotin reagent and subsequent 2DE coupled with LC-MS/MS to identify age-related differences in the $CD4^+$ T cell membrane proteome. To our knowledge this represents the first non-hypothesis driven approach to study age-related protein expression differences on the cell surface of $CD4^+$ T cells.

2. MATERIALS AND METHODS

2.1 Participants

Young male adults (18-35 years old) and older male adults (50-70 years old) were recruited for this study. Several physiological changes are reported from the age of 50 years, around the age of menopause for women; it has been proposed that health is preserved evolutionarily to this age to allow successful reproduction (2). To avoid oestrogenic hormonal variability alone influencing cell surface markers, T cells from males only were analysed. These volunteers were healthy, non-smokers and were not taking any disease modifying or anti-inflammatory medication or nutritional supplements. Participants provided informed written consent and ethical approval was obtained from the Aston University Ethics Committee.

2.2 Blood measurements and processing

After an overnight fast, 40 mL whole blood was drawn from the antecubital vein of each participant and collected into EDTA coated tubes (Greiner Bio-One Ltd, UK) between 8:00 and 10:30 am. Anthropometric measurements were recorded and biochemical indices (glucose, total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides) were measured by pipetting 30 µL whole blood onto Reflotron strips (Roche Diagnostics, Indianapolis, USA) then determined using a Reflotron blood analyser (Boehringer Mannheim GmbH, Germany). Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (22). Peripheral blood mononuclear cells (PBMC) were isolated immediately using density gradient centrifugation as previously described (23). Briefly, whole blood (20 mL) was mixed 1:1 with PBS supplemented with 0.1% w/v BSA, layered onto Lymphoprep (11 mL, Axis-Shield, UK)

and centrifuged at 160 x g for 15 minutes. Plasma (5-10 mL) was removed and the preparation centrifuged for a further 20 minutes at 350 x g. PBMC (1-2 x 10^7) were pipetted into a clean centrifuge tube (Corning, UK) and washed twice with PBS with 0.1% w/v BSA to remove residual Lymphoprep. $CD4^+$ T cells were isolated from PBMC using a negative isolation kit (Life Technologies, UK) yielding 5-7 x 10^6 cells. For analysis of gene expression, mRNA was harvested from $CD4^+$ T cells using Trizol® reagent (Invitrogen, Carlsbad, CA). For the purpose of flow cytometry for cell surface antigens, whole blood (50µL) was fixed by addition of 500µL OptiLyse C (Beckman Coulter) for 2 hours and then stored at -80°C prior to analysis. To determine total intra- and extra-cellular protein expression, fixed whole blood was incubated with 0.5% saponin in PBS, treated for 10 minutes prior to staining with appropriate antibodies.

2.3 Membrane protein preparation

Primary human $CD4^+$ T cells (5 x 10^6 cells) were washed three times in ice cold PBS (1mL; pH 8) and cell surface proteins labelled with 0.5 mg/mL biotin (Sulfo-NHS-SS-Biotin, Thermo Scientific, UK) for 20 minutes at 4 ºC on a rotary mixer. Cells were washed twice with glycine (10 mM) to quench the biotinylation reaction and washed once with PBS (pH 8). Cells were lysed on ice for 30 minutes in MNE (25mM MES (pH 6.5), 150mM NaCl, 2mM EDTA) lysis buffer supplemented with 1mM sodium orthovanadate, 1% v/v Triton X-100 and 0.1% v/v protease inhibitor cocktail (Sigma Aldrich, UK), genomic DNA was sheared using a 21 G needle (Terumo, UK) and centrifuged at 2,000 xg for 5 minutes to obtain a post-nuclear supernatant (PNS). The PNS (1mL) was incubated for 30 minutes at room temperature with 200 µL of pre washed Magnabind Streptavidin (SA) beads (Thermo Scientific, UK), an unbound fraction was collected and beads were washed twice in MNE lysis buffer and twice in PBS (pH 8). Cell

surface proteins were eluted into extraction buffer (8M urea, 2M thiourea, 2% w/v CHAPS, 1% v/v DeStreak reagent (GE Healthcare, Amersham, UK) and 0.2% v/v Bio-Lytes (Bio-Rad)) for 30 minutes at room temperature. PNS, unbound and elution fractions were stored at -80 ºC for SDS-PAGE analysis and western blotting. The yield of CD4+ T cell membrane proteins eluted ranged from 8-20µg for the 19 subjects studied.

2.4 Confocal microscopy

Primary $CD4^+$ T cells were biotinylated as described above or left unlabelled and prepared as described by Peirce et al. (19). Cells were washed three times with PBS and allowed to adhere to poly-L-lysine microscope slides (VWR, UK) for 20 minutes. Adherent $CD4^+$ T cells were washed with PBS, fixed for 15 minutes with 1% v/v formaldehyde, 1% w/v BSA in PBS at room temperature, rinsed in PBS and either permeabilised with 0.1% v/v Triton X-100 in PBS or incubated with PBS for 30 minutes at 4 ºC. Cells were rinsed in PBS, blocked with 1% w/v BSA in PBS for 30 minutes at 4ºC and incubated with a 1:1000 dilution of 2mg/mL SA-Alexa Fluor® 488 (Life Technologies, UK) for 30 minutes at 4 ºC. Cells were rinsed in PBS and visualized using confocal microscopy (Leica, UK).

