Exploring the potential of oxidative stress-related biomarkers of ageing in a population-based study of the very old

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Abstract

There are considerable differences in the timing, type and extent of age-related decline between individuals who share the same chronological age, which may be driven by a combination of genetic, stochastic and environmental factors. Biomarkers of ageing (BoA) that can discriminate between individuals who differ in their biological age will therefore be useful to understand biological mechanisms, develop and test interventions and allow the prediction of age-related events so interventions can be implemented. In recent years, a variety of mechanistic candidate BoA have been discovered on the basis of a greatly improved understanding of the cellular and molecular biology of ageing. These include various measures of oxidative stress, which is thought to contribute causally to the ageing of organisms via its acceleration of cellular senescence. However, their reliability and validity as BoA, especially within population based cohorts are scarce. This study therefore focused on various oxidative stress-related measures as candidate BoA including: reactive oxygen species (ROS) production from dysfunctional mitochondria, by measuring superoxide levels, mitochondrial mass and mitochondrial membrane potential in blood mononuclear cells by flow cytometry; and also markers of lipid peroxidation, F_2-isoprostanes, by measuring 8-iso Prostaglandin $F_{2\alpha}$ by Automated Dissociation Enhanced Lanthanide Fluorescence Immunoassay (AutoDELFIA). Despite providing evidence of experimental reliability for all measures and also some evidence of construct validity for ROS production from dysfunctional mitochondria in terms of: associations with chronological age, associations with some markers of oxidative stress-induced cellular senescence, validation in a dietary restricted animal model of ageing and a role in an immunosenescent phenotype; there was no evidence of predictive validity in terms of longevity or age-related health outcomes in a population based cohort of the very old, the Newcastle 85+ study. This questions the predictive validity of these parameters as candidate BoA in the very old population.

Acknowledgements

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Publications

Published papers in peer-reviewed journals

- **Wiley, L.,** Ashok, D., Martin-Ruiz, C., Talbot, D.C.S., Collerton, J., Kingston, A., Davies, K., Chinnery, P.F., Jagger, C., Kirkwood, T.B.L., von Zglinicki, T. (2014) ‘Reactive oxygen species production and mitochondrial dysfunction in white blood cells are not valid biomarkers of ageing in the very old’, *PLoS ONE*, 9(3), pp e91005.

- Gardner, M., Bann, D., **Wiley, L.,** Cooper, R., Hardy, R., Nitsch, D., Martin-Ruiz, C., Shiels, P., Sayer, A.A., Barbieri, M., Bekaert, S., Bischoff, C., Brooks-Wilson, A., Chen, W., Cooper, C., Christensen, K., De Meyer, T., Deary, I., Der, G., Diez Roux, A., Fitzpatrick, A., Hajat, A., Halaschek-Wiener, J., Harris, S., Hunt, S. C., Jagger, C., Jeon, H. S., Kaplan, R., Kimura, M., Lansdorp, P., Li, C., Maeda, T., Mangino, M., Nawrot, T.S., Nilsson, P., Nordfjall, K., Paolisso, G., Ren, F., Riabowol, K., Robertson, T., Roos, G., Staessen, J. A., Spector, T., Tang, N., Unryn, B., van der Harst, P., Woo, J., Xing, C., Yadegarfar, M.E., Park, J.Y., Young, N., Kuh, D., von Zglinicki, T., Ben-Shlomo, Y., Halcyon study team. (2013) ‘Gender and telomere length: systematic review and meta-analysis’, *Experimental Gerontology*, 51, pp 15-27.

- Martin-Ruiz, C., Jagger, C., Kingston, A., Collerton, J., Catt, M., Davies, K., Dunn, M., Hilkens, C., Keavney, B., Pearce, S., den Elzen, W., Talbot, D.C.S., **Wiley, L.,** Bond, J., Mathers, J., Eccles, M., Robinson, L., James, O., Kirkwood, T.B.L., von Zglinicki, T. ‘Assessment of a large panel of candidate biomarkers of ageing in the Newcastle 85+ study’, *Mechanisms of Ageing and Development* 2011, 132(10), pp 496-502.

Published abstracts from conference presentations

- **Wiley, L.,** Ashok, D., Martin-Ruiz, C., Talbot, D.C.S., Collerton, J., Kingston, A., Davies, K., Chinnery, P.F., Jagger, C., Kirkwood, T.B.L., von Zglinicki, T. (2014) **‘**Exploring the potential of oxidative stress-related biomarkers of ageing in a population-based study of the very old’, *Mutagenesis*, 29, pp e3.

Table of Contents

List of Figures

List of Tables

Abbreviations

ADL	Activities of daily living
ADP	Adenonine diphosphate
ATP	Adenosine triphosphate
AU	Arbitrary units
AUROC	Area under the receiver operating curve
AutoDELFIA	Automated Dissociation Enhanced Fluorescence Immunoassay
BoA	Biomarker of ageing
bp	Base pairs
CD3+/CD8+	Cytotoxic T Lymphocytes
$CD4^+$	Helper T Lymphocytes
CD8+/CD56+	Natural Killer T lymphocytes
CD8+CD56-CD27-	Senescent Memory T lymphocytes
CD8+CD56-CD27+	Naïve and Non-Senescent Memory T lymphocytes
COPD	Chronic obstructive pulmonary disease
CoQ	Coenzyme Q
CRP	C reactive protein
CV	Coefficient of variation
Cyt c	Cytochrome c
DHE	Dihydroethodium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ERPHO	Eastern Region Public Health Observatory
EU^+	Europium
FADU	Fluorometric analysis of DNA unwinding
FBS	Fetal bovine serum
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
FL1	Green fluorescent channel
FL3	Red fluorescent channel
FSC	Forward scatter (cell size)

GCMS	Gas Chromatography Mass Spectroscopy
Gy	Grays
H_2O	Water
HDL	High density lipoproteins
HLE	Healthy life expectancy
TERT	Telomerase reverse transcriptase
IAH	Institute for Ageing and Health
IL-6	Interleukin 6
IQR	Inter quartile range
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide
LCMS/MS	Liquid Chromatography Tandem Mass Spectroscopy
LDL	Low density lipoproteins
LE	Life epectancy
LiHep	Litium heparin
LOD	Limit of detection
m/z	Mass to charge ratio
mtDNA	Mitochondrial DNA
n/a	Not available
NADH	Nicotine adenine dinucleotide
NAD	Nicotinamide adenine dinucleotide
O_2	Oxygen
^oC	Degrees centigrade
ONS	Office For National Statistics
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polyerase chain reaction
QC	Quality control
PEFR	Peak expiratory flow rate
r	Correlation coefficient
RIA	Radio immunoassay
ROS	Reactive oxygen species
rpm	Revolutions per minute
SASP	Senescence associated secretory phenotype

SD	Standard deviation
SMMSE	Standardized Mini-Mental State Examination
SSC	Side scatter (cell shape/granularity)
TNF-α	Tumour necrosis factor-alpha
TR-FIA	Time-resolved fluoro immunoassay
u/k	Unknown
UK	United Kingdom
US	United States
UN	United Nations
$	Truncation
adj	Adjacent
n/t	Not tested
NT-pro BNP	N-terminal pro b-type natriuretic peptide
TUG	Timed up and go
Free T3	Free triiodothyronine
Free T4	Free thyroxine
HbA1c	Glycosylated haemoglobin
RhF	Rheumatoid factor

Chapter 1. Introduction

1.1. Concerns of increasing life expectancy

1.1.1. Population ageing

It is well acknowledged that the human population worldwide is increasing. For example, in England alone for males and females respectively there was an estimated population of 23.21 and 24.49 million in 1990, which rose to 24.03 and 25.20 million in 2000, and 25.76 and 26.48 million in 2010 (Figure 1.1. A.) (Office for National Statistics (ONS), 2011a). The major reason for the increase in population is increasing life expectancy. For example, the average life expectancy at birth in England for males and females respectively was 72.85 and 78.43 years in 1990, which rose to 75.29 and 80.12 years in 2000, and 78.31 and 82.33 years in 2010 (Figure 1.1. B.) (ONS, 2011b). The age structure of the population worldwide is therefore changing where older individuals are becoming a proportionally larger share of the total population known as population ageing. For example, the proportion of the very old population in England (those aged 85 years and older) are currently the lowest however they are the fastest growing sector of the population (Figure 1.2.) (ONS, 2011c; ONS, 2011d; ONS, 2011e). Although population ageing is experienced more in developed countries, it is becoming more apparent in the developing world however at varying levels and in different time frames (Stefansson, 2005). It is predicted that by 2050 the proportion of the older population (those aged 60 years or older) in the world will exceed the younger population (those aged 15 years or under) for the first time in history (United Nations (UN), 2002).

1.1.2. Scenarios of increasing life expectancy

Since the older population are more vulnerable to longstanding illnesses and disabilities and report the worst self-reported health (Figures 1.3. A. and 1.3. B.), a major concern is an increase in the number of morbid years towards the end of life (ONS, 2010). There are however three possible scenarios of increasing life expectancy (Figure 1.4.) (Fries, 1996).

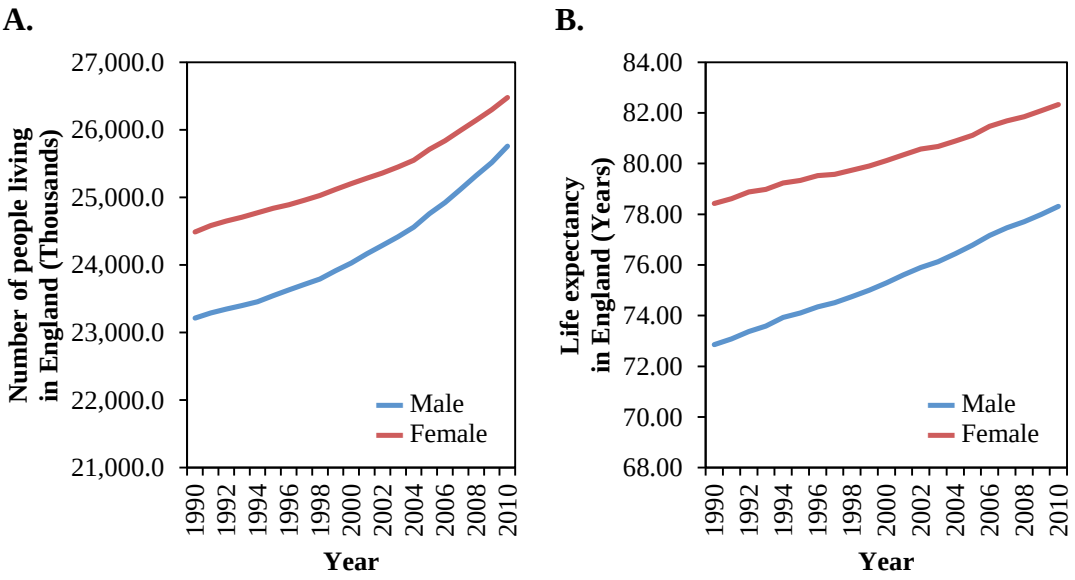


Figure 1.1. A. Mid-year population estimates and B. life expectancy at birth in England by gender between 1990-2010. (ONS, 2011a; ONS, 2011b). *(Chart was created from data tables provided)*

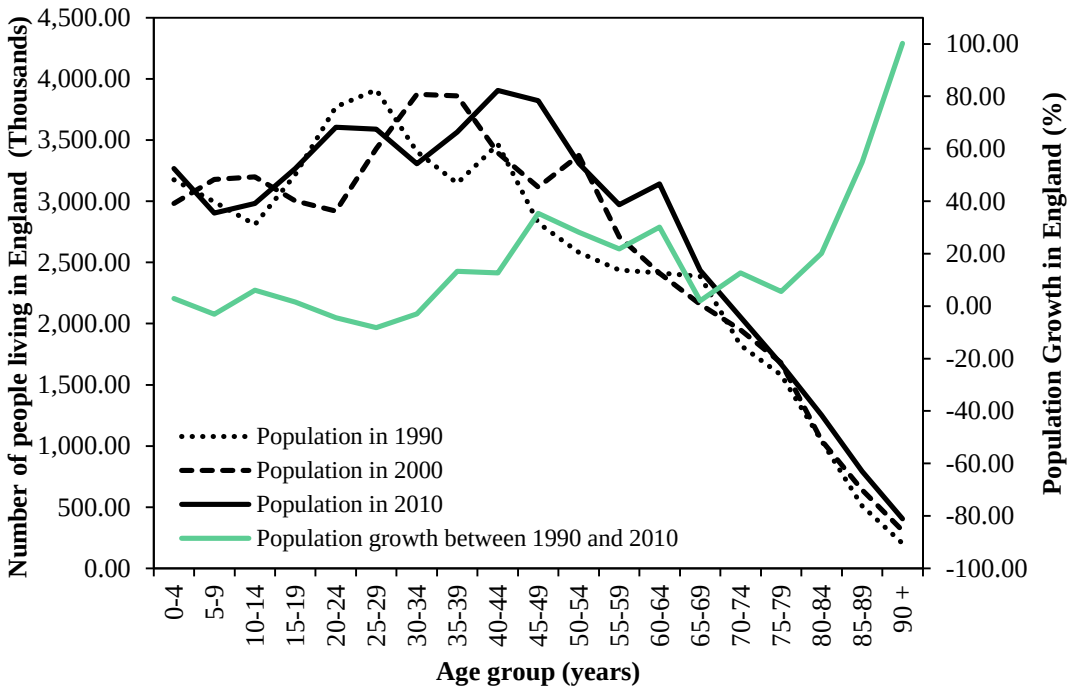


Figure 1.2. Mid-year population estimates and population growth in England by age group between 1990, 2000 and 2010. (ONS, 2011c; ONS, 2011d; ONS, 2011e). *(Chart was created from data tables provided)*

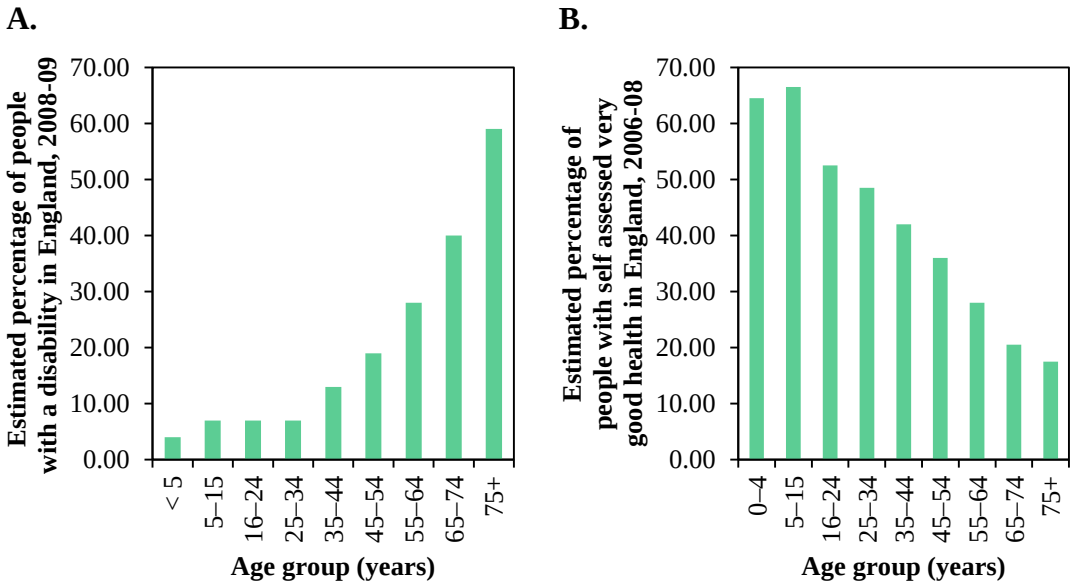


Figure 1.3. A. The percentage of people with a disability (including those with a limiting longstanding illness/es) in England by age group in 2008-09 and B. the percentage of people with self-reported 'very good health' in England by age group in 2006-08. (ONS, 2010). *(Chart was created from data tables provided)*

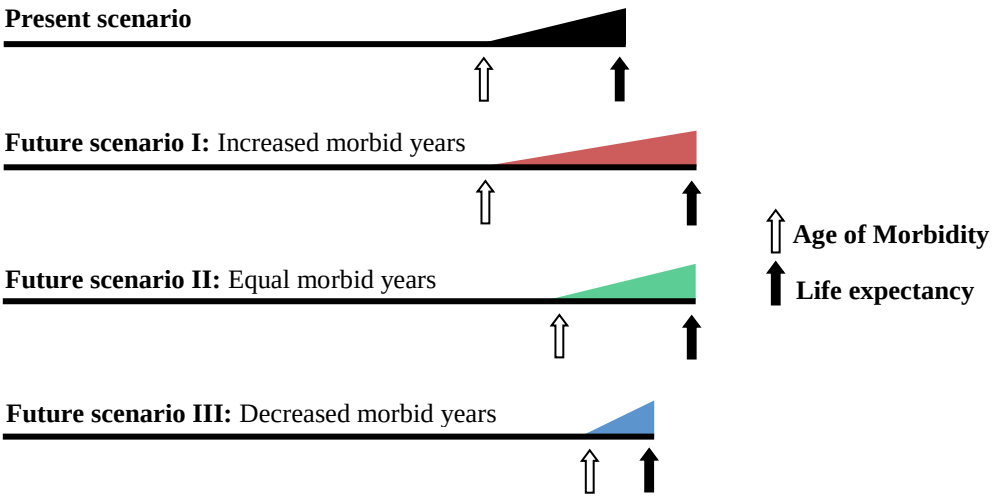


Figure 1.4. Future scenarios of increasing life expectancy. (Adapted from (Fries, 1996)).

Scenario I suggests the age of morbidity has not changed or is increasing slower than life expectancy so there is an expansion in the number of morbid years. Scenario II suggests the age of morbidity is increasing at the same rate as life expectancy and so the number of years of morbidity does not change. Scenario III suggests that the age of morbidity is increasing faster than life expectancy and so there is compression in the number of morbid years.

1.1.3. Current scenario within the United Kingdom (UK)

Demographic data shows that scenario III (compression of morbidity) has taken place in the United Kingdom (UK) between 2005-07 to 2008-10 (Figure 1.5. A.) however shows different scenarios for each constituent country of the UK (ONS, 2012). England and Wales showed scenario III (compression of morbidity) (Figures 1.5. B. and 1.5. C. respectively) where as Scotland (males only) and Northern Ireland showed scenario I (expansion of morbidity) (Figures 1.5. D. and 1.5. E. respectively) (ONS, 2012). Different scenarios for other countries are also shown. For example, scenario III (compression of morbidity) took place in Austria between 1978 and 1998 (Doblhammer and Kytir, 2001) where as scenario I (expansion of morbidity) took place in Australia between 1980 and 2003 and in the United States (US) between 1998 and 2008 (Lynch *et al.*, 2007; Crimmins and Beltran-Sanchez, 2011). The global outcome of increasing life expectancy is therefore uncertain and differs between population groups.

1.1.4. Implications in the expansion of morbidity

An expansion in the number of morbid years as shown for some countries will have great impacts on many economic, social and political factors. The retirement window will become larger putting higher financial demands on the working-age population. This is because larger contributions will be needed for taxes, pensions and investments to secure their future and support the current elderly population. This will also include contributions to increased medical costs and demands on health care services. It will impact socially on younger generations where longer care of elderly family members will be needed. There will be the need for improved information and analysis of the impact of an ageing population for potential policy changes which could involve

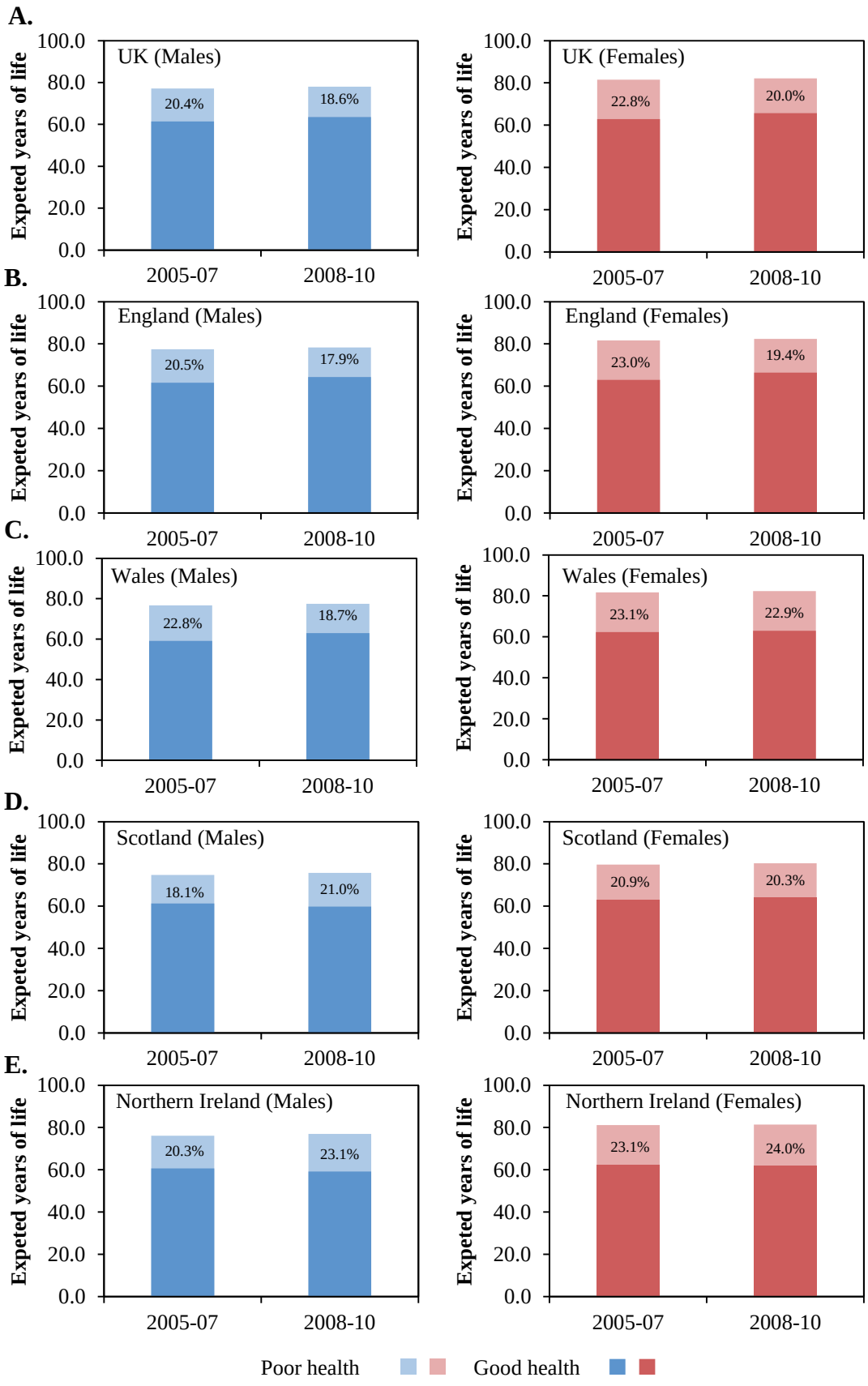


Figure 1.5. Life expectancy (LE) and healthy life expectancy (HLE) at birth in the UK and constituent countries in 2005-07 and 2008-10. (ONS, 2012). *(Chart was created from data tables provided)*

finances, transportation, housing, health services and infrastructure. This is "to ensure that people everywhere will be enabled to age with security and dignity and continue to participate in their societies as citizens with full rights" (UN, 2002). There is therefore a great importance in understanding the complex biology of ageing and its association with frailty and disease (Kirkwood, 2008). The aim is to acquire scientific insights that can help ensure the extra years of life we are gaining are as healthy, productive and enjoyable as possible.

1.2. Current understanding of the biology of ageing

The biological process of ageing is considered complex involving numerous genetic and non-genetic factors and to date, no overall definition exists. It is however generally accepted that biological ageing is characterised by the gradual decline of functional capacity over time increasing vulnerability to age-related frailty, disability, disease and eventually death (Kirkwood, 2008). This is the result of the lifelong accumulation of damage at the cellular and molecular level, which gradually overwhelms defence, repair and maintenance systems (Zglinicki, 2004). Recent advances in ageing research has identified potential mechanisms and products of this damage, such as those discussed in Section 1.4., which could potentially be used to measure ageing.

1.3. Biomarkers of ageing (BoA)

1.3.1. Definition of BoA

There are considerable differences between individuals with respect to the timing, type and extent of age-related decline driven by a combination of genetic, stochastic and environmental factors (Kirkwood, 2008). There is therefore the need to find biological measurements that can discriminate between individuals who share the same "chronological age" but differ in their "biological age". These so-called biomarkers of ageing (BoA) were first defined by Baker and Sprott in 1988 (Baker and Sprott, 1988) as "biological parameters of an organism that either alone or in some multivariate composition will, in the absence of disease, allow better prediction of functional capability at some late age than will chronological age". Understanding why individuals differ in their biological age through measuring BoA will be useful to understand

biological mechanisms, develop and test interventions, and allow the prediction of age-related events so that interventions can be implemented (Figure 1.6.). Overall, BoA will have the potential to help make sure the extra years of life we are gaining are spent in good health.

1.3.2. Investigating the reliability and validity of BoA

There are various steps to consider when investigating candidate BoA (Figure 1.6.). Firstly, the degree of measurement reliability needs to be established. This is greatly important in population studies to ensure the variability of candidate BoA reflects genuine inter-individual differences and not measurement error, especially when only one measurement may be obtained. Evidence of experimental and intra-individual stability should therefore be shown. Experimental stability concerns various handling factors during preparation and measurement, where significant effects can be controlled during experimentation. Intra-individual stability concerns the day-day behaviours or environmental conditions of an individual that may change or fluctuate candidate BoA and therefore impact on their day-day repeatability (Hershberger and Moskowitz, 2001; Nesselroade and Ram, 2004). These factors could be identified and controlled before experimentation or by using statistical approaches during data analysis (Hershberger and Moskowitz, 2001; Nesselroade and Ram, 2004). Examples of factors that could affect the intra-individual variability of candidate BoA include changes in diet, smoking, alcohol consumption, medication, sleep quality, physical activity, use of aids and appliances, social events and seasonal/environmental exposures. Secondly, evidence of construct validity should be shown which is defined here as the ability of a measurement to reflect the construct being investigated, in this case biological ageing. This is important to understand biological mechanisms and to develop and test interventions. BoA should therefore correlate but change independently of chronological age, reflect known mechanisms of ageing and be associated with age-related outcomes (Sprott, 2010). Analysis here could be confounded by inter-individual variation defined as individual differences among different people (Hershberger and Moskowitz, 2001). This could be fixed variables including age, gender, ethnicity, genetic differences such as mitochondrial haplogroup or genetic disease(s) or disorder(s), age of natural mother/fathers death, having offspring, having siblings,

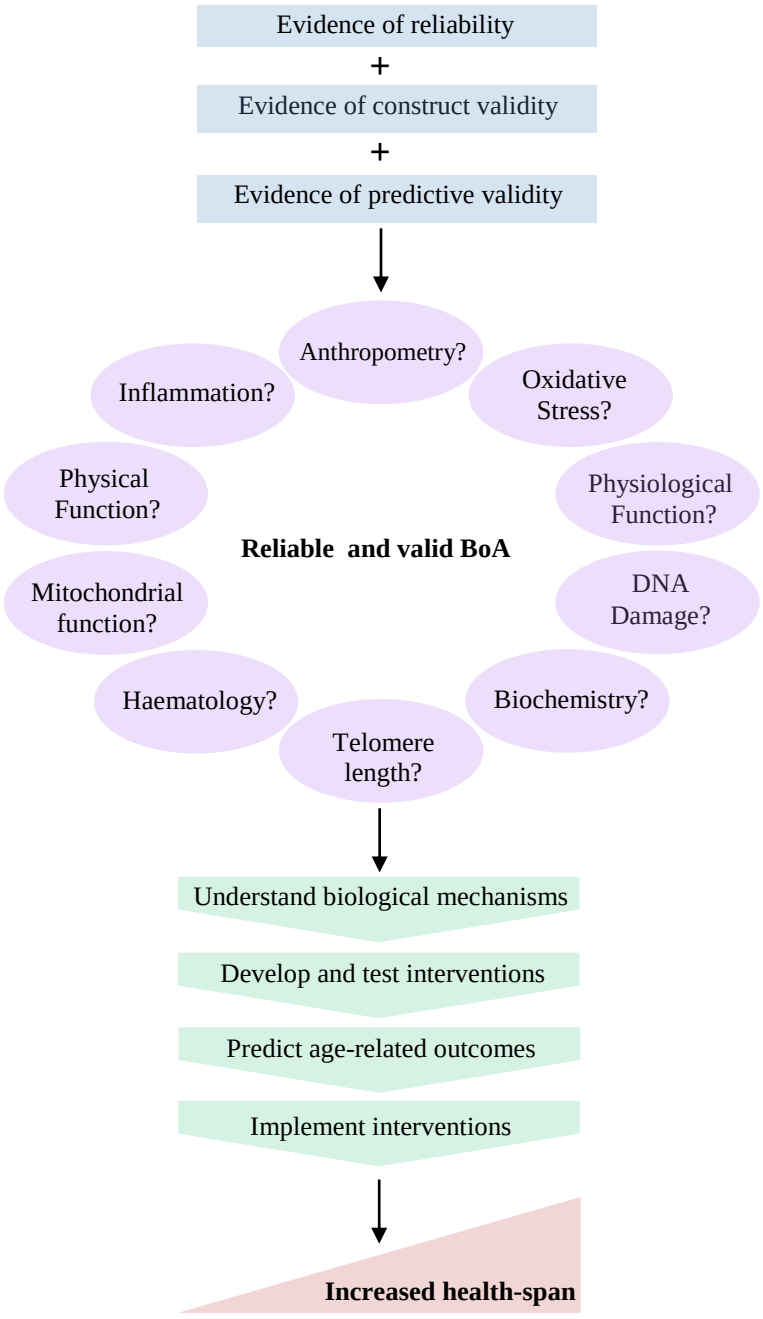


Figure 1.6. The identification and value of biomarkers of ageing (BoA).

education, employment history and/or life history events or could be changeable variables (as mentioned for inter-individual stability) such as diet, smoking, alcohol consumption, medication, sleep quality, physical activity, use of aids and appliances, social events and seasonal/environmental exposures. Again these factors could be identified and controlled before experimentation or by using statistical approaches during analysis (Hershberger and Moskowitz, 2001). The third and most important step when investigating candidate BoA is that they should be able to predict the years of remaining good health and the trajectory towards a wide range of age-related outcomes and mortality better than chronological age (Sprott, 2010). This is important to determine when the implementation of an intervention may be useful. Another criterion of BoA is that they should cause no harm when tested repeatedly. For example, analysis of blood sample or an image. They should also be technically simple enough to measure so most clinical laboratories can perform the test accurately and reproducibly without the need for specialized equipment or techniques.