2.5 2DE, gel staining and image analysis

Samples were prepared using a 2DE clean-up kit (GE Healthcare, UK) following the manufacturer's instructions. Protein pellets were solubilised overnight at 20ºC in solubilisation buffer (8M urea, 2M thiourea, 4% w/v CHAPS) with gentle agitation. Urea/thiourea solutions were deionised before use by incubation with a 1% w/v Amberlite mixed bed resin (GE Healthcare, UK) for 10 minutes and then filtered. Protein concentration was determined by

Bradford assay (Sigma, UK). Purified membrane proteins (8µg) from each of the young donors (n=10) and older donors (n=9) were pooled to generate a "young" and "older" adult T cell membrane protein pool respectively. To examine differences in protein expression, 2DE was undertaken independently on pooled young and older adult membrane proteins, which were run in either triplicate (older) or quadruplicate (younger) i.e. three or four repeat 2DE analysis was undertaken of the same pool. For 2DE gels, 17-cm ReadyStrip IPG strips (pH 3–10 non-linear) were loaded with 15 µg total protein from the appropriate pool of membrane proteins. IPG ReadyStrips were rehydrated for 18 hours in the presence of proteins in a volume of 300 µL rehydration solution (8M urea, 2M thiourea, 2% w/v CHAPS, 1% v/v DeStreak Reagent, 0.2% v/v Bio-Lytes). IEF was performed with a PROTEAN i12 IEF apparatus (Bio-Rad) at 150 V for 11 h, followed by stepwise application of 1000 and 10000V for a total of 25,000 Vh. After IEF ReadyStrip IPG strips were incubated for 15 minutes in equilibration buffer (6M urea, 2% w/v SDS, 30% v/v glycerol, 1% w/v DTT, 50mM Tris-HCl, pH 8.6) and a further 15 minutes in equilibration buffer with 4.7% w/v iodoacetamide, applied to 8 –16% gradient SDS-PAGE gels (Jules. Inc, USA) and sealed with 1% w/v agarose (Sigma Aldrich, UK). Electrophoresis was undertaken using a PROTEAN II multi-cell (Bio-Rad) with electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 0.2% w/v SDS) and a current of 40 µA was applied to each gel for 5 hours or until the gel front had migrated fully. Gels were electrophoresed in parallel and were imaged using a PharosFX fluorescent scanner (Bio-Rad). Gels were fixed in 10% v/v acetic acid and 40% v/v ethanol for 24 hours and stained with Flamingo fluorescent stain for 8 hours and scanned using PharosFX scanner (Bio-Rad). Spot matching and data analysis was performed using Progenesis SameSpots software (Nonlinear Dynamics Limited, Newcastle upon Tyne, UK). Reproducibility for spot identification between gels was CV≤10%. Fold-differences (≥ 1.4

fold) in the intensity of spots within triplicate gels were defines as significantly different between pooled samples of younger and older male adult groups when $p < 0.05$, analysed by ANOVA.

2.6 LC-MS/MS

Protein spots were prepared for protein identification using in gel digestion with trypsin (24). Flamingo fluorescence-stained spots were excised, divided into ~2 mm^3 cubes and destained with acetonitrile followed by 100 mM ammonium bicarbonate. This cycle was repeated until gel pieces were destained. Gel pieces were dried (vacuum centrifugation; 5 min) and rehydrated in 10 mM DTT and 100 mM ammonium bicarbonate and reduced at 60°C for 15 min. The liquid was removed and replaced with 50mM iodoacetamide and 100mM ammonium bicarbonate. Gel pieces were incubated at room temperature in the dark for 45 min and then washed with 100mM ammonium bicarbonate. After drying the gel (vacuum centrifugation; 5 min) 0.3 µg trypsin gold (Promega, WI, USA) was added and shaken at room temperature for 30 min, before dilution with 100 mM ammonium bicarbonate. Hydrolysis was allowed to occur overnight (~16 h) at 37 °C. Peptides were extracted with the initial solution of 2% acetonitrile and 0.1% formic acid in water was added and shaken for 30 minutes. Supernatant was removed to a clean plate. A second peptide extraction was performed using 40% acetonitrile and 0.1% formic acid in water, shaken for 30 minutes at room temperature. The supernatant was removed, pooled with the previous extracted peptides and dried in an evaporator. The samples were re-suspended in 0.1% formic acid in water.

UltiMate® 3000 HPLC series (Dionex, Sunnyvale, CA USA) was used for peptide concentration and separation. Samples were separated in Nano Series™ Standard Columns 75 µm i.d. x 15 cm,

packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA). The gradient used was from 3.2% to 44% solvent B (0.1% formic acid in acetonitrile) for 30 min. Peptides were eluted directly (~ 350 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a LTQ Orbitrap Velos ETD mass spectrometer (ThermoFisher Scientific, Germany). The data-dependent scanning acquisition was controlled by Xcalibur 2.1 software. The mass spectrometer alternated between a full FT-MS scan (m/z 380 – 1600) and subsequent collision-induced dissociation MS/MS scans of the 7 most abundant ions. Survey scans were acquired in the Orbitrap with a resolution of 60 000 at m/z 400 and automatic gain control $1x10^6$. Precursor ions were isolated and subjected to collision induced dissociation in the linear ion trap with automatic gain control $1x10^5$. Collision activation for the experiment was performed in the linear trap using helium gas at normalized collision energy to precursor m/z of 35% and activation Q 0.25. The width of the precursor isolation window was 2 m/z and only multiply-charged precursor ions were selected for MS/MS.