1.3.3. Current status of efforts in BoA development

Research into the development of BoA began in the late 1960's where Alex Comfort first described the necessity and possibility of developing a technique to measure biological ageing rate in humans. This would then allow the identification and development of interventions that will increase health-span (Comfort, 1969). Since then the field of BoA development has exponentially increased (Figure 1.7.). Many candidate BoA have been proposed including various anthropometric (St-Onge, 2005), physical (Guralnik *et al.*, 1989), physiological (Masoro, 1988), haematological (particularly in relation to immunology) (DelaRosa *et al.*, 2006) and biochemical measurements (Stadtman, 1988) which will be termed here as "classical" BoA and also various cellular and molecular measures discovered by the advancing mechanistic aspects of biological ageing (Cristofalo, 1988) termed here as "newer" BoA. The proposal for many of these candidate BoA comes from evidence of construct validity including changes with chronological age, reflecting known mechanisms of ageing and/or be associated with age-related outcomes. However although the gold standard criteria of BoA, the predictive validity, has also been investigated for many of the proposed BoA in longitudinal population studies (Figure 1.8.) there are many inconsistencies in their findings. Therefore to date, no measure meeting the full criteria

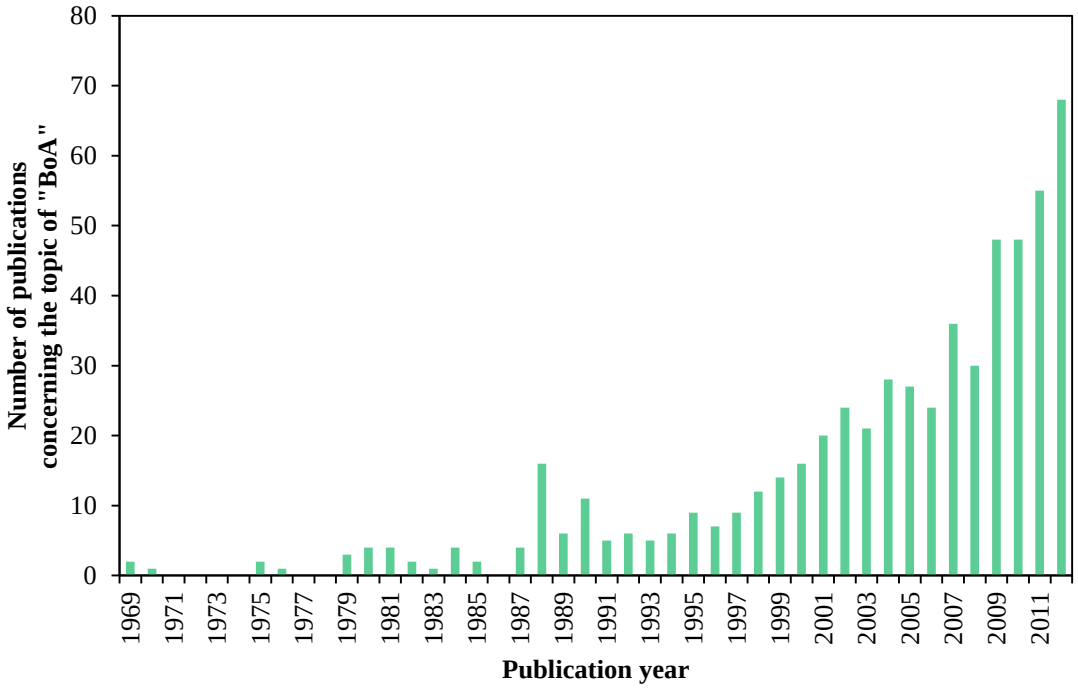


Figure 1.7. The exponential growth in the field of BoA development. The electronic database MEDLINE via the database provider OvidSP was searched up to 2012 for studies on the topic of BoA using the following search terms: [ageing adj marker$] OR [ageing adj biomarker$] OR [ageing adj measur$] OR [marker$ adj of adj ageing [biomarker$ adj of adj ageing [measur$ adj of adj ageing] OR [aging adj marker$] OR [aging adj biomarker$] OR [aging adj measur$] OR [marker$ adj of adj aging] OR [biomarker$ adj of adj aging] OR [measur$ adj of adj aging] OR [marker$ adj of adj biological adj ageing] [biomarker$ adj of adj biological adj ageing] OR [measur$ adj of adj biological adj ageing] OR [marker$ adj of adj biological adj aging] OR [biomarker$ adj of adj biological adj aging] OR [measur$ of adj biological adj aging] OR [marker$ adj of adj biological adj age] OR [biomarker$ adj of adj biological adj age] OR [measur$ adj of adj biological adj age] restricted to abstracts, studies in humans and from 1946 onwards. (adj: adjacent, $: truncation)

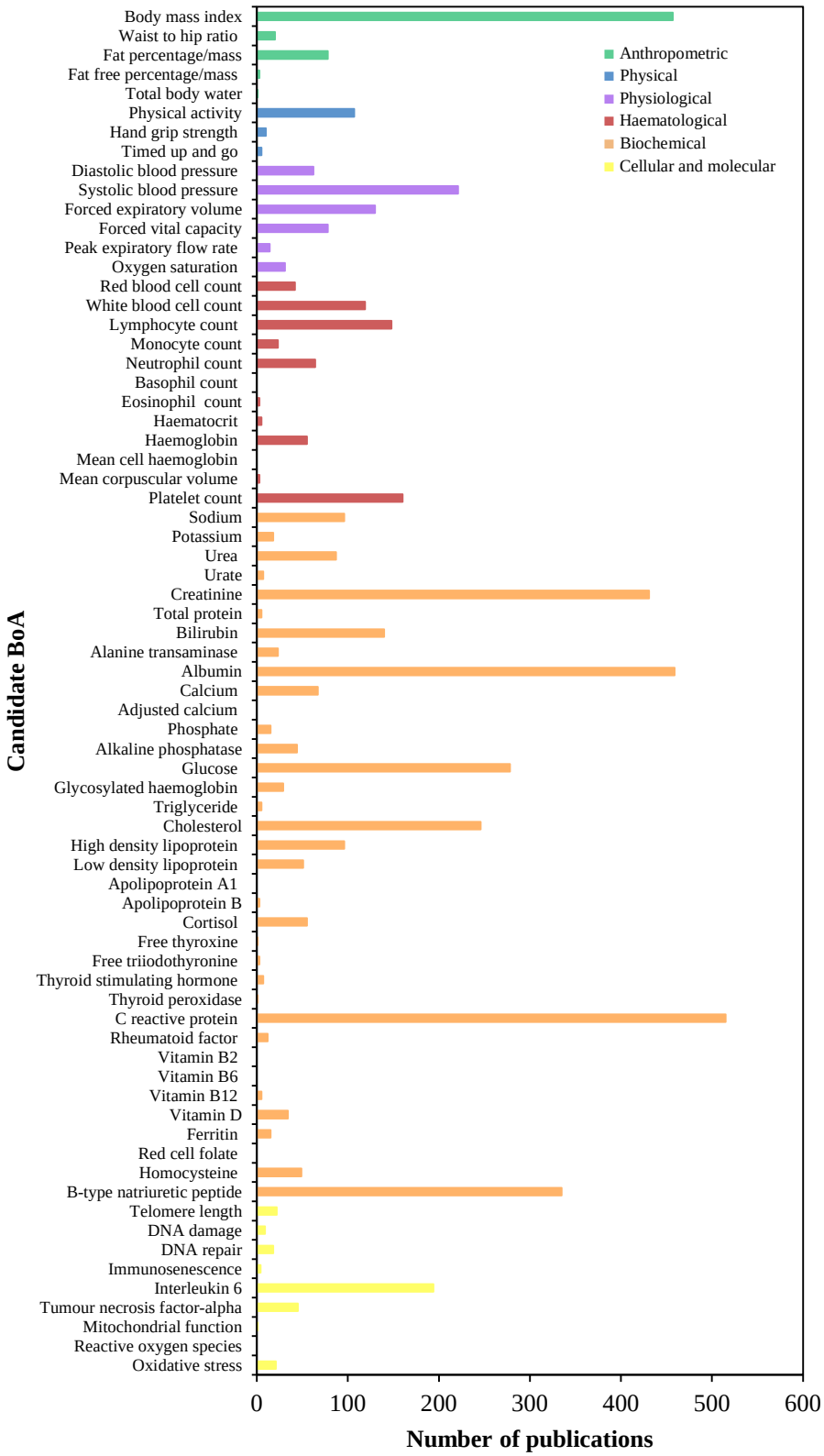


Figure 1.8. The number of studies investigating the predictive validity of various candidate BoA. The electronic database MEDLINE via OvidSP was searched up to November 2012 using specific search terms: [Candidate BoA* (*total of 71 using a combination of appropriate words/truncations)] AND [predict$] AND [survival OR death OR mortality OR $morbid$ OR disabil$ OR cognit$] restricted to abstracts, studies in humans and from 1946 onwards. Full details of the search terms are shown in Appendix A.

of BoA has been identified (Sprott, 2010). For example, this is shown for the most frequently investigated candidate BoA in the six main groups which include body mass index, physical activity, systolic blood pressure, platelet count, C reactive protein and interleukin-6 (Table 1.1.). Some studies find statistically significant predictions of age-related outcomes with these candidate BoA but others do not and some even find opposing directions of associations. The ideal BoA is one that can be applied to all members of the population at any given age however this data suggests that this is unlikely and the predictive power of a BoA changes between different populations. Identifying and providing explanations for these discrepancies will be important in BoA research. On one hand it could be differences in the quality of the studies such as sample size, the use of appropriate statistical techniques, adjustment of appropriate confounders, the reliability of the experimental technique that was used to measure the candidate BoA or it could be differences between population groups included such as age, gender, lifestyle habits, environmental influences and presence of disease(s) etc. For example, body mass index was positively associated and predictive of age-related outcomes in participants of the Melbourne Collaborative Cohort Study (Wong *et al.*, 2012), was negatively associated and predictive of age-related outcomes in participants of the PROTEGER study (Zhang *et al.*, 2012), however was not associated or predictive of age-related outcomes in another population-based cohort (Thrift *et al.*, 2012). This was also the case for other candidate BoA. Differences between these studies that could reflect their inconsistencies include age of the participants, ratio of males to females, follow up length, age-related outcome investigated, statistical analysis used, types of confounders adjusted for or health/disease status of the participants. Further studies are needed to determine which variables are the reasons for the inconsistencies between studies. For example, a particular variable such as age could be investigated by dividing studies into different age groups and determining the predictive value of the candidate BoA in each age group. The value of the candidate BoA investigated may change throughout the lifespan and thus although may be predictive of age-related events early in life it may not be predictive later on in life and vice versa (McClearn, 1992; Sprott, 2010). The reasons for this are not well understood but could be due to different intra- and extra-organismic environmental influences on the candidate BoA that confound its association with age-related outcomes within different age groups (McClearn, 1992). It may therefore be necessary to define BoA that are valid early in life and others that are

Candidate BoA	Age (years)	Gender	n	Follow up (years)	Age-related outcome predicted	Association	Statistical analysis	Adjustments	Study	Reference
Body mass index	Mean 64.0 SD 3.3	38% male	7,142	14	Disability	▲	AUROC	Age, education and smoking status	Community-based cohort (Melbourne Collaborative Cohort Study)	(Wong *et al.*, 2012)
	Mean 86.8 SD 6.9	26% male	331	1	All cause mortality	▼♀	Cox proportional hazard models	Age, smoking status, total-to-high density lipoprotein cholesterol ratio and plasma glucose	Hospital-based cohort of patients with cardiovascular disease (PROTEGER study)	(Zhang *et al.*, 2012)
	18-79	89% male	783	6.4	All cause mortality	x	Cox proportional hazard models	Age, gender, pre-treatment tumour stage, treatment and presence of comorbidities	Population-based cohort of participants with esophageal adenocarcinoma or gastroesophageal junction adenocarcinoma	(Thrift *et al.*, 2012)
Physical activity	≥20	49% male	67,740	16	Cardiovascular mortality	▲♂ (obese only)	Cox proportional hazard models	Age, smoking status, ever use of blood pressure medication, use of alcohol, marital status, and education	Community-based cohort (The HUNT study)	(Vatten *et al.*, 2006)
	Mean 78.9 SD 2.2	38% male	1,021	8.34	All cause mortality	▼♀	Cox proportional hazard models	Age, baseline vulnerability and grade of disability	Community-based cohort (Brønshøj-Husum Study)	(Schultz-Larsen *et al.*, 2012)
	35-79	100% male	9,824	u/k	Prostate cancer mortality	x	Multivariate logistic regression	Age, education, urban-rural residence, smoking status and body mass index	Population-based cohort (The Puerto Rico Heart Health Program)	(Crespo *et al.*, 2008)
Systolic blood pressure	≥25	45% male	8,534	3	All cause mortality	▲♂	Cox proportional hazard models	Age	Population-based cohort	(Stevenson *et al.*, 2012)

	Mean 67 SD 13	70% male	500	2	All cause mortality	▼	Cox proportional hazard models	Age, gender, B-blocker therapy, hyponatremia, uric acid, electrocardiographic readings and six-minute walking distance	Outpatient-based cohort of patients with heart failure	(Zafrir *et al.*, 2012)
	≥80	u/k	287	u/k	All cause mortality	x	Cox proportional hazard models	Age, gender, weight, mobility at entry, smoking status, bacteriuria, electrocardiographic readings, serum cholesterol levels, haematocrit levels and socioeconomic status (two u/k)	Population-based cohort of elderly entering a residential home	(Dontas *et al.*, 1996)
Platelet count	16-99	54% male	40,797	6	All cause mortality	▲	Cox proportional hazard models	Age, gender, ten pre-existing comorbid states, categories of dialysis, primary insurance, marital status, standardized mortality ratio of the dialysis clinic during entry quarter, dialysis dose, presence or absence of a dialysis catheter and residual renal function during the entry quarter	Population-based cohort of patients with end-stage renal disease with a history of hemodialysis	(Molnar *et al.*, 2011)
	14-92	63% male	793	2.8	All cause mortality	▼	Cox proportional hazard models	Dynamic International Prognostic Scoring System risk, unfavourable karyotype and red cell transfusion dependent	Population-based cohort of patients with primary myelofibrosis	(Gangat *et al.*, 2011)
	21-68	u/k	111	5	All cause mortality	x	Cox proportional hazard models	u/k	Hospital-based cohort of chemotherapy treated patients	(Wang *et al.*, 2012)
C reactive protein	u/k	u/k	281	7.25	All cause mortality	▲	Cox proportional hazard models	None	Population-based cohort of patients on haemodialysis	(Suzuki *et al.*, 2012)
	41-85	64% male	296	5.75	All cause mortality	x	Cox proportional hazard models	Age and gender	Population-based cohort of patients with suspected myocaridal infarction	(Lyngbaek *et al.*, 2012)

Interleukin 6	Mean 73.0 SD 3.0	58% male	972	9.4	All cause mortality	▲	Cox proportional hazard models	Age, gender, race, clinic site, body mass index, smoking status and pack-years smoked, adiponectin, plasminogen activator inhibitor 1, visceral fat area and hypertension	Community-based cohort of older adults (Health ABC Study)	(van den Borst *et al.*, 2012)
	65-94	45% male	638	3	All cause mortality	x	Multivariate logistic regression	Age, gender, education, high waist circumference, high blood pressure, low high-density lipoprotein cholesterol, high low-density lipoprotein cholesterol, high triglycerides, C-reactive protein, hospital stay in previous years, alcohol intake, smoking in pack-years, low physical activity, liver disease, coronary heart disease, diabetes mellitus, depression, and cancer	Community -based study of older adults (InCHIANTI Study)	(Alley *et al.*, 2007)

Table 1.1. Inconsistencies between studies investigating the most popular studied candidate BoA as predictors of age-related outcomes. (SD: standard deviation, ▲: positive association, ▼: negative association, x: no association, ♂: males only, ♀: females only, AUROC: area under the receiver operating curve, u/k: unknown, n/a: not available/no data identified)

valid throughout different stages of the lifespan and treat each measurement occasion as a spearate predictive event.

Longitudinal regional birth cohort studies are advantageous for BoA investigation since this will reduce the effects of intra- and extra-organismic environmental influences and thus confounding in the association between candidate BoA and age-related outcomes and thus their prediction. This is because there is the elimination of a chronological age effect and participants will be expected to have a similar timings of life history events, lifestyle factors and seasonal/environmental exposures. The variation in these factors within the study group could be investigated first to determine if this is the case. Valid BoA specific to different age groups could be defined. Since there is a wide variability in age-related outcomes amongst the elderly and some individuals preserve high levels of health and functional ability (Collerton *et al.*, 2009), a longitudinal birth cohort of the elderly is likely to be informative in the validation of candidate BoA. Another advantage is that it will allow the prediction of morbidity and mortality factors in a shorter time scale than in a younger population group. Disadvantages to birth cohorts however is that they will only identify BoA of a narrow age group and more studies will be needed to cover all age ranges.

Although some classical BoA may have been validated as predictors of age-related outcomes in specific population groups they still don't provide a meaningful insight into the mechanistic aspects of biological ageing. This limits the development of potential interventions that will increase health-span. However, their identification is still important since they can be informative by their association and thus construct validation of "newer" mechanistic cellular and molecular BoA. This is especially so when longitudinal studies are now identifying newer candidate BoA where measurement may have taken place sometime after the start of the study and only one measurement may be available. Validating them with informative BoA will allow the decision of whether further measurements of cellular and molecular BoA should take place or not which has timing, material use and financial implications.

1.4. The role of reactive oxygen species (ROS) in ageing

1.4.1. Free radicals and ROS

A free radical is an independent molecular species that contains one or more unpaired valence electrons not contributing to intermolecular bonding. Most free radicals are highly reactive because their unpaired electron/s will either accept or donate an electron from other appropriate molecular species which could be another radical or a non-radical. If the other molecule is a non-radical then a chain reaction is initiated until two free radicals react to form a two-electron bond in which the chain reaction is then stopped. Oxygen free radicals take an electron away from the target molecule to pair with their single free electron, which is known as oxidation. Highly reactive oxygen free radicals are a major cause of structural changes and thus damage to molecules. There are other related oxygen molecules that do not fit the definition of a free radical but contribute to their production or are able to oxidise molecules such as singlet oxygen and hydrogen peroxide. The term "reactive oxygen species" (ROS) is therefore used to refer to both these oxidants and the oxygen free radical.

1.4.2. ROS generation

ROS are generated by sources endogenous and exogenous to cells. Endogenous production includes on-going metabolism, especially from the respiratory chain during oxidative phosphorylation in the mitochondria which is thought to be the major source of ROS production (Turrens and Boveris, 1980; Turrens, 2003). The enzyme complexes I and III are thought to be the major sites of ROS production in the mitochondria (Figure 1.9.). Other various enzymatic reactions leading to the production of ROS include xanthine oxidase (McCord, 1985), cytochrome P450, (Ekstrom and Ingelman-Sundberg, 1989), nitric oxide synthase (Giulivi *et al.*, 1998), lipoxygenase (Kukreja *et al.*, 1986), cyclooxygenase (Swindle *et al.*, 2007) and quinine reductase (Cadenas *et al.*, 1992). Endogenous ROS are also produced from the auto-oxidation of neurotransmitters, norepinephrine and dopamine, and the auto-oxidation of catecholes to quinines (Seacat *et al.*, 1997; Manini *et al.*, 2007). Exogenous factors can also influence and enhance endogenous ROS production such as infection and chronic

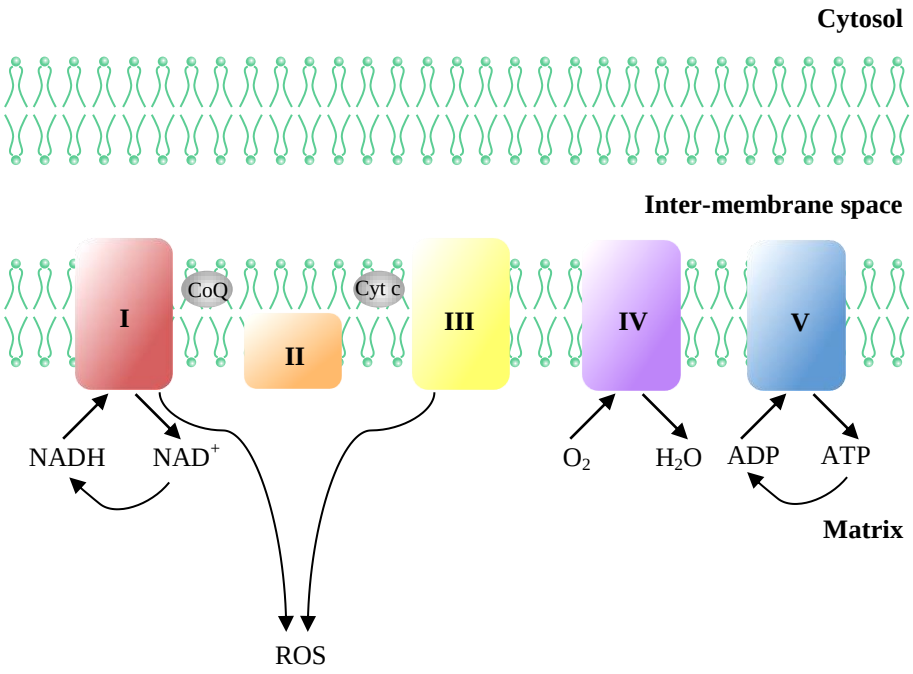


Figure 1.9. The generation of ROS in the mitochondrial respiratory chain. The main function of the mitochondria is the production of metabolic energy in the form of adenosine triphosphate (ATP) from adenonine diphosphate (ADP). Oxygen (O_2) consumed by a cell drives the translocation of electrons through a series of enzyme complexes (complex I to V) and shuttle proteins, coenzyme Q (CoQ) and cytochrome c (Cyt c). From glycolysis within the cell, pyruvate enters the mitochondrion which is converted to acetyl-CoA initiating the citric acid cycle. Nicotine adenine dinucleotide (NADH), which is generated from the citric acid cycle, is oxidised and releases its electron pairs as hydrogen atoms in the first proton pump of the electron transport system in complex I. From here to the end of the chain enough energy is generated to synthesize ATP. Nicotinamide adenine dinucleotide (NAD) is regenerated to allow the cycle to continue. Although most of the oxygen consumed by a cell is reduced to water (H_2O), a small proportion (0.2-2%) is converted to ROS in the form of superoxide, mainly documented in complex I and III (Chance and Williams, 1956; Boveris and Chance, 1973; Balaban *et al.*, 2005; Li *et al.*, 2013).

inflammation involving various cytokines and growth factors (Lo and Cruz, 1995; Floyd *et al.*, 1999; Kohchi *et al.*, 2009), ultraviolet light (Masaki *et al.*, 1995), ionizing radiation (Leach *et al.*, 2001), various chemotherapeutic agents (Gille and Nohl, 1997) and hyperthermia (Salo *et al.*, 1991). Examples of ROS include the superoxide radical, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite.

1.4.3. ROS damage

ROS are unstable and have the tendency to capture electrons from neighbouring molecules to attempt to restore their stable resting state. Extracting an electron from a nearby molecule causes a series of chain reactions as molecules obtain electrons from one another. ROS are important in many biological processes such as the regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes, activation of oxidative stress responses that ensure the maintenance of redox homeostasis (Droge, 2002) and also fighting off invading bacteria and viruses in immune cells during infection (Kohchi *et al.*, 2009). However when overwhelmed by high ROS levels, membranes are damaged by lipid peroxidation (Niki *et al.*, 2005), cytosolic enzymes and proteins are functionally compromised (Stadtman and Levine, 2000), and DNA is directly or indirectly damaged (Richter *et al.*, 1988; Cooke *et al.*, 2003). ROS induce damage to lipids by oxidising polyunsaturated fatty acids residues which leads to alterations in cellular membrane fluidity/macroviscosity and consequently the function of receptors, ion pumps and transport of metabolites. This can have detrimental effects *in vivo* including mitochondrial dysfunction (Lucas and Szweda, 1998) and thus decreased respiratory control (Rafique *et al.*, 2001) and has been implicated in cardiovascular and neurological damage (Semchenko *et al.*, 1983; Meerson *et al.*, 1987). ROS may also induce modification to the amino acids of proteins through oxidation of the protein backbone, formation of protein cross linking, oxidation of amino acid side chains and protein fragmentation which results in loss of function or enzymatic activity due to the loss of secondary or tertiary structure of the protein. Protein oxidation has been associated with alterations of ion pumps and changes in membrane potential and has been implicated in various cardiac, metabolic and neurological disorders (Wolff and

Dean, 1987; Richards *et al.*, 1988; Smith *et al.*, 1991; Ylaherttuala *et al.*, 1995). ROS induce damage to DNA of the nucleus and the mitochondria by creating a wide range of different structural lesions which affects either the base or the sugar residue of DNA including oxidised purines, oxidised pyrimidines, abasic sites, single strand breaks and double strand breaks leading to instability and mutations affecting its function. For example, mutations in mitochondrial DNA results in the decline of functional respiratory chain proteins. This leads to the reduction in the bioenergetics capacity of the tissue ultimately resulting in age-related pathology, senescence and possibly more mitochondrial DNA mutations (Wallace, 1999). An increase in oxidative DNA lesions have been found in many tumours (Matsui *et al.*, 2000) indicating a role in the etiology of cancer and also other age-related pathological conditions such as neurodegenerative disease (Alam *et al.*, 1997), inflammatory disease (Frenkel *et al.*, 1993) and cardiovascular disease (Collins *et al.*, 1998). The damage to lipids, proteins and DNA depends on which ROS is involved, its reactivity, its localization, the oxidisable molecules present, the ratio of ROS/oxidant, the antioxidant defences available and available repair. Lipid peroxidation may also initiate other molecules to lipoxidation-derived damage since chain reactions create hydroperoxides and endoperoxides which are involved in producing reactive intermediates including alkanals, alkenals, hydroalkenals, glyoxal, and malondialdehyde which induce DNA and protein damage (Ueda *et al.*, 1985; Park *et al.*, 2002).

1.4.4. Antioxidant defences

Cells have antioxidant defence systems that help protect from the destructive effect of ROS by either inhibiting its generation or directly removing it. They are therefore involved in the maintenance of cellular homeostasis. Effective antioxidant defences may be achieved through the upregulation of genes that encode antioxidants and systems involved. Antioxidants may be enzymatic or non-enzymatic. The importance and efficacy of various antioxidants defences depends on which ROS is involved, how it is generated, where it is generated and which target of damage is selected. However in some situations, antioxidants may protect against ROS in one system but fail to protect in another system (Burton and Ingold, 1984; Palozza *et al.*, 1995; Edge *et al.*, 1997). For example, antioxidants have been shown to become pro-oxidants when iron and copper are not in their normal non-catalytic state (Bagnati *et al.*, 1999) as shown in some

disease states such as hemochromatosis (Britton and Brown, 1995). Important enzymatic antioxidants are superoxide dismutase, catalase and glutathione peroxidases and non-enzymatic defence systems include glutathiones and vitamins such as vitamin A, C and E.

1.4.5. Repair/elimination of ROS damage

Antioxidants are unable to completely remove ROS and therefore mechanisms have evolved that can monitor and repair the damage to lipids, proteins and DNA, or if damage is too extensive remove the cell or prevent it from dividing. This serves to maintain intracellular homeostasis and thus genomic stability for healthy functioning and survival. Different cellular responses are triggered depending on the type and extent of oxidative damage. Repair of oxidative modification to double bonds in phospholipid acyl chains is made by deacylation followed by rapid and selective reacylation of lyso-phospholipids (Kuypers, 2007). This is accomplished by an adenosine triphosphate (ATP)-dependent process in which fatty acids are taken up from plasma, activated to acyl Coenzyme A and used to generate phospholipid. Long-chain acyl Coenzyme A synthetases have been shown play a crucial role in plasma membrane phospholipid turnover (Kuypers, 2007). Little is known about the repair of protein modification by oxidative damage however it is classified as either direct or indirect. Direct repair involves specific enzymatic systems which catalyse the reduction of oxidative products of sulphur-containing amino acids including: the glutaredoxin/glutathione/glutathione reductase and the thioredoxin/thioredoxin reductase systems which reverse the oxidation of disulfide bridges and cysteine sulfenic acids; and the methionine sulfoxide reductases which reduce methionine sulfoxide back to methionine within proteins (Petropoulos and Friguet, 2006). Indirect repair involves the recognition, removal, degradation and replacement of the damage protein by triggering *de novo* protein synthesis. Various DNA repair systems are involved in the removal of damaged DNA by ROS depending on the type of lesion induced including: base excision repair which removes oxidised purines, pyrimidines and basic sites; repair of single and double strand breaks; repair of mitochondrial DNA damage; and the repair of bulky DNA lesions by nucleotide excision repair (Lu *et al.*, 2001).

1.4.6. The oxidative stress theory of ageing

The oxidative stress theory of ageing is one of the most studied mechanistic insights into the biological ageing process. Denham Harman in 1956 first postulated that free radicals play a role in biological ageing when he recognised that ionizing radiation of living organisms, which induces the formation of free radicals, induces mutations, cancer and physiological changes seen with in age-related outcomes giving rise to the "free radical theory of ageing" (Harman, 1956). This theory suggests that ROS are involved in the progressive loss of homeostatic regulation of biological function over time due to damage caused by them at the cellular and molecular level increasing vulnerability to detrimental health outcomes and thus mortality. Further evidence that free radicals are produced endogenously was made by the discovery of the highly specialized enzyme superoxide dismutase which is able to convert superoxide into water and hydrogen (McCord and Fridovich, 1969). This suggested that ageing could be accelerated by the failure in the antioxidant defence system or by pro-oxidant generation. Harman in 1972 then modified the "free radical theory of ageing" to the "mitochondrial free radical theory of ageing" when he suggested that mitochondria are the major sources of ROS production and are also a major target of ROS resulting in their damage (Harman, 1972). This is because mitochondria lack nucleotide excision repair mechanisms and are therefore inefficient in repairing DNA damage caused by ROS. The mitochondrial nuclear genome also lacks histone protection and is therefore at an increased risk of damage by ROS. The "mitochondrial free radical theory of ageing" was then modified to the "oxidative stress theory of ageing" to include other related oxygen molecules that do not fit the definition of a free radical but contribute to their production or are able to oxidise molecules such as singlet oxygen and hydrogen peroxide (Yu and Yang, 1996).

1.4.7. Cellular senescence

In 1961, Hayflick and Moorhead proved that somatic cells have a limited lifespan when they discovered that embryo-derived fibroblasts can only divide for a limited number of times in culture known as the Hayflick limit (Hayflick and Moorhead, 1961). When

cells reach the Hayflick limit they become irreversibly arrested in the cell cycle and are therefore unable to continue cell proliferation known as cellular senescence. Cellular senescence is not limited to fibroblasts but is also observed in other cell types such a keratinocytes (Rheinwald and Green, 1975), endothelial cells (Mueller *et al.*, 1980), lymphocytes (Tice *et al.*, 1979), adrenocortical cells (Hornsby and Gill, 1978) and chondrocytes (Evans and Georgescu, 1983). Since cell lines from tumours never reach replicative senescence and are therefore immortal it is thought that cellular senescence evolved as a mechanism to supress tumour progression and therefore prevent the development of cancer. However there is increasing evidence that cellular senescence contributes to biological ageing where an increasing number of studies have shown that senescent cells can be found *in vivo* and are associated with the detrimental outcomes associated with ageing (Martin *et al.*, 1970; Dimri *et al.*, 1995; Paradis *et al.*, 2001; Going *et al.*, 2002; Minamino *et al.*, 2003). It is therefore thought that an accumulation of senescent cells will disrupt normal tissue function thus leading to age-related pathology.

1.4.8. The role of ROS production from dysfunctional mitochondria in cellular senescence and other cellular senescence phenotypes

In addition to growth arrest, senescent cells show other phenotypic changes distinct from their replicative counterparts which provide mechanistic insights into the causes and/or consequences of cellular senescence. These phenotypes may therefore serve as potential BoA. In 1990, Harley *et al.* demonstrated using fibroblasts that during each round of cellular division the DNA-protein structures that cap the ends of linear chromosomes, known as telomeres which prevent DNA repair machineries recognizing them as DNA damage, get shorter and are therefore a possible mechanistic cause of cellular senescence (Harley *et al.*, 1990). In 1995, von Zglinicki discovered that ROS accelerated the onset of replicative senescence by increasing the rate of telomere shortening (von Zglinicki *et al.*, 1995; von Zglinicki, 2002). In 1998, Bodnar *et al.* supported this mechanism when they found that the expression of the catalytic subunit of telomerase (hTERT), an enzyme able to elongate telomeres, leads to life-span extension of human cells (Bodnar *et al.*, 1998). It was suggested that telomeres act as a biological clock and once they reach a critical length then cellular senescence will be triggered. Telomere length has therefore been extensively studied as a potential BoA

however there are many conflicting studies which, as like other "classical" BoA, may be because of the diminishing association between telomere length and mortality with age (Martin-Ruiz *et al.*, 2005). In fact, other characteristics of telomere dysfunction independent of telomere length may also be important initiators of cellular senescence (Hewitt *et al.*, 2012). This could include the disruption of telomeric repeat-binding factors (TRF1, TRF2, POT1 etc.) which are essential in the formation of t-loops that cap and protect telomeres (de Lange, 2005). In 2003, di Fagagna *et al.* demonstrated that the main initiator of cellular senescence is a DNA damage response when telomeres become uncapped which includes induction of DNA damage foci and induction of DNA repair and damage checkpoint factors (di Fagagna *et al.*, 2003). There is also evidence for a telomere-independent DNA damage response that drives cellular senescence (Nakamura *et al.*, 2008). Cellular senescence however is much more complex than solely a DNA damage response and other key players are required to maintain the cellular senescence state. Studies found that senescent cells express many genes differentially than non-senescent cells (Shelton *et al.*, 1999). The most prominent genes involved in senescence are those involved in inflammatory signalling known as the senescence associated secretary phenotype (SASP) (Coppe *et al.*, 2008) and those involved in mitochondrial dysfunction (Passos *et al.*, 2007a). ROS production from dysfunctional mitochondria is thought to be the driving force of cellular senescence which links these key players by increasing the rate of telomere shortening (von Zglinicki *et al.*, 1995; von Zglinicki, 2002), as well as feeding into a continuous DNA damage response that sustains the SASP; indicated by the accumulation of inflammatory mediators, and mitochondrial dysfunction; indicated by the enhanced production of superoxide together with (frequently) increased mitochondrial mass and decreased mitochondrial membrane potential. This maintains an irreversible state of persistent damage in senescent cells and may even induce senescence in neighbouring cells (Acosta *et al.*, 2008; Kuilman *et al.*, 2008; Passos *et al.*, 2010; Nelson *et al.*, 2012). The subsequent dysregulation of physiological function contributes to age-related pathology and/or carcinogenesis (Tchkonia *et al.*, 2010; Baker *et al.*, 2011)**.** It is therefore hypothesized that the main players, ROS production from dysfunctional mitochondria, are potential BoA (Figure 1.10.). However their reliability and validity have not been investigated in large population studies, especially within the very old population.