The MS and MS/MS scans were searched against NCBI database using the Mascot algorithm (Matrix Sciences). Variable modifications were deamidation (N and Q), oxidation (M), carboxyamidomethylation (C) and protein linker (K). The precursor mass tolerance was 10 ppm and the MS/MS mass tolerance was 0.8Da. Two missed cleavage was allowed and were accepted as a real hit protein with at least two high confidence peptides.

2.7 Western blotting analysis

Proteins (5 µg) were separated by SDS-PAGE using 10 % polyacrylamide gels and transferred to Hybond-P membrane (GE Healthcare, UK) as previously described (24). Membranes were

blocked for one hour at room temperature, or overnight at 4ºC with either 3% w/v BSA (Sigma Aldrich, UK) or 5% w/v non-fat milk and incubated with rabbit polyclonal anti-cluster of differentiation 3 (CD3; 1: 2000, ab5690, AbCam, Cambridge, UK) and mouse monoclonal anti-lactate dehydrogenase (LDH) (1: 1000; sc-133123, Santa Cruz Biotechnology, USA) primary antibodies, and subsequently with peroxidase conjugated goat anti-rabbit (1: 5000; A6154, Sigma Aldrich, UK) and goat anti-mouse (1: 20000; A0168, Sigma Aldrich, UK) secondary antibodies respectively. Antibody incubations were either for 2 hours at room temperature or overnight at 4ºC. After incubation steps membranes were washed three times for 10 minutes in TBS with Tween 20 (0.05%). Bands were visualized using enhanced chemiluminescent detection (GE Healthcare, UK).

2.8 Flow cytometry analysis

Peripheral blood leukocytes were washed four times in cold wash buffer (PBS supplemented with 1% w/v BSA) and left on ice in blocking buffer (0.3M glycine, 1% w/v BSA and 10% w/v goat serum (PAA) in PBS) for 30 minutes. Following four washes in cold wash buffer, cells were incubated with mouse monoclonal anti-α-enolase (ab54979) or IgG1 isotype control (ab91353) on ice for 30 minutes. After a further four washes, cells were incubated with goat polyclonal to mouse IgG1 conjugated FITC (ab97239) on ice for 30 minutes, washed four more times and then incubated with mouse monoclonal CD4 conjugated phycoerythrin (PE; ab1155) or mouse IgG2a conjugated PE isotype (ab91363) on ice for 30 minutes. Finally, cells were washed four times in cold buffer and analysed in duplicate on a Cytomics FC 500 flow cytometer (Beckman Coulter, Wycombe, UK). We prepared a pooled leukocyte preparation from multiple donors to act as a single internal quality control (QC). This QC was fixed in Optilyse C, stored frozen in aliquots

and a new aliquot was labelled with antibodies freshly each analysis day. We analysed leukocytes in batches of 10 in the following order; first from an older in duplicate (1,2), followed by younger leukocytes in duplicate (3,4), then repeated this on other older and younger cells (5-8) before analysing the pooled QC leukocytes (9-10). If the variance between duplicates or QC batches was greater than 10%, samples were re-analysed. Positive staining for each sample was defined as fluorescence with no more than 0.5% overlap into the respective isotype negative control. Viable cells were gated for CD4 expression, then α-enolase expression was analysed in 2000 $CD4^+$ T cells.

2.9 Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was used for cDNA synthesis. Real-time qPCR reactions were performed using cDNA (1ug) amplified in a standard 40-cycle SYBR® green real-time PCR reaction. Optimised, sequence-specific pre-validated primers for α-enolase and YWHAZ commercially available from Qiagen. YWHAZ was used for normalization. Relative fold difference in gene expression using the comparative CT method and statistical analysis were determined using the freely available Relative Expression Software Tool (REST 2009, www.qiagen.com).

2.10 Case-control cardiovascular disease cohort

Ten consecutive patients (mean age 57.5 years) admitted to hospital with proven symptomatic coronary artery disease (CAD) were recruited with ten age-matched controls (mean age 60.5 years). CAD was defined as the presence of one or more >50% stenosis on invasive coronary angiography, previous myocardial infarction (MI) and/or previous coronary revascularisation. Within the hospital, healthy controls were recruited who were free from CAD (determined either

by invasive coronary angiography or computed tomography [CT] coronary angiography and calcium scoring). Patients and controls were selected from City Hospital, Birmingham; and Sandwell General Hospital, West Bromwich, West Midlands, United Kingdom.

Non-fasting peripheral venous blood samples were obtained from all of the study subjects with a 21-gauge needle with minimal stasis into commercial tubes (Greiner Bio-One, Gloucestershire, United Kingdom) containing EDTA (for flow cytometric analysis). Samples were immediately fixed by adding 50µl blood into 500µl Optilyse and were then stored at -80^{o}C until analysis. The study was performed in accordance with the Helsinki Declaration and approved by the Warwickshire Research Ethics Committee. All of the participants provided written, informed consent.

2.10 Data analysis

For comparison of values between young and older adult male groups, data from each group was checked for normality using the Kolmogorov-Smirnov test. A parametric independent samples t-test was applied to the data and significance set at $p < 0.05$. Where necessary, data were log transformed. Data was analysed using SPSS statistical package version 20.0 for Windows (SPSS Inc, USA).