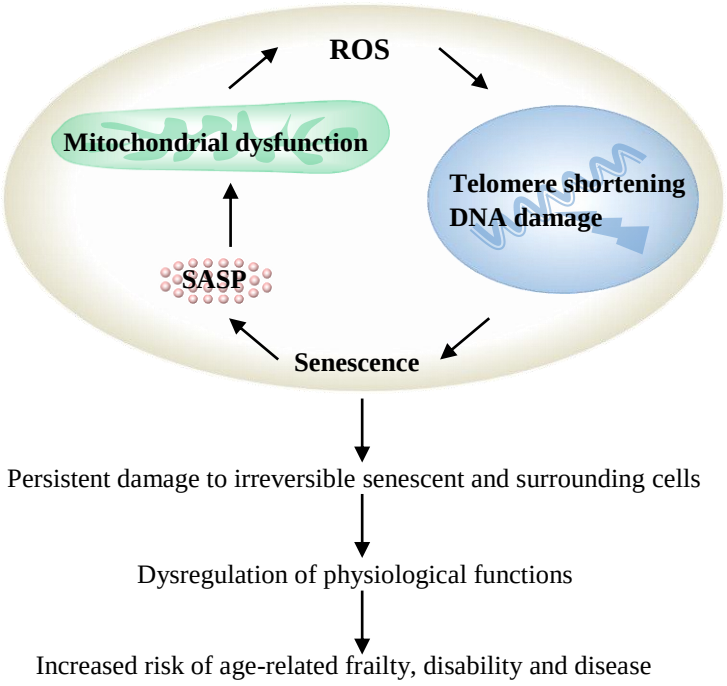


Figure 1.10. ROS production from dysfunctional mitochondria is hypothesized to be the driving force of cellular ageing and is therefore a potential BoA.

1.5. Potential oxidative stress-related BoA

1.5.1. Superoxide production, mitochondrial mass and mitochondrial membrane potential as markers of mitochondrial ROS production and dysfunction and their measurement by flow cytometry

Mitochondria are semi-autonomous organelles that contain their own genome. Although most mitochondrial proteins are encoded by genes in the nucleus, some components of the electrol transport chain are encoded by the mitochondrial DNA (mtDNA). The main function of the mitochondria is the production of ATP by oxidative phosphorylation (as mentioned previously) however they are also involved in a variety of other important functions including various signalling events, apoptosis and ROS production. The function of mitochondria is reflected in their dynamic nature. They are able to continuously fuse and divide when adapting to changes in physiological conditions which serves to maintain their function (Seo *et al.*, 2010).

Fusion and fission is thought to conserve healthy mitochondria by sustaining the integrity of mtDNA. A single cell can contain both mutant and wild-type mtDNA, known as mtDNA heteroplasmy however when a threshold of 70-90% mutant mtDNA is reached then cellular dysfunction occurs (Wallace and Fan, 2009; Seo *et al.*, 2010). One hypothesis is that efficient fusion of mitochondria enables the complementation of mutant mtDNA since this will be diluted by the introduction of wild-type mtDNA, preventing the threshold being reached (Chan, 2006; Seo *et al.*, 2010). A balance of fusion and fission will therefore allow the distribution of healthy mitochondria. If the protein machinery involved in fusion and fission were to become damaged, which may occur directly or indirectly by oxidative stress, then this will cause substantial changes in mitochondrial dynamics, notably in mitochondrial mass and membrane potential. Changes in these parameters therefore reflects mitochondrial dysfunction. This would then results in further ROS production, resulting in a vicious cycle of self-amplifying damage. Since ageing is thought be the result of increasing mtDNA mutations and the duplication and turnover of mitochondria is an ongoing process, measuring mitochondrial ROS production, mass and membrane potential may serve as potential BoA.

It has previously been shown that in human fibroblasts and other tissues that high mitochondrial ROS production, high mitochondrial mass and low mitochondrial membrane potential are associated with cellular senescence (Passos *et al.*, 2007a; Passos *et al.*, 2007b; Passos *et al.*, 2010; Wang *et al.*, 2010). While the association of increased mitochondrial mass in cellular senescence has been contested (Sahin *et al.*, 2011), increases in ROS and decreases in mitochondrial functionality, typically associated with low mitochondrial membrane potential, have commonly been found in ageing and senescence (Hagen *et al.*, 1997; Hagen *et al.*, 1999; Kokoszka *et al.*, 2001; Moiseeva *et al.*, 2009). It is therefore hypothesized that these measurements reflecting mitochondrial dysfunction are potential oxidative stress-related BoA (Figure 1.11.).

The use of fluorescent probes that bind to the mitochondria independent or dependent of mitochondrial membrane potential have been widely used to measure mitochondrial ROS production, mass and membrane potential. These can can be detected using fluorescence microscopy or flow cytometry however the latter is a preferred technique due to its rapid measurement on individual cells, especially when large number of samples are to be measured. Flow cytometers are laser based and measure cellular parameters within a fluid stream based on light scatter and fluorescence. The light scatter from the laser can be measured as forward scatter (FSC) which is an approximation of cell size or as side scatter (SSC) which is an approximation of granularity or shape. FSC and SSC parameters are first used to identify the cell population of interest. When cells are labeled with a flurochrome the emitted light can be detected by florescent detectors for which many cellular properties can be measured. These may include specific cellular components or cellular functions depending on the type of probe used. There are various probes available that permit the measurement of ROS production, mitochondrial mass and mitochondrial membrane potential. Dihydroethodium (DHE) is a widely used probe for detecting intracellular superoxide levels. Within the cytosol it exhibits a blue fluorescence but when it becomes oxidized by superoxide it forms ethidium which intercalates with DNA and emits a red fluorescence. The specificity of DHE to superoxide has been demonstrated by various studies and it has been reported as the “gold standard” for superoxide detection (Rothe and Valet, 1990; Bindokas *et al.*, 1996). Probes that measure mitochondrial mass need

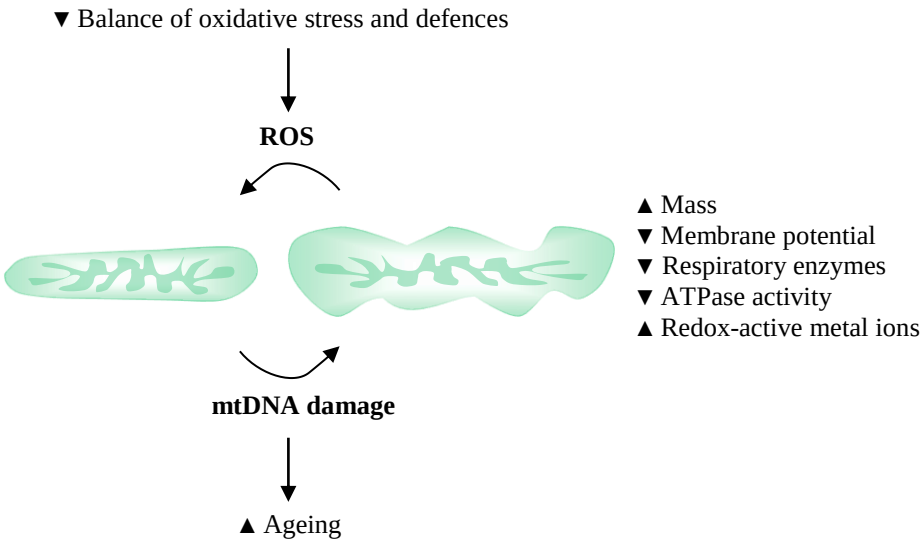


Figure 1.11. Proposed model and potential markers of mitochondrial dysfunction. (Adapted from (Seo *et al.*, 2010)).

to be independent of membrane potential. MitoTracker probes including MitoTracker Green FM are cell-permeant mitochondrion-selective probes that contain a mildly thiol-reactive chloromethyl moiety. The chloromethyl group appears to be responsible for keeping the probe associated with the mitochondria (Presley *et al.*, 2003). To measure the mitochondrial membrane potential fluorescent probes with dual fluorescent signal emissions were developed that are sensitive to a switch in membrane potential. One of these probes is 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). It exhibits potential-dependent accumulation in mitochondria that is detectable by a fluorescent shift from green to orange. Membrane potential is measured by the orange to green fluorescent intensity ratio. JC-1 is one of the most stable of the mitochondrial membrane probes.

1.5.2. F_2-isoprostanes as markers of lipid peroxidation and their measurement by automated dissociation enhanced fluorescence immunoassay (AutoDELFIA)

Since ROS are often present at low concentrations or are short lived in biological systems an alternative oxidative stress-related BoA is to measure the damage caused or products of the damage. Isoprostanes are prostaglandin-like molecules formed from non-cyclooxygenase oxidative modifications of arachidonic acid from the free radical attack of cellular membrane phospholipids or circulating low density lipoproteins. The first class of isoprostanes identified were the F_2-isoprostanes (Figure 1.12). They are derived from the precursor arachidonic acid which undergoes hydrogen and oxygen insertion. Four different peroxyl radicals are formed depending on the site of insertion. Endocylization of the radicals takes place and further oxidation yields endoperoxide regioisomers. These are then reduced to form F_2-isoprostanes. These can be designated 5, 12, 8 or 15 series depending on the carbon to which the side chain hydroxyl is attached. Although some compounds are favoured evidence that all four are produced has been shown (Liu *et al.*, 1999). Isoprostanes are released by cellular activation and circulate in the plasma either as free form or esters in phospholipids and are excreted in the urine. The F_2-isoprostanes are the most studied and have been shown to be accurate markers of oxidative stress *in vivo,* including measurements in plasma and urine (Morrow et al., 1990a; Morrow et al., 1990b; Morrow and Roberts, 1997; Morrow and Roberts, 2002; Cracowski and Durand, 2006; Morrow, 2006; Montuschi et al., 2007).

Several methods have been developed to analyse F_2-isoprostanes including gas chromatography mass spectroscopy (GCMS) (Liu *et al.*, 2009), liquid chromatography with tandem mass spectroscopy (LCMS/MS) (Liang *et al.*, 2003; Sircar and Subbaiah, 2007), radio immunoassay (RIA) (Basu, 1998) and enzyme immunoassay (EIA) (Sasaki et al., 2002). Although GCMS is the preferred the adaptation of this method, LCMS/MS, is also widely used. The advantage of this technique is that it has a high resolution of separation, high specificity and sensitivity and determines multiple F_2--isoprostane isomers at a time. However it is not the method of choice when analysing a large number of samples since it is laboriously intensive, requires expensive equipment, skilled operators and is time consuming. Alternatively EIA have also been developed to quantify F_2-isoprostanes using antibodies generated against some selected isomers such as 8-isoprostanes. EIA are low cost and easy to use. A disadvantage to the EIA method is that questions remain on the specificity of the antibody to specific isomers. Time-resolved fluorescence immunoassays (TR-FIAs) including automated dissociation enhanced lanthanide fluorescence immunoassay (AutoDELFIA) offers greater sensitivity, specificity and wide linear range than conventional EIA. TR-FIAs utilize lanthanide chelates which have unique fluorescence properties, a large stoke shift, high florescent intensity and long decay times giving this method high sensitivity and specificity (PerkinElmer, 2006). TR-FIA can be performed manually or high throughput on a full automated walkway which performs all sample and reagent handling and all assay stages including measurements automatically. This therefore brings cost and time saving. Some studies comparing the GCMS method with EIA have reported good agreement however not identical results (Soffler *et al.*, 2010) where as others have not shown any agreement (Il'yasova *et al.*, 2004). The adaptations of these methods, LCMS/MS and DELFIA, therefore need to be investigated for their agreement.

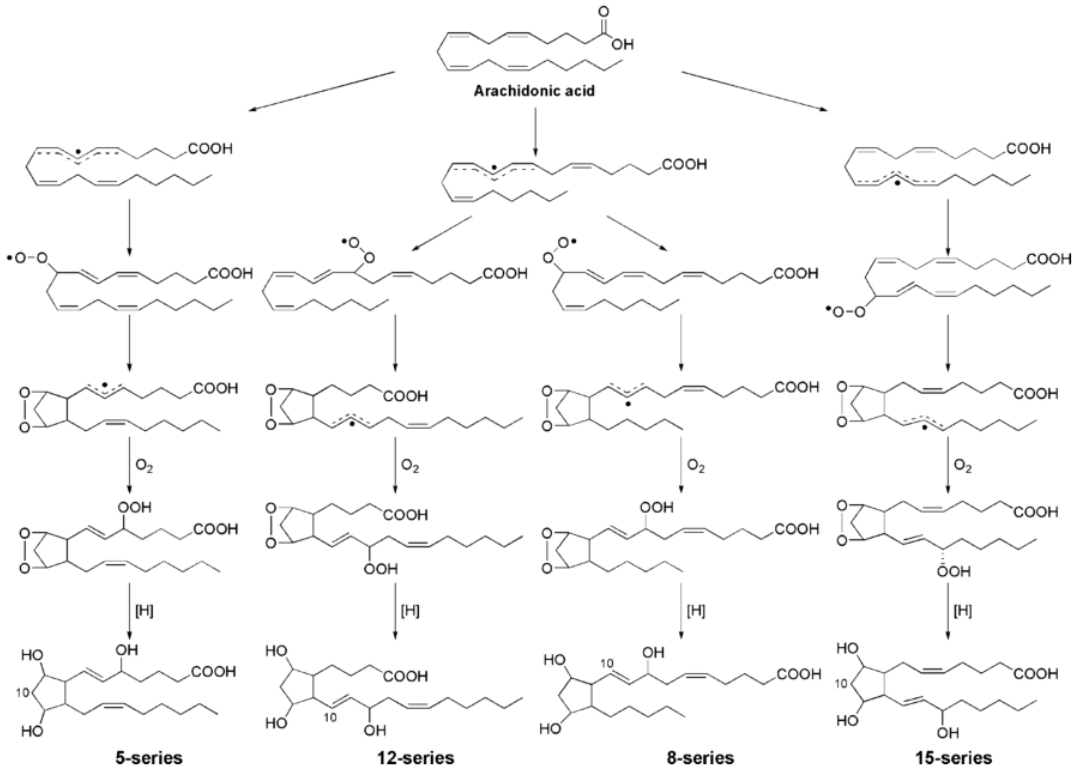


Figure 1.12. Formation of the F_2-isoprostane series. (Milne *et al.*, 2005).

1.5.3. Peripheral blood as a surrogate tissue for measuring potential oxidative stress-related BoA in population studies

A criteria of a BoA is that it should cause no harm when tested repeatedly within individuals and it should also be technically simple enough to measure so most clinical laboratories can perform the test accurately and reproducibly, without the need for specialized equipment or techniques. This therefore must be taken into consideration when deciding which are the most suitable markers and methods for measuring potential oxidative stress-related BoA within a large population studies. There are various surrogate tissues available that can easily be obtained which are assumed to be informative of other inaccessible tissues. Surrogate tissues that would enable the direct measurement of mitochondrial ROS production and dysfunction within cells include blood cells, buccal cells, hair follicle, and skin. Oxidative stress could also be measured indirectly by measuring products of oxidative damage, such as F_2-isoprostanes, which may be present in blood plasma, urine, stool, saliva, sputem, nasal lavage, cerumen, hair shaft, or nail (Rockett *et al.*, 2004). Despite all of these being easy assessable, blood is the most appropriate surrogate tissue since it can be obtained in large quantities enabling greater sensitivity and specificity and also it can be used to compare and validate potential oxidative stress related BoA measured directly in blood cells and those measured indirectly in plasma. Limitations however to using peripheral blood are that this would only be a reflection of one type of tissue. Tissues may age at different rates, as reflected by different timings and types of age-related diseases, and thus oxidative stress-related BoA may be tissue dependant. However many studies, although found in different levels, have shown agreements between various cellular senescence and oxidative stress-related markers in peripheral blood and other tissues suggesting they may be tissue independent (Table 1.2). Peripheral blood therefore offers a significant promise for the measurement of potential BoA as a substitute for tissues that are not easily assessable due to practical and ethical barriers.

Reference	Tissue	Mean (p value)	R^2 (p value)	n
Telomere length				
(Friedrich *et al.*, 2000)	Leukocytes[a]	6546±519SD bp		9
	Skin tissue[b]	7792±596SD bp (<0.01[a+b])	0.79 (0.017)[a+b]	9
	Synovial tissue[c]	7910±420SD bp (<0.001[a+c])	0.54 (0.038)[a+c]	9
DNA damage adducts				
(Godschalk *et al.*, 1998)	Monocytes[a]	2.9(0.7-0.1 range) x10^8n/t		10
	Skin tissue[b]	0.28(0.25-0.81 range) x10^8n/t	0.74 (0.006)[a+b]	10
DNA repair				
(Herrera *et al.*, 2009)	Lymphocytes[a]	0.60±0.29SD		23
	Colon tissue[b]	3.78±5.53SD (0.001[a+b])	0.53 (0.034)[a+b]	23

Table 1.2. Agreement between various cellular senescence and oxidative stress related biomarkers in peripheral blood and other tissues of humans. (bp: base pairs, n/t: not tested, SD: Standard deviation).

1.6. Overall aims

The overall aim of this study is to assess the reliability and validity of potential oxidative stress-related BoA in the very old population specifically ROS production and mitochondrial dysfunction in PBMCs by flow cytometry and plasma F_2-isoprostanes by AutoDELFIA

The specific questions asked are:

- Does the predictive value of various classical BoA change in different age groups and if so which are predictive in the very old population? This is so they can be used to validate potential oxidative stress-related BoA in this age group.

- Are potential oxidative stress-related BoA experimentally reliable?

- Are potential oxidative stress-related BoA valid in the very old population?

- Are potential oxidative stress-related BoA valid in a mouse model of ageing?

- Are potential oxidative stress-related BoA associated with age-related outcomes in the very old population?

Chapter 2. Materials and Methods

2.1. The Newcastle 85+ study

2.1.1. Study design

A full description of the Newcastle 85+ study design, a community-based cohort study of health and ageing in the very old, has previously been published (Collerton *et al.*, 2007; Collerton *et al.*, 2009; Davies *et al.*, 2010). Briefly the study invited all surviving adults born in 1921 who turned 85 in 2006 when the study commenced and were permanently registered with a participating general practice in Newcastle upon Tyne and North Tyneside (North-East England). All eligible individuals were invited to participate including those in institutions and those with cognitive impairment. The study excluded only those with end stage terminal illness and those who might pose a safety risk to a nurse visiting alone. Participants were visited by a research nurse at their place of residence (i.e. their own home, care home: nursing or residential) at baseline (phase 1: 2006-7, n=854), 18 months (phase 2: 2007-9, n=631) and 36 months (phase 3: 2009-10, n=484). Attrition between phases 1 and 3 was mainly due to deaths (62.7%, 232/370) with the remainder due to drop out. At each phase a multi-dimensional health assessment was undertaken including questionnaires, physical measurements, function tests and a blood sample. Bloods were drawn between 7:00am and 10:30am with great attention to getting the blood samples to the laboratory as quickly as possible. 95% of samples were received for processing within 1 hour of venepuncture. Blood processing was carried out at the Biomarkers Laboratory, Edwardson Building, Institute for Ageing and Health (IAH), Newcastle University, Newcastle upon Tyne, UK by Carmen Martin-Ruiz, Claire Kolenda, Craig Parker, Paul Collier, Sam Jameson, Hanna Curtis and Anna Tang. A review of general practice records was also undertaken during phase 1 and phase 3 assessments by trained research nurses. Ethical approval was obtained from the Newcastle and North Tyneside Research Ethics Committee (reference number 06/Q0905/2). Written informed consent was obtained from either participants or from a consultee, usually a relative or carer, when participants lacked capacity to consent. The study population at baseline was sociodemographically representative of the local (Newcastle upon Tyne and North Tyneside) and wider (England and Wales) population (Collerton *et al.*, 2009). A summary of the study recruitment is shown in Figure 2.1.

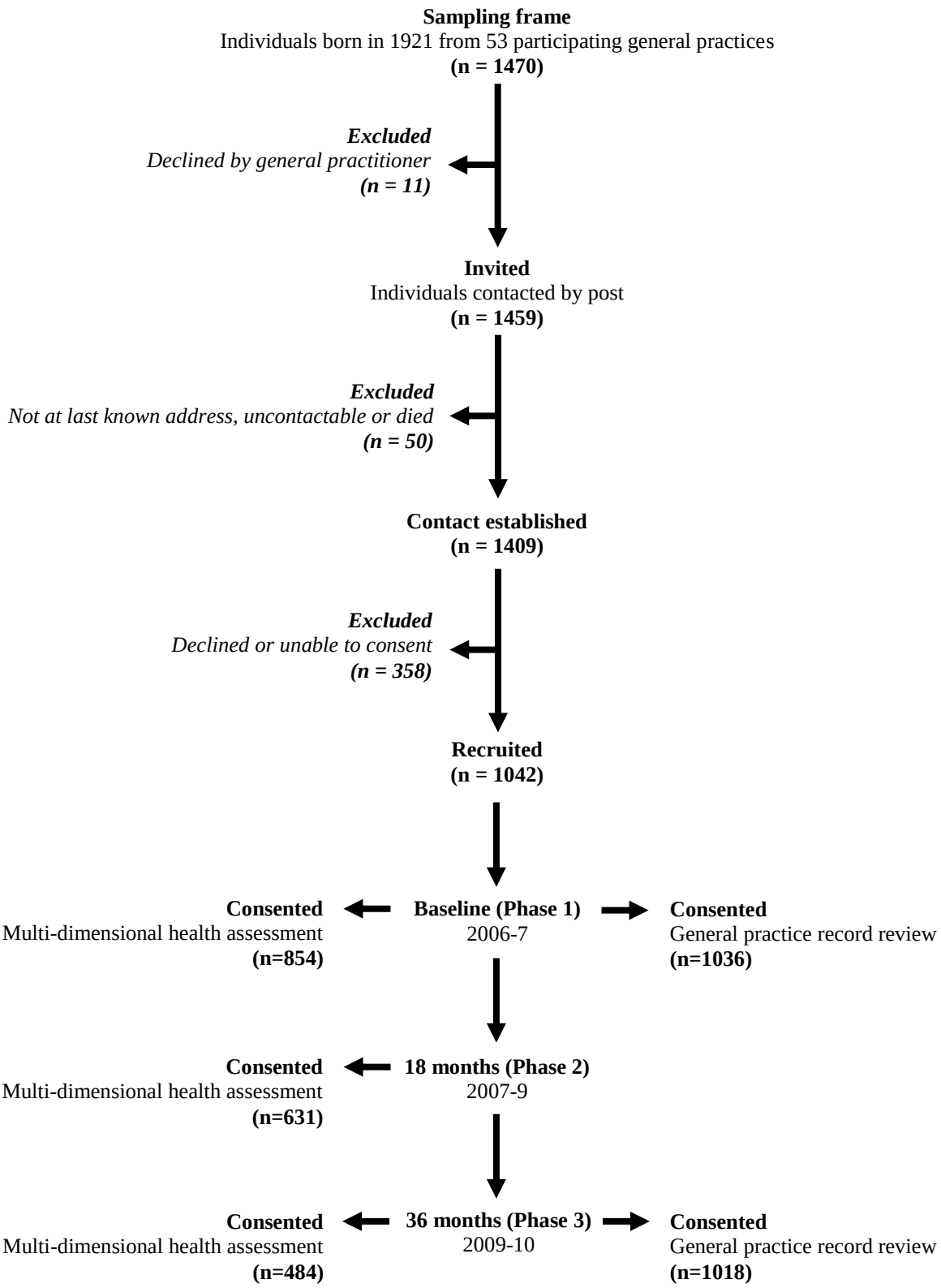


Figure 2.1. Recruitment of the Newcastle 85+ study. *(Figure was adapted from data kindly supplied by Karen Davies, Senior Clinical Research Nurse Manager of the Newcastle 85+ Study)*

All data reported in this study are from the phase 3 assessments (participants aged 88-89 years) unless otherwise stated within the result sections.

2.1.2. Characteristics

Gender, ethnicity, age of natural mother and/or fathers death, place of birth, has/had sibling(s), any full-time higher education, number of years in main job/role, has offspring, marital status, living alone, housing type, alcohol consumption, smoking status, non-prescribed supplementation use, prescribed supplementation use, aids and appliance use, social participation, important key events and blood fasting status were assessed using questionnaires at participants place of residence by trained research nurses.

2.1.3. Mitochondrial haplogroups

Mitochondrial haplogroups were determined using a stepwise algorithm (Torroni *et al.*, 1996) by primer extension of multiplex polymerase chain reaction (PCR) products with the detection of the allele specific extension products by matrix-associated laser desorption/ionization time of flight (Sequenom MassARRAY, San Diego, California, US) at the Wellcome Trust Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK by Deepthi Ashok as described in (Collerton *et al.*, 2013*).*

2.1.4. Anthropometry

Anthropometry was measured at participant's place of residence by trained research nurses. Weight, body fat %, body fat mass, fat free mass and total body water were measured using an electronic body composition analyser (Tanita Europe B.V., Middlesex, UK). Height was calculated using the average of two right arm demi-span measures (1.35 multiplied by demi-span plus 60.1 for females and 1.40 multiplied by demi-span plus 57.8 for males) due to difficulties measuring height in the elderly.

2.1.5. Haematology

Full blood counts (red blood cells, white blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils) were measured by analysts at the Freeman Hospital, Newcastle upon Tyne, UK.

2.1.6. Biochemistry

Electrolytes, urate, urea, creatinine, liver panel (total protein, bilirubin and alanine transaminase), bone panel (albumin, calcium, albumin-adjusted calcium, phosphate and alkaline phosphatase), glucose, glycosylated haemoglobin (HbA1c); lipid profile (cholesterol, triglycerides, high and low density lipoproteins, apolipoproteins (A1 and B)), serum cortisol, thyroid function (free triiodothyronine (T3), free thyroxine (T4), thyroid stimulating hormone and thyroid peroxidase antibody), high-sensitivity C-reactive protein (CRP), rheumatoid factor (RhF) and N-terminal pro b-type natriuretic peptide **(**NT-pro BNP) were measured by analysts at the Royal Victoria Infirmary, Newcastle upon Tyne, UK as described in (Collerton *et al.,* 2013).

2.1.7. Nutrition

Plasma total homocysteine, plasma vitamin B2, and plasma vitamin B6 were measured by the Nutritional Biomarker Analysis team from the Elsie Widdowson Laboratory at the MRC Human Nutrition Research Unit, Cambridge, UK and serum ferritin, red cell folate, vitamin B12 and serum vitamin D were measured at the Royal Victoria Infirmary, Newcastle upon Tyne, UK as described in (Martin-Ruiz *et al.*, 2011a).

2.1.8. Inflammatory response

Inflammatory response was measured at the Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University, Newcastle upon Tyne, UK by Claire Kolenda and Craig Parker. Lithium heparin (LiHep) blood samples were stimulated with ultrapure lipopolysaccharide (Autogenbioclear, Nottingham, UK) and serum supernatants were incubated for 24 hours at 37˚C. Il-6 and TNF-α were measured by electrochemiluminescence on a 96-well Multi-SPOT Meso Scale Discovery assay using

the SECTOR Imager 6000 (Meso Scale Diagnostics, LLC. Gaithersburg, Maryland, US) as decribed in (Martin-Ruiz *et al.*, 2011a).

2.1.9. Lymphocyte subpopulations

Lymphocyte subpopulations were measured at the Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University, Newcastle upon Tyne, UK by Anna Tang and Sam Jameson. Blood samples stained with fluorescently-labelled antibodies (BD Biosciences Oxford, UK) to determine the % of B lymphocytes (all (CD19 positive (+)), Memory (CD27+) and Naive (CD27 negative (-))), Cytotoxic T lymphocytes (all (CD3+/CD8+), Memory (CD45RO+/CD27-) and Naive (CD45RO-/CD27+)) and Helper T lymphocytes (all (CD4+), Memory (CD45RO+/CD27-) and Naive (CD45RO-/CD27+)) were measured by 4-colour flow-cytometry (Becton Dickinson FACScan Flow Cytometery system, BD Biosciences, California, US) as described in (Martin-Ruiz *et al.*, 2011a).

2.1.10. Telomere length

Telomere length was measured at the Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University, Newcastle upon Tyne, UK by Carmen Martin-Ruiz and Craig Parker. The abundance of telomeric template versus a single gene (glyceraldehyde 3-phosphate dehydrogenase) was measured by quantitative real-time polymerase chain reaction (PCR) (7900HT Fast Real Time PCR system, Applied Biosystems, California, US) as described in (Martin-Ruiz *et al*., 2011a).

2.1.11. DNA damage and repair

DNA damage and repair were measured at the Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University, Newcastle upon Tyne, UK by Craig Parker. DNA damage (gamma ray-induced DNA strand breakage) and DNA repair capacity of peripheral blood mononuclear cells (PBMCs) were measured by automated Fluorometric analysis of DNA unwinding (FADU) analysis. PBMCs from LiHep blood were isolated by density gradient centrifugation using Leucosep® tubes (Greiner, North Carolina, US) and kept in culture with RPMI 1640 medium (R0883, Sigma, Poole, UK) plus 10% foetal bovine serum, 1% penicillin/streptomycin and 2% glutamine at 37˚C

with 5% carbon dioxide overnight. Three 50 microliter (µl) aliquots of $5x10^5$ cells were prepared per participant: control cells (C), damaged cells (D) which were treated with 5 grays (Gy) of gamma-irradiation (a dose estimated to halve the integrity of the DNA through single strand breaks), and repaired cells (R) which after 5Gy of gamma-irradiation were kept for 1 hour at 37˚C to allow for the damage to be repaired. Cell suspensions were kept on ice at all times. Samples were then treated with 10mM of sodium phosphate, 0.25M of meso-inositol, 1mM of magnesium chloride and pH7.2 then applied onto 96-well black microplates with six repeats for each of the conditions described above. By means of an epMotion liquid handling robot with thermal modules (Eppendorf UK Ltd, Histon, UK), cells were sequentially treated as follows: 12 minutes at 4˚C with lysis buffer (9M urea, 10mM of sodium hydroxide, 25mM of trans-1,2-diaminocylohexan-N,N,N′N′-tetraacetic acid; 0.1% sodium dodecyl sulfate), 15 minutes at 4˚C plus 90 minutes incubation at 30˚C with alkaline buffer (40% lysis buffer in 0.M sodium hydroxide), 10 minutes at room temperature with neutralising buffer (1M glucose, 15mM beta-mercaptoethanol) and 7 minutes at room temperature in the dark with 1xSYBR Green (diluted in 48mM sodium hydroxide). The plates were then measured for fluorescence (excitation 485nm, emission 535nm) using the TECAN SPECTRAFluor Plus fluorimeter (TECAN UK, Reading, UK). Each plate included an internal control of sheared salmon sperm DNA, 60 ng/µl. Gamma ray-induced DNA damage (D) was estimated as the percentage of fluorescence per signal intensity in samples (C) that is lost in samples (D): D = 100 x (1 – (average signal for samples D)/(average signal for samples C)), the breakage remaining after 1 hour repair (B) was estimated as the percentage of fluorescence signal intensity in samples (C) that is lost in samples (R): B = 100 x (1 – (average signal for samples R)/(average signal for samples C)), while DNA repair was estimated as % damage that has been recovered after 1 hour of repair: 100 x (D – B)/D. *(This was described in (Martin-Ruiz et al., 2011a).*

2.1.12. F_2-isoprostanes by liquid chromatography-tandem mass spectrometry (LCMS/MS)

F_2-isoprostanes by LCMS/MS were measured at Institute of Cellular Medicine, Framlington Place, Newcastle University, Newcastle upon Tyne, UK by Michael Dunn. Isoprostanes $iPF_{2\alpha}$-III (8-iso Prostaglandin $F_{2\alpha}$) and iPF_{2a}-I (5-$iPF_{2\alpha}$-VI) were extracted from ehtylenediaminetetraacetic acid (EDTA) plasma samples (300µl). Internal standard, isoprostanes $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-I and the deuterated analogue $iPF_{2\alpha}$-III–D4