3 RESULTS

3.1 **Biotinylation of primary CD4$^+$ T cells using Sulfo-NHS-SS Biotin reagent**

Using streptavidin SA-Alexa Fluor® 488 to visualize protein biotinylation on non-permeable and permeabilised T cells, the absence of signal on non-biotinylated CD4$^+$ T cells treated with SA-Alexa Fluor® 488 (Figure 1A) demonstrates that non-specific binding of SA-Alexa Fluor® 488 to T cell membranes is negligible. Sulfo-NHS-SS-Biotin labelled CD4$^+$ T cells with SA-Alexa Fluor® 488 detection (Figure 1B) clearly show concentrated signal at the cell surface and absence of intracellular fluorescence indicating successful specific biotinylation of CD4$^+$ T cell exofacial membrane proteins only. Permeabilized CD4$^+$ T cells (Figure 1C) co-stained with Sulfo-NHS-SS-Biotin and SA-Alexa Fluor® 488 show signal concentrated to the cell surface with minimal intracellular labelling. These data are in agreement with others (19) and infer that biotin-labelled CD4$^+$ T cell proteins are at the exofacial cell surface of the plasma membrane.

3.2 **Purification of T cell plasma membrane proteins**

To confirm the specificity of the method described by Zhao and colleagues (20) for recovery of plasma membrane but not cytosolic proteins, unlabelled cells from a Jurkat T cell line were incubated with streptavidin magnetic beads as a negative control. In these experiments no purified membrane or total protein was observed (data not shown). CD3, LDH and β-actin were used as markers of CD4$^+$ T cell plasma membrane, cytosolic and cytoskeletal proteins respectively. As shown in Figure 2, after labelling exofacial proteins with biotin and purification using SA beads, CD4$^+$ T cell plasma membrane proteins (e.g. CD3) are present in the PNS and SA-bead elution fraction but absent in the unbound fraction, whereas cytosolic proteins (e.g.

LDH) are present in the unbound fraction and absent in the SA-bead eluate. Cytoskeletal proteins (e.g. β-actin) are prominent in PNS and unbound fraction and greatly reduced in the eluate from SA beads. Together these data suggest that $CD4^+$ T cell plasma membrane proteins are considerably separated from cytosolic and cytoskeletal contaminants using this technique.

3.3 **Age associated differences in human $CD4^+$ T cell membrane proteome**

Healthy, non-smoking young (aged 18-35 years old) and older adult males (50-70 years old), were recruited for analysis of the human $CD4^+$ T cell membrane proteome. Biochemical and anthropometric measurements confirmed that there were no significant differences observed between the two age groups for blood glucose, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides or body mass index (Table 1).

To assess age related differences between young and older adult males, $CD4^+$ T cells were isolated from whole blood and membrane proteins extracted (described in experimental procedures section). Equal amounts of membrane proteins from young and older adult T cells were pooled into two age-defined groups and separated by 2DE (Figure 3). Differences to seventeen protein spots (ten spot densities increased and seven decreased in the older adult group when compared to the young adult group) were observed between young and older adults when criteria $p < 0.05$ and > 1.4 fold difference was applied. Four discrete and abundant spots which could be accurately excised, were analysed LC-MS/MS and protein identification was achieved for two spots as summarized in Table 2, including human α-enolase.

3.4 **Validation of age related differences to CD4$^+$ T cell membrane α-enolase and total enolase expression by flow cytometry and qPCR**

To validate the protein expression difference identified by 2DE analysis in whole blood, cell surface levels of α-enolase on CD4$^+$ T cells between an independent group of young (n=8) and older (n =11) male adults was analysed by flow cytometry. There were no significant differences observed between the two age groups for blood glucose, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides or body mass index and anthropometric data for these samples can be found in supplementary Table 1. Cell surface α-enolase was measured in the CD4$^+$ T cell positive population (Figure 4A and 4B). A reduction in α-enolase on CD4$^+$ T cells was demonstrated in the older compared to young male adult group (young group; 50.47 ± 13.26 Vs. older group; 38.98 ± 10.25 median fluorescence, p = 0.048, Figure 4C). The majority of CD4$^+$ cells were α-enolase positive; 3.1% of CD4$^+$ T cells from younger donors were α-enolase negative compared to 8.7% of CD4$^+$ T cells from older donors. Total intra- and extra-cellular α-enolase expression was also lower in a subset of six older (51-55 years) Vs younger (24-30 years) subjects (p=0.03, Figure 4D). The decrease in cellular α-enolase expression in older adults was evident at the level of mRNA expression in older adults (p=0.029, Figure 4E).

3.5 **Cardiovascular associated differences in CD4$^+$ T cell membrane α-enolase**

To investigate whether the loss in α-enolase on CD4$^+$ T cells during ageing was related to health status and knowing that α-enolase is a plasminogen activator, we examined α-enolase expression in a case-control study of age-matched cardiovascular disease patients. There was no significant difference in age between case and control groups. A significant reduction (p=0.018) in α-enolase on CD4$^+$ T cells was demonstrated in cardiovascular disease patients (Figure 4F).

4 DISCUSSION

To our knowledge this is the first study to demonstrate (a) primary $CD4^+$ T cell membrane protein enrichment and isolation using a biotinylation reagent; (b) age-related differences in α-enolase expression; and (c) cardiovascular disease-associated loss of α-enolase at the $CD4^+$ T cell membrane. Ageing and cardiovascular disease are associated with a decline in specific acquired immune response. Recent studies have indicated an immune-modulatory role for surface α-enolase (25). Our findings suggest that further investigation of surface α-enolase function on specific subsets of $CD4^+$ T cells during these conditions is merited to explore the potential contribution of α-enolase-dependent function immune decline.

Using a previously described biotinylation enrichment method (19-20), we labelled lysine residues at the $CD4^+$ T cell membrane with an impermeable biotinylation reagent which contains a disulphide bond. This disulphide bond becomes reduced in the intracellular environment, and thus on cell entry, no biotin is bound to lysine residues present on intracellular proteins. We provide evidence of a membrane protein fraction devoid of cytosolic protein contamination from primary $CD4^+$ T cells by western blotting and confocal microscopy. The main purpose of this study was to identify age-related differences to the $CD4^+$ T cell membrane proteome by 2DE. Protein expression profiles for $CD4^+$ T cell membrane proteins were compared between young and older male adult groups. In total, seventeen protein spots were altered and from four protein spots which could be accurately excised, one protein, α-enolase which was identified by LC-MS/MS, was decreased significantly in the older age group.

Intracellularly, α-enolase is known as a key glycolytic enzyme, responsible for catalysing the dehydration of 2-phosphoglycerate to phosphoenolpyruvate in the final steps of glycolysis. Aside from this well documented glycolytic activity, α-enolase has also more recently been implicated in many other biological and physiological processes (for a comprehensive review the reader is directed to Diaz-Ramos et al. (26)). α-Enolase has been detected on the surface of vascular endothelium (27) various cell types including monocytes, macrophages, B cells and T cells (28) and is recognized as a plasminogen receptor.

Interaction between plasminogen and α-enolase at the cell surface has been shown to enhance its activation and concentrate plasmin-associated protease activity. A previous study undertaken by Aillaud and colleagues (29) in 20 young and 20 elderly individuals reported an age associated increase in the plasma level of plasminogen activator inhibitor-1, which inhibits plasmin production from plasminogen, with a likely increase in propensity for clotting; there is evidence that this is further increased in healthy centenarians compared to healthy elderly individuals (30). Another reported function for plasmin lies in the processing of integrins and therefore a decrease in localised plasminogen activation, following from loss of α-enolase on older adult T cells, may affect cell migration (31).

The surface difference in α-enolase expression identified by 2DE approaches was verified in whole blood taken from volunteers of different ages by flow cytometry. The loss of enolase spot volume by 2DE may be in part due to a post-translational modification which altered enolase migration in the gel. However, we confirmed that there was significantly lower surface enolase expression in a second independent cohort of 8 young and 11 older adults. This proof of concept

was undertaken by analysis of individual subject CD4+ T cells using an antibody-based, flow cytometric method which did not require any pre-extraction of sample. The distribution of α-enolase was generally lower in older adults with a noticeable loss of $CD4^+$ cells that expressed high levels of α-enolase. This may reflect an age-associated difference in specific $CD4^+$ subset frequency (32) which merits future investigation for association with specific T cell subsets and effects on function. While it could reflect a more generalised failure to traffic α-enolase to the surface in ageing T cells, this is less likely as our data suggests that α-enolase mRNA transcription is lower in T cells from older adults. Lower α-enolase expression with ageing implies a more refractory phenotype as T cells tend to tune down glycolysis in a resting state because higher energy flux with ATP production is not required for non-proliferating cells (33). The association of α-enolase with caveolar proteins, present in lipid rafts, regulates its subcellular localization (34). In ageing T cells fewer proteins are associated in rafts in the resting state but there is a failure of protein mobility within rafts during activation (35). This contrasts with senescent endothelial cells which showed significantly increased numbers of caveolae and associated proteins and promotion of anti-inflammatory pathways (36).

The health-status of the young and older populations studied here were self-declared and based on lack of regular medication, however, the α-enolase expression was not always lower than the 5^{th} percentile of young adult expression. To investigate whether this is associated with vascular disease, we explored the expression of T cell surface α-enolase in cardiovascular disease patients compared to age-matched controls. In common with ageing, the presence of vascular disease in patients aged between 50 and 72 years was also associated with even lower surface α-enolase expression compared to age-matched healthy controls. There is increasing interest in the role of

T cells within the arterial wall and they have been implicated in the genesis of hypertension, vascular dysfunction, and cardiac fibrosis (37, 38). Indeed, Tregs were shown to ameliorate hypertension-driven vascular dysfunction through a paracrine-dependent mechanism raising a possible role for T cell-mediated therapeutics for vascular disease (14, 38).

In addition to a difference in α-enolase expression, several other proteins were identified as altered between the young and older groups and excised for LC-MS/MS analysis. One protein spot appears to be two proteins of similar molecular weights and isoelectric point; both pro-interleukin-16 and T-complex protein 1 subunit alpha were identified with high peptide cleavage and sequence coverage. A further two excised spots were unidentified. To improve speed of peptide extraction, recovery and coverage from excised membrane proteins we used an acid-labile surfactant during tryptic digestion. However, the frequency of arginine and lysine residues is less in hydrophobic membrane proteins, resulting in fewer potential tryptic cleavage sites which may have contributed to our difficulty in identifying them. This was likely further compounded by labelling of lysine residues with biotin reagent for membrane protein enrichment of samples thereby reducing the available number of cleavage sites.

Here, reduced α-enolase levels at the $CD4^+$ T cell surface were observed in a healthy older compared to young male group and in a cardiovascular disease group compared to age-matched controls. Lower α-enolase levels in older adults may impact on plasminogen concentration at the T cell surface, plasminogen activation, plasmin proteolytic activity and plasmin-mediated intracellular signalling. Since lower surface α-enolase is evident during healthy ageing and is

lowered further in cardiovascular disease patients, its role in predisposition to vascular disease in older adults merits further investigation.

5 CONCLUSION

Using subcellular fractionation and gel-based proteomics, we have shown that cell surface expression of α-enolase is lower in older adults compared to younger people and in cardiovascular disease. This is the first study to identify an alteration in the expression of α-enolase on the $CD4^+$ T cell surface. Whether this impacts on the plasmin/plasminogen proteolytic system and T cell function remains to be investigated.