(Cayman Chemical Ann Arbor, Michigan, US), were diluted in LC/MS/MS grade methanol. Calibration curve standards and EDTA plasma samples (300µl) were spiked with 2ng of $iPF_{2\alpha}$-III–D4, treated with 500µl of aqueous 1M potassium hydroxide and incubated at 37˚C for 14 hours. After incubation, the pH of the samples was adjusted to pH3 with 2% orthophosphoric acid and centrifuged at 13000 revolutions per minute (rpm). Samples were then applied for purification onto 96 well Bond Elute Plexa solid phase extraction cartridges (100mg), Varian, Harbor City, California, US) preconditioned with water and methanol. After washing with 5% methanol, the isoprostanes were eluted with methanol. Eluates were evaporated to dryness under a stream of nitrogen and reconstituted in 100µl of 90:10 water:methanol. A seven point linear calibration curve was established over a range of 0.1-10ng/ml for both $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-I. The seven calibration standards were run both before and after the participants' samples. Every 20 samples, an internal control sample (10% methanol spike with $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-I) was run to monitor intra-assay and inter-assay variability. Quantitation of plasma $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-I was performed using a Quantum Ultra triple quadrupole mass spectrometer coupled to a Surveyor LC system operating with Xcalibur software, version 2.0 (Thermo Fisher Scientific, Hemel Hempstead, UK). A Rheodyne 10 port switching valve was used for on-line solid phase extraction (2mm) x 20mm Strata C18-E 20 µm cartridge, Phenomenex, Macclesfield, UK). Samples were loaded onto the on-line extraction cartridge with 80:20 water:methanol at a flow of 1ml/minute. Liquid chromatographic separation was carried out on a 2.1mm x 50mm x 2.5µm XBridge C18 column (Waters, Manchester, UK), with gradient elution comprising solvent A of 20mM ammonium bicarbonate/ammonium carbonate in water and solvent B of 20mM ammonium bicarbonate/ammonium carbonate in 95:5 methanol: water, at pH9.2 and a flow rate of 200µl/minute. A solvent gradient of 90% A, 10% B to 40% A and 60% B over 20 minutes, followed by 100% B for a further 5 minutes was employed. The analytical runs were performed at 40^{o}C. The mass spectrometer was operated using multiple reaction monitoring in negative ion mode, with a heated electrospray ionisation source with argon as collision gas. Precursor, product ions and collision energy were determined after optimising of MS/MS conditions by infusion of 100µg)/ml solutions of $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-I and $iPF_{2\alpha}$-III–D4 in methanol. Both $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-I had a parent ion mass-to-charge ratio (m/z) of 353.2, with $iPF_{2\alpha}$-III giving a diagnostic product ion m/z of 193.1, while $iPF_{2\alpha}$-I gave a diagnostic product ion m/z of 115.1. The internal standard, $iPF_{2\alpha}$-III–D4, had a

parent ion m/z of 357.2, giving a diagnostic product ion m/z of 197.1. *(This was described in (Martin-Ruiz et al., 2011a).*

2.1.13. Health assessments and function tests

Health assessments and function tests were measured at participant's place of residence (except general practice record reviews) by trained research nurses. Disability score (calculated from 17 self-reported activities of daily living (ADL) with participants scoring 1 for each activity in which they required help or were only independent with difficulty), cognitive impairment (using the Standardised Mini-Mental State Examination (SMMSE) with scores ranging from 0 to 30 (26-30 normal, 22-25 mildly impaired, 18-21 moderately impaired and 0-17 severely impaired), self-reported physical activity and sleep quality were assessed by questionnaires. A simple disease count score (0-18) was derived from selected chronic diseases (hypertension, ischemic heart disease, cardiovascular disease, peripheral vascular disease, heart failure, arterial fibrillation, arthritis, osteoporosis, chronic obstructive pulmonary disease, other respiratory disease, diabetes, hypothyroidism or hyperthyroidism, cancer in past five years, eye disease, dementia, parkinsonism and renal impairment) reviewed within general practice records. Hand grip strength was measured using a digital handgrip dynamometer (Takei A5401, Chasmors Ltd., London, UK). Four measurements were obtained alternating the first with the participants right hand then the left hand, then again with the right hand and finally again with the left hand. A mean value using the highest value from each hand was then calculated and recorded. Timed up and go (TUG) test was measured using a stop watch recording the time it took the participant to walk three meters from a seated position and back using a standardised chair (46cm to seat 64 cm to arm). Time commenced from the point where the participant started to move until the participant sat back down. The use of a walking aid or not was also recorded. Spirometry measures including forced expiratory volume in 1 second (FEV_1) were measured using a spirometer (Microlab spirometer, CareFusion, Basingstoke, UK) connected to a tablet computer for which data was recorded using the Spida 5 software program.

2.2. Younger control participants

2.2.1. Study design

Blood samples were taken from 19 volunteers in total and were used for experimental investigations, to examine age-related changes and to compare with the very old from the Newcastle 85+ study. The date of birth was known for 12 of the volunteers with an age range of 22-55 years.

2.3. Animal model of ageing study

2.3.1. Study design

Since dietary restriction is known to delay ageing rate, extend survival, delay the appearance of most age-related diseases and is becoming increasing recognised to slow the rate of accrual of age-related oxidative stress (Merry, 2004), this was used as a validated model of ageing. C57Bl/6 mice were fed an *ad libitum* (AL) diet since birth or 60% of an *ad lib* diet since 3 months of age (dietary restricted (DR)). Peripheral blood samples were taken at 25 month of age (AL (n=10) and DR (n=11)) and bone marrow samples were taken at 2 month of age (AL (n=6)), 25 months of age (AL (n=12) and DR (n=11)) and 32 month of age (AL (n=10) and DR (n=10)). Mice were housed in cages in groups of up to 6 which did not change from weaning (56 x 38 x 18 cm, North Kent Plastics, Kent, UK). Each mouse was identified with an ear notch. Mice were provided with sawdust and paper bedding and had free access to water. Mice were housed at 20 ± 2^{o}C under a 12 hour light/dark photoperiod with lights on at 7am.

2.4. Quantification of ROS production and mitochondrial function by flow cytometry

2.4.1. Human peripheral blood mononuclear cells (PBMC) isolation

PBMCs from LiHep blood collected at phase 3 (n=362) of the Newcastle 85+ study and from control participants (n=20) were isolated by density gradient centrifugation using Leucosep® tubes and suspended in culture with RPMI 1640 medium (R0883) plus 10% foetal bovine serum, 1% penicillin/streptomycin and 2% glutamine at 37˚C with 5%

carbon dioxide at the Biomarkers Laboratory, Edwardson Building, Newcastle University by the Biomarkers team. Flow cytometry of human PBMCs was performed at the Biogerontology Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University by myself and also partially by Deepthi Ashok who introduced me to the technique at the beginning of phase 3 of the Newcastle 85+ study.

2.4.2. Mouse PBMC isolation

PBMCs from EDTA blood was centrifuged at 300rpm for 15 minutes at 4^{o}C. Plasma was transferred to a new tube and the remaining blood was diluted in cold 1x PBS in a 1:1 ratio. Diluted blood was pipetted slowly on top of half its volume of lymphoprep^{TM} (1114544, Axis-Shield, Oslo, Norway) making sure they did not mix. Blood was centrifuged at 800rpm for 15 minutes at room temperature with no brake applied. PBMC layer was brought to a new tube and the volume was adjusted to 1-2ml with cold PBS. PBMCs were centrifuged at 800rpm for 15 minutes at 4^{o}C. Supernatant was removed and the pellet was re-suspended in 1ml of RPMI 1640 medium (R7509, Sigma, Poole, UK). Since the volume of blood collected for some mice was too little for analysis individually, some mice blood had to be combined which were grouped by gender, age, dietary group and to the best ability pathology. Mouse PBMCs were isolated and analysed by flow cytometry at the Biogerontology laboratory, Edwardson Building, IAH, Newcastle University by myself.

2.4.3. Mouse bone marrow isolation

The hind limbs were collected from mice and kept in cold PBS until analysis. All hair and skin was removed from the tibia and femur using sharp small scissors and pointed curve forceps and the foot was cut off. The muscles of the tibia were moved down to the knee with scissors whilst tightly holding the leg from the knee and the tibia was dislocated with blunt forceps and put into cold PBS. The muscles from the femur were removed upto the hip bone whilst tightly holding the femur and cut with scissors from the hip joint and put into cold PBS. The ends of the bones were cut and, using a 1ml syringe and needle, were flushed with PBS to make a cell suspension. The clumps were broken by pipetting up and down with a syringe and needle in a petri dish. The cells were transferred to a 15ml falcon tube. Cells were centrifuged at 1300rmp for 5 minutes at 4^{o}C. The supernatant was removed and the pellet was re-suspended in 1ml red blood

cell lysis buffer for 5 minutes, stopping the reaction with 10ml of PBS. Cells were centrifuged at 1300rpm for 5 minutes at 4^{o}C. Supernatant was removed and the pellet was re-suspended in 1ml of RPMI 1640 medium (R7509). Mouse bone marrow was isolated and analysed by flow cytometry at the Biogerontology laboratory, Edwardson Building, IAH, Newcastle University by myself.

2.4.4. Human PBMC subpopulation isolation

Populations of Helper T Lymphocytes *(CD4+),* Cytotoxic T Lymphocytes *(CD3+/CD8+),* Natural Killer T lymphocytes (CD8+CD56+), Naïve and Non-Senescent Memory T cells (CD8+CD56-CD27+) and Senescent Memory T Cells (CD8+CD56-CD27-) were isolated by immunomagnetic sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) out of 20ml blood samples from four volunteers (age range 30-50 years). This was decribed in (Spyridopoulos *et al.*, 2009).

2.4.5. Quantification of superoxide levels, mitochondrial mass and mitochondrial membrane potential

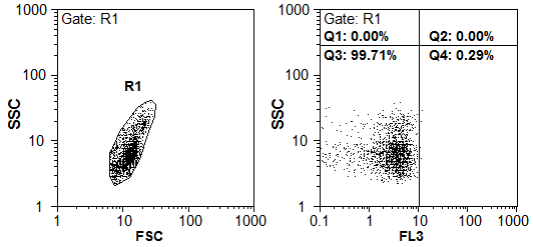
The appropriate volume of cell suspension containing 1x10^{5} cells was centrifuged at 800rpm for 5 minutes. Supernatant was removed and cells were incubated with 10μM DHE (D-23107, Life Technologies, Invitrogen, Paisly, UK) to measure superoxide levels, 100nM MitoTracker Green FM (M-7514, Life Technologies, Invitrogen, Paisly, UK) to measure mitochondrial mass or 7.7μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidaz olylcarbocyanine iodide (JC-1) (T-3168, Life Technologies, Invitrogen, Paisly, UK) to measure mitochondrial membrane potential in 1ml of RPMI-1640 medium (R7509) at 37^{o}C for 30 minutes, shortly vortexing for 10 seconds before incubation. RPMI-1640 medium (R7509) alone was used as an unstained control. After incubation 3ml of RPMI-1640 (R7509) was added and cells were centrifuged for 5 minutes at 800rmp. Supernatant was removed and 2ml of RPMI-1640 medium (R7509) was added. Samples were analysed immediately. Flow cytometry data acquisition and analysis were performed using the Partec PAS particle analysing system (Partec, Munster, Germany). A small amount of Fluoresbrite calibration grade 3.0 micron YG microspheres (18861-1, Polysciences, Eppelheim, Germany) was used to check fluorescence was maintained at 487nm in all fluorescent channels (FL). 1x10^{4} events (cells) were analysed per sample. DHE and MitoTracker Green FM were

analysed in the SSC vs. the red fluorescent channel (FL3) and JC-1 in the green fluorescent channel (FL1) vs. FL3 channel (Figure 2.2. A. i., B. i and C. i. respectively). Cells of interest were first identified based on their size (FSC) and density (SSC) in the unstained control using a polygon to gate cells. Cells were then identified in the appropriate channel cytogram, as described above, in the unstained control by applying the polygon and a quadrant was used to gate all unstained cells. The mean fluorescence of unstained cells was recorded. The unstained polygon and quadrant was then applied to the stained cells in which the mean fluorescence was recorded. Normalized mean fluorescence was then quantified, which was used to represent either mean PBMC superoxide levels, mitochondrial mass or mitochondrial membrane potential in arbitrary units (AU). Since the density gradient centrifugation method used isolates PBMC subpopulations lymphocytes and monocytes only, we were also able to quantify measures in these two subpopulations based on their SSC, since lymphocytes have a lower granularity than monocytes (Fleisher and Marti, 2001). To confirm that the cell population remaining unstained in all the stained samples were red blood cells, the analysis was repeated however lysing the red blood cells before staining (Figure 2.2. A. ii., B. ii and C. ii). This was also confirmed by staining unlysed and lysed red blood cell samples with with 2µl PerCP Mouse Anti-Human CD45 to identify PBMCs (lymphocytes sand monocytes) (557513, BD Pharmingen™, San Jose, California, US) in 1ml RPMI medium (R7509) (Figure 2.2. D. i. and ii respectively). This unstained population was removed in all lysed red blood cell samples, confirming that some red blood cells remain after the density gradient method using Leucosep® tubes. Since red blood cell lysis affects the stability of the parameters, as investigated in Chapter 4, it is not appropriate to remove red blood cells from the samples by this method, since this will cause experimental variability within the sample. Gating the red blood cells out is therefore more appropriate.

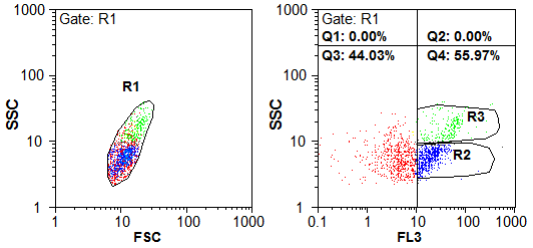
A.

i. Unlysed

Unstained

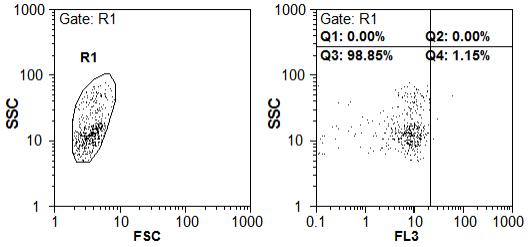


DHE stained

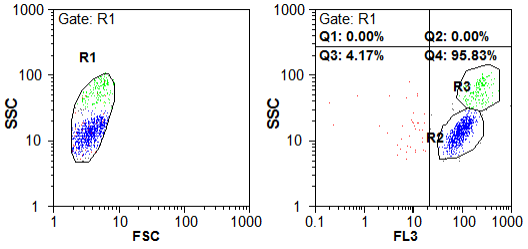


ii. Lysed

Unstained



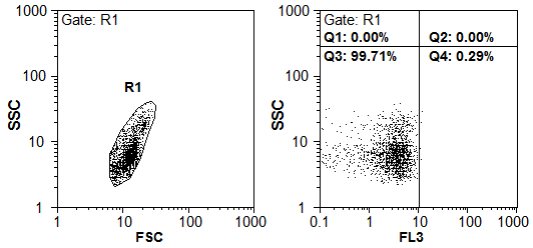
DHE stained



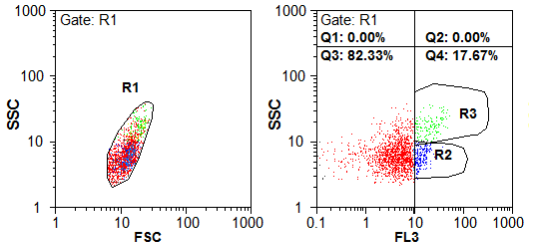
B.