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FIGURE LEGENDS

Figure 1. Biotinylation of CD4$^+$ T cell membrane proteins using Sulfo-NHS-SS-Biotin. CD4$^+$ T cells were stained with SA-Alexafluor488 in the absence of Sulfo-NHS-SS-Biotin reagent (A). CD4$^+$ T cells incubated with Sulfo-NHS-SS-Biotin reagent followed by no permeabilisation (B) or permeabilisation with Triton X-100 (C) prior to staining with SA-Alexa Fluor® 488. These data are representative of 3 independent experiments with T cells from male subjects between 22 and 56 years where the scale bars shown are 5μm.

Figure 2. Membrane protein purity. CD4$^+$ T cell plasma membrane protein purity was assessed by measuring abundance of cytoplasmic protein (lactate dehydrogenase; LDH), plasma membrane protein (cluster of differentiation 3; CD3) and cytoplasmic associated protein (β-actin) markers in post nuclear supernatant (PNS), unbound (UB) and eluted fractions (E) by western blot analysis (one gel representative of three independent experiments with T cells from male subjects between 22 and 56 years).

Figure 3. CD4$^+$ T cell membrane protein separation by 2DE. Representative 2DE image of human CD4$^+$ T cell membrane proteins (n=7, from two pools). Membrane proteins were extracted and then separated on a pH 3-10 immobilised gradient and resolved on 8-16% SDS-PAGE gels. Gels were visualised with Flamingo fluorescent total protein stain. Spot matching and data analysis was performed using Progenesis SameSpots software. Arrows indicate four discrete and abundant spots, altered between young and older groups, which were excised for protein identification by LC-MS/MS. Spot numbers correspond to spot identification numbers in Table 2.

Figure 4. Cell surface α-enolase levels are decreased on primary $CD4^+$ T cells with age. Cell surface α-enolase levels on $CD4^+$ T cells were examined by flow cytometry. (A) Cells were separated by forward and side scatter and α-enolase levels measured on $CD4^+$ T cells (white and filled peaks represent isotype control and anti-CD4 antibodies in the upper panel and isotype control and anti-α-enolase antibodies in the lower panel). (B) The comparative distribution of surface α-enolase on younger (red) versus older (blue) male $CD4^+$ T cells relative to isotype control (black). (C) The age dependent difference in cell surface α-enolase levels on $CD4^+$ T cells from 2DE analysis were validated in a younger (n = 8, mean age 27 years) and older (n =11, mean age 59 years) group. Values are expressed as median fluorescence and data are presented as a box plot. Percentiles are represented by the box (25th and 75th) and whisker (10th and 90th). * $p = 0.048$. (D) Total and surface α-enolase levels on $CD4^+$ T cells in six younger and older adults were analysed by flow cytometry. The box represents 25th and 75^{th} percentiles and the whisker, 10th and 90th. * $p < 0.05$. (E) $CD4^+$ T cell α-enolase mRNA expression relative to YWHAZ calculated using the ΔΔCt method. (F) Cell surface α-enolase levels are decreased on primary $CD4^+$ T cells from patients with cardiovascular disease (CVD; mean age = 57.5 years) compared to healthy controls (Mean age 60.5 years). Values are expressed as median fluorescence and data are presented as a box plot with whiskers representing the 95% confidence intervals. * $p = 0.018$.

Figure 1.

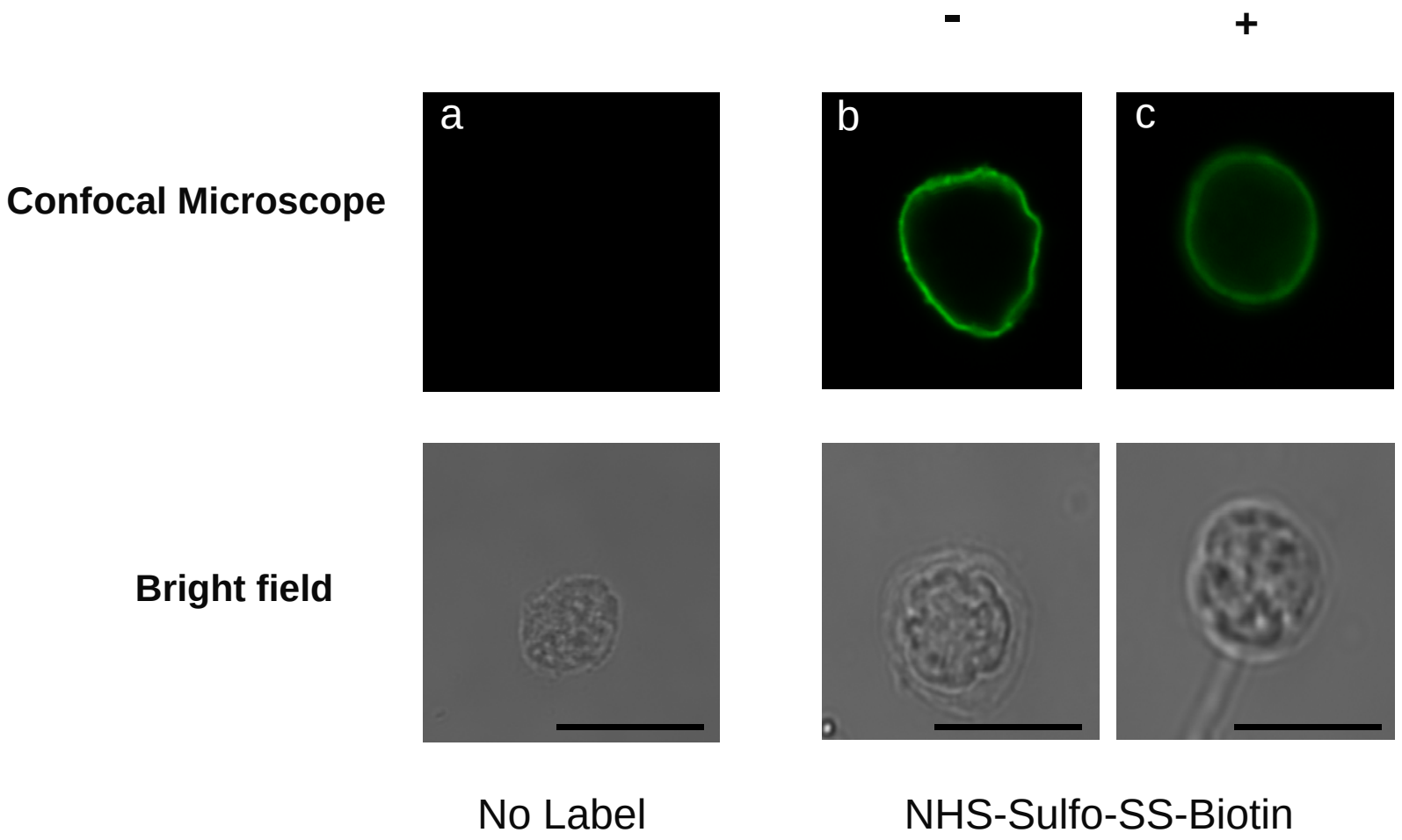
-
+
Confocal Microscope
a
b
c
Bright field
No Label
NHS-Sulfo-SS-Biotin

Figure 2.

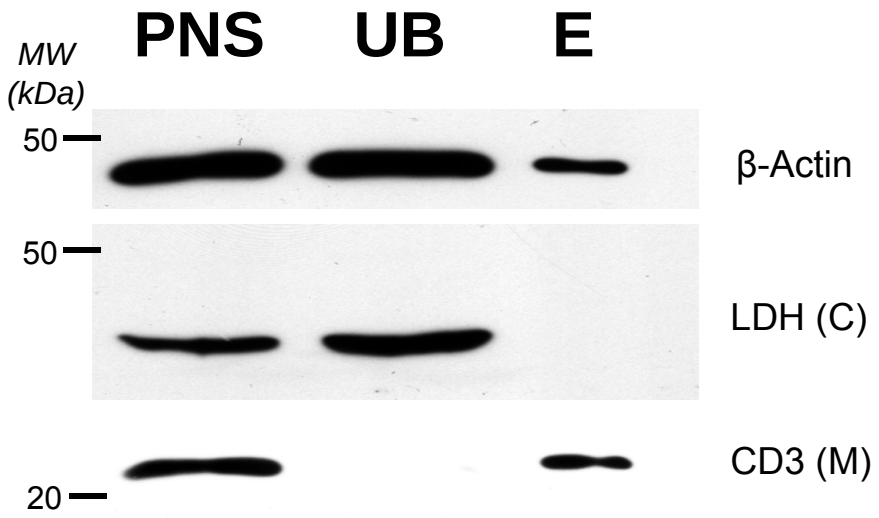

Figure 3.

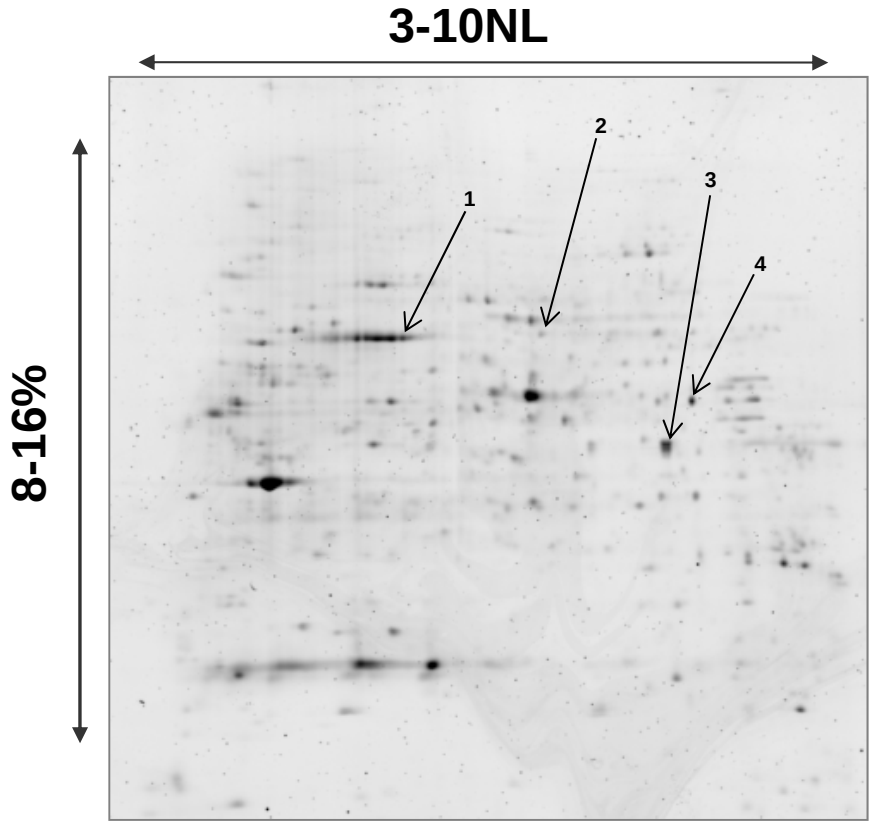

Figure 4. Bennett et al.