i. Unlysed

Unstained

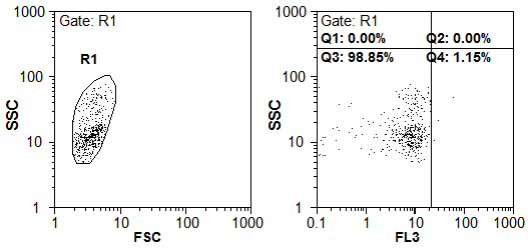


MitoTracker Green FM stained

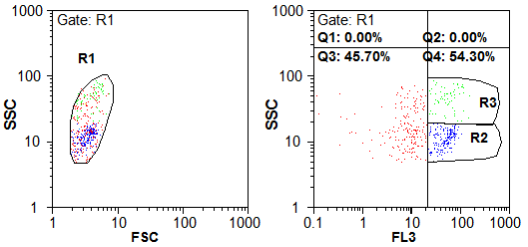


ii. Lysed

Unstained



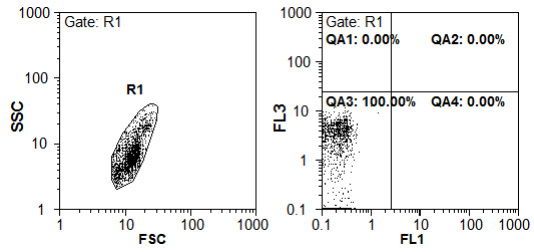
MitoTracker Green FM stained



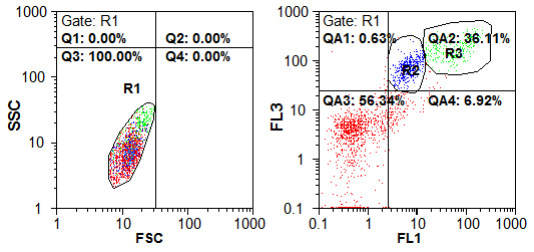
C.

i. Unlysed

Unstained

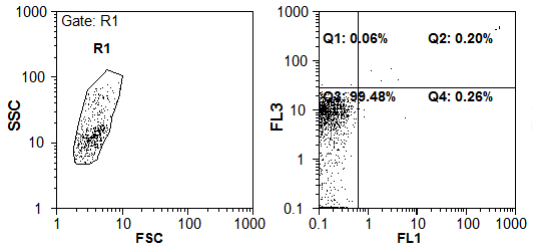


JC-1 stained

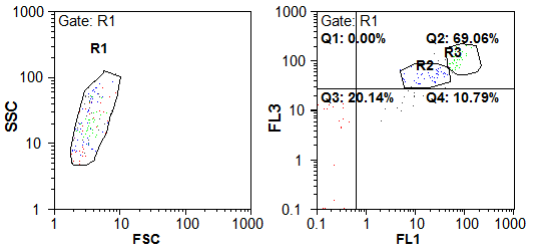


ii. Lysed

Unstained



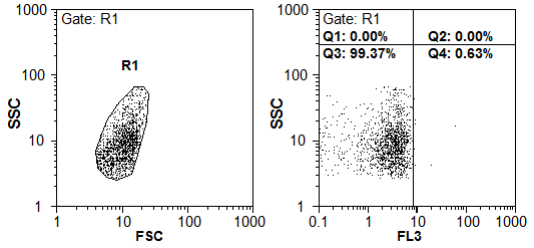
JC-1 stained



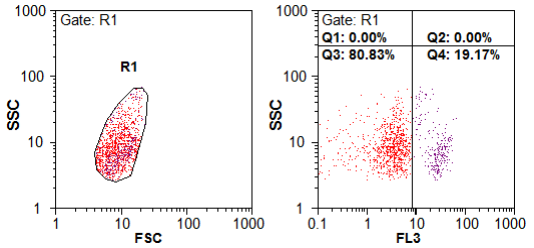
D.

i. Unlysed

Unstained

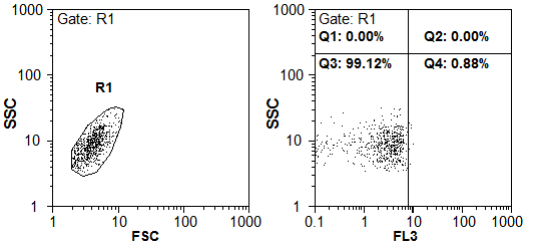


PerCP Mouse Anti-Human CD45 stained

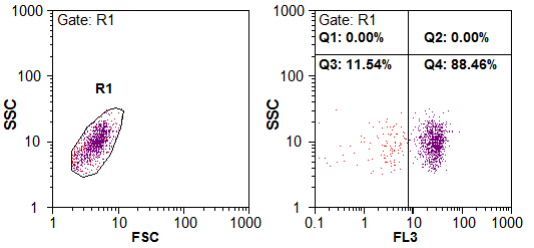


ii. Lysed

Unstained



PerCP Mouse Anti-Human CD45 stained



E.

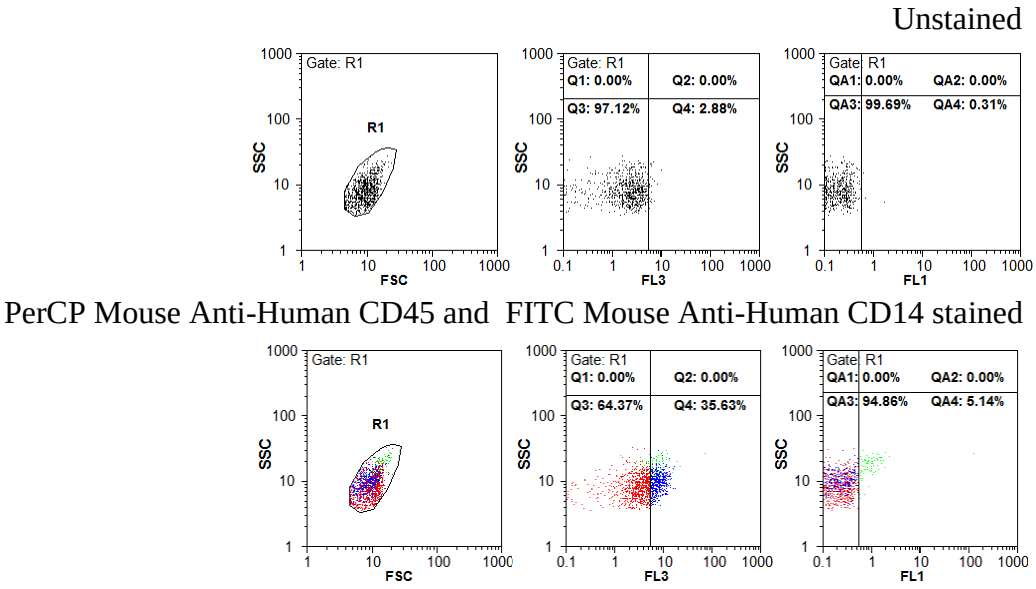


Figure 2.2. Quantification of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMCs and cell subpopulations by flow cytometry. A. Superoxide levels: unstained cells and DHE stained cells in i. unlysed and ii. lysed samples, B. Mitochondrial mass: unstained cells and MitoTracker Green FM stained cells in i. unlysed and ii. lysed samples, C. Mitochondrial membrane potential: unstained and JC-1 stained cells in i. unlysed and ii. lysed samples, D. CD45 expression: unstained cells and PerCP Mouse Anti-Human CD45 stained cells in i. unlysed and ii. lysed samples E. Confirmation of PBMC subpopulations: unstained cells and PerCP Mouse Anti-Human CD45 and FITC Mouse Anti-Human CD14 stained cells. (●: Red blood cells (CD45-CD14-), ●: PBMCs (CD45+), ●: Lymphocytes (CD45+CD14-), ●: Monocytes (CD45+CD14+))

2.4.6. Confirmation of PBMC subpopulations

Correct distinction between lymphocytes and monocytes by size/granularity was confirmed with a parallel experiment performed in a subset of control samples where $1x10^5$ cells were incubated with 2µl PerCP Mouse Anti-Human CD45 in 1ml RPMI medium (R7509) to identify both lymphocytes and monocytes and 6µl FITC Mouse Anti-Human CD14 (555397, BD Pharmingen™, San Jose, CA, USA) in 1ml RPMI medium (R7509) to identify monocytes only (Figure 2.2. E.)

2.5. Quantification of F_2-isoprostanes by AutoDELFIA

2.5.1. Plasma sample collection

Plasma samples from LiHep blood collected at phase 1 (n=654), phase 2 (n=500) (of which n=438 participated in both phase 1 and phase 2) and phase 3 (n=379) (of which n=320 participated in both phase 1 and phase 3, n=323 participated in phase 2 and phase 3 and n=287 participated in phase 1, phase 2 and phase 3) of the Newcastle 85+ study and from younger control participants (n=19), were prepared at the Biomarkers Laboratory, Edwardson Building, IAH, Newcastle University by the Biomarker team and stored at –70^{o}C. AutoDELFIA 1235 (Perkin Elmer Massachusetts, US) isoprostane analysis was performed at Unilever Discover, Colworth Science Park, Sharnbrook, Bedford by myself and Duncan Talbot.

2.5.2. Reagent preparation

A 1/2706 dilution of an 8.2 mg/ml anti-8-iso Prostaglandin $F_{2\alpha}$ antibody (Prepared by Colworth monoclonal group, clone 6514:14) was prepared in DELFIA assay buffer (1244-111, Perkin Elmer, Massachusetts, US) and 0.2 micrometre (µm) filtered (expiry date 2 weeks hence). A 1/160 dilution of a europium labelled ovalbumin-8-iso Prostaglandin $F_{2\alpha}$ (Labelled by Dave Tolley, Unipath, Batch DT5) was prepared in tracer stability buffer containing 50mM Tris, 0.9% sodium chloride, 0.1% sodium azide, pH7.8 and purified 0.1% bovine serum albumin (CR84-100, Perkin Elmer, Massachusetts, US) which was free from heavy metals and 0.2µm filtered (expiry date 2 weeks hence). *(Assay utilised in (Hall et al., 2008; Carter et al., 2013).*

2.5.3. Preparation of F_2-isoprostane standards and quality control

1mg of 8-iso Prostaglandin $F_{2\alpha}$ (1635, Cayman Chemical, Michigan, US) was weighed into a glass vial in an enclosed Mettler AE160 four figure balance and dissolved in 1ml dimethylformamide. The standard and quality control (QC) solutions (Figure 2.3.) were then prepared in clean graduated round-bottomed flasks by weighing on a Mettler PE 1600 two-figure balance. Solutions were mixed and stored at 4^0C overnight prior to testing. The following day the standards and QC samples were allowed to warm to room temperature, mixed and assayed. They were then aliquotted into labelled 2.5ml and 3ml standard vials respectively. Standards and QC samples were then stored at -20^0C. *(This was described in (Talbot and Butlin)).*

2.5.4. Quantification of 8-iso Prostaglandin $F_{2\alpha}$

50µl standard, QC or plasma sample (phase 1 and 2 matched on same plate, phase 3 on independent day) were dispensed, in replicate, into dry solid yellow low fluorescence anti-mouse plates (AAAND-003, Perkin Elmer, Massachusetts, US). The AutoDELFIA further diluted the anti-8-iso Prostaglandin $F_{2\alpha}$ antibody 1/100 in assay buffer (i.e. a final antibody dilution of 1/270,600: 0.03 µg/ml). 100µl antibody in assay buffer was then added to each well of the plate. The AutoDELFIA 1235 automatic immunoassay system diluted the europium (Eu^{3+}) labelled ovalbumin-8-iso Prostaglandin $F_{2\alpha}$ tracer a further 1/100 in assay buffer (i.e. a final tracer dilution of 1/16,000). 50µl diluted tracer was then added to each well of the plate. The plate was incubated with shaking for 60 minutes. The plate was washed 6 times with 400µl diluted (250ml in 6 litres pure H_2O) wash buffer (1244-114, Perkin Elmer, Massachusetts, US) 200µl of enhancement solution (1244-105, Perkin Elmer, Massachusetts, US) was added to each well. The plate was shaken for 5 minutes and the fluorescent counts read by the AutoDELFIA.

Intermediate 1 (1000 ng/ml)
- 0.5g 1mg/ml 8-iso Prostaglandin $F_{2\alpha}$ to 500g with phosphate buffered saline (PBS) + 0.5% ovalbumin

Intermediate 1 (1000 ng/ml)
- 0.5g 1mg/ml 8-iso Prostaglandin $F_{2\alpha}$ to 500g with PBS + 0.5% ovalbumin

Standard F (100 ng/ml)
- 50g intermediate 1 to 500g with buffer

Standard E (25 ng/ml)
- 12.5g intermediate 1 to 500g with buffer

Standard D (5 ng/ml)
- 125g intermediate 2 to 500g with buffer

Standard C (1 ng/ml)
- 25g intermediate 2 to 500g with buffer

Standard B (0.25 ng/ml)
- 6.25g intermediate 2 to 500g with buffer

Standard A (0 ng/ml)
- 500g buffer

QC - High (50 ng/ml)
- 25 g intermediate 1 to 500g with buffer

QC - Medium (2 ng/ml)
- 50g intermediate 2 to 500g with buffer

QC - Low (0.5 ng/ml)
- 12.5g intermediate 2 to 500g with buffer

Figure 2.3. F_2-isoprostane standards and QC samples. *(Taken from (Talbot and Butlin)).*

The concentration values were calculated from the standard curve in the AutoDELFIA Multicalc programme. Figure 2.4. shows the AutoDELFIA 8-iso Prostaglandin $F_{2\alpha}$ assay format. Figure 2.5. shows the 8-iso prostaglandin $F_{2\alpha}$ AutoDELFIA assay schedule. To ensure there was no carry-over during sample pipetting, alternate solutions of high standard, buffer, low standard and buffer were dispensed across a 96 well plate and assayed. This formed part of the routine AutoDELFIA maintenance. The expectation was that sample position and carry-over during pipetting was insignificant and would have no effect on assay precision. *(This was described in (Talbot and Butlin)).*

2.6. Experimental reliability assessments

2.6.1. Experimental handling variables

To investigate the effects of experimental handling on PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential and plasma 8-iso prostaglandin $F_{2\alpha}$ directly, control samples were subjected to various experimental handling variables specific to the method used as shown in Figure 2.6. and as described in Table 2.1. and Table 2.2. respectively. For red blood cell lysis, after cells were centrifuged at 800rmp for 5 minutes in the first step of Section 2.4.5, the supernatant was removed and the pellet was re-suspended in 1ml red blood cell lysis buffer for 5 minutes, stopping the reaction with 10ml of PBS. Cells were centrifuged at 800rpm for 5 minutes at 4^{o}C. Supernatant was removed and the pellet was re-suspended in 1ml of RPMI 1640 medium (R7509). The method described in Section 2.4.5 was then followed.

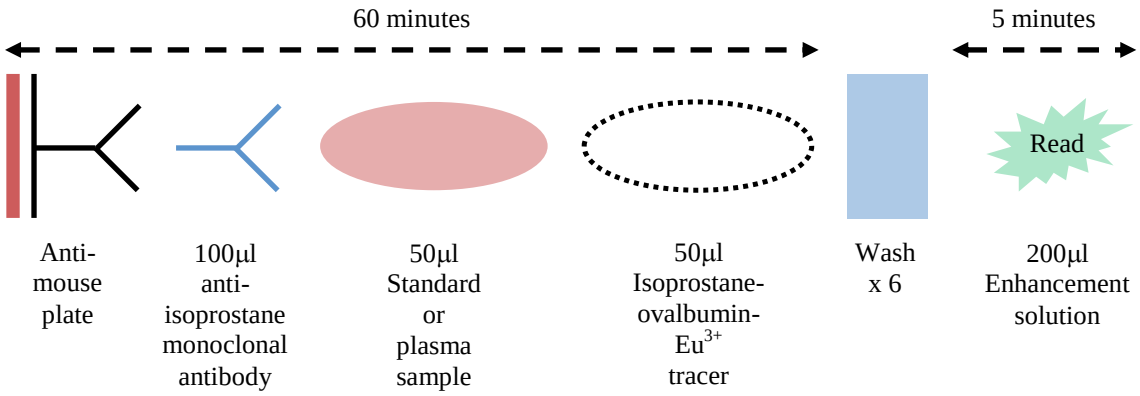


Figure 2.4. AutoDELFIA 8-iso Prostaglandin $F_{2\alpha}$ assay format. *(Adapted from (Talbot and Butlin)).*

```
# PGF.ASS
# ************************************
# Cassette types and volumes (ul) in reag. bottles
# in 1 plate and 4 plate kits.
# variables: cassType,Buff1Vol,TracerVol,AntibodyVol,Buff2Vol
#call DspDefs1 1  50000 1400 750 0
#call DspDefs4 3 180000 1400 750 0
DATA DSPDATA
# ************************************
BEFORENEXT LOAD SAMPDIL