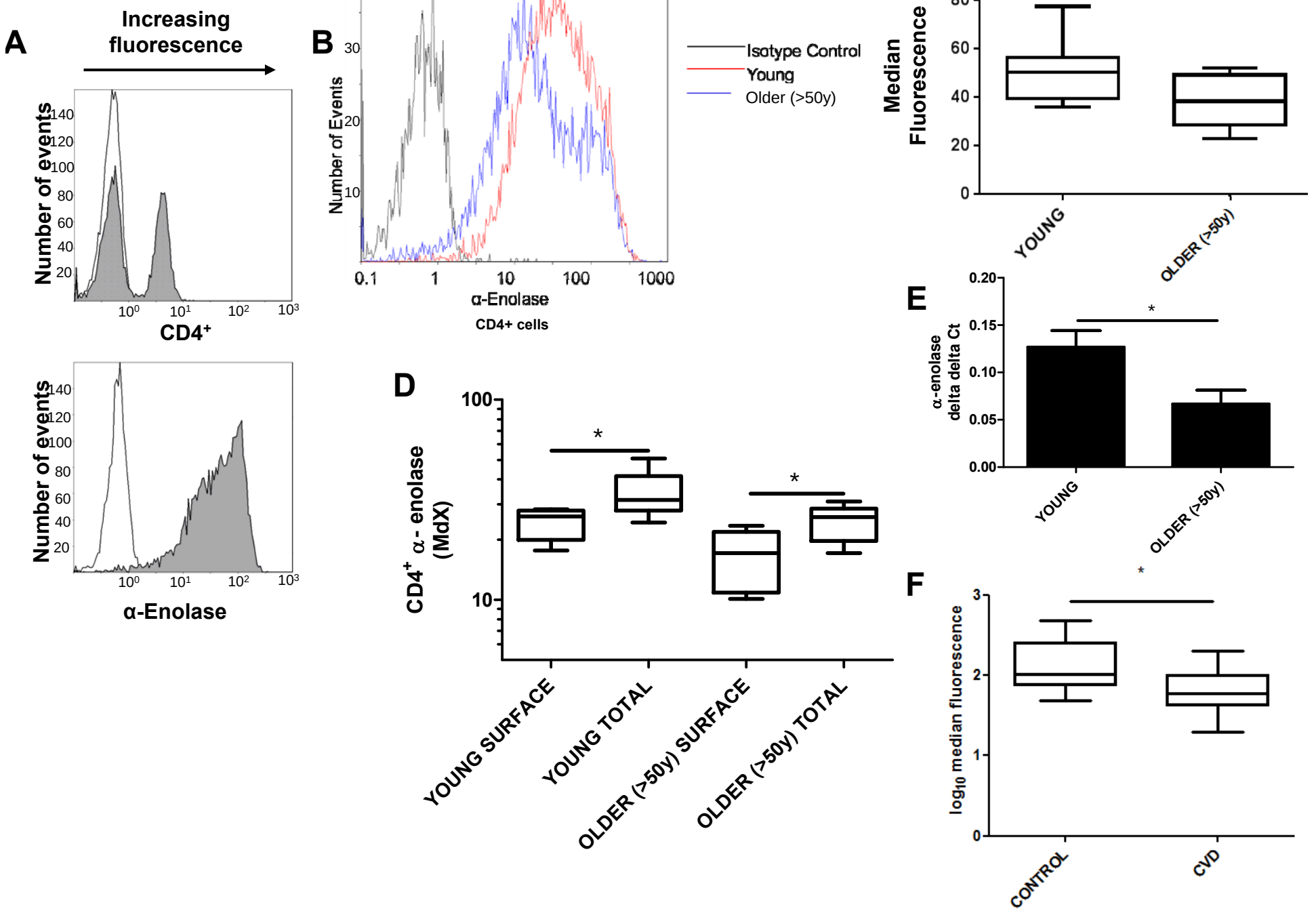

Table 1. Biochemical and anthropometric measurements in volunteers for 2DE study.

	Young adult (n=10)	Older adult (n=9)
Age (years)	26 ± 2	56 ± 5*
BMI	24.0 ± 3.3	25.0 ± 2.1
Waist to hip ratio	0.90 ± 0.06	0.94 ± 0.05
Glucose (mmol/L)	5.21 ± 0.44	5.30 ± 0.57
Triglycerides (mmol/L)	1.11 ± 0.10	1.12 ± 0.06
Cholesterol (mmol/L)	4.26 ± 1.19	4.93 ± 0.95
LDL Cholesterol (mmol/L)	2.89 ± 1.18	3.43 ± 0.98
HDL Cholesterol (mmol/L)	1.00 ± 0.21	1.11 ± 0.27

Values expressed as mean ± SD; * difference relative to young ($p < 0.001$).

Table 2. Protein identification by LC-MS/MS

Spot Number	Fold Change (relative to younger; p<0.05)	Protein	Accession Number (NCBInr database)	Δ Score	Unique Peptides Identified	Amino acid Coverage (%)
1	↑ 1.4	pro-IL 16 isoform 1	27262655	660.96	17	46.28
		T-complex protein 1 subunit α	57863257	610.68	15	47.66
3	↓ 1.5	human Enolase-1	203282367	470.94	17	73.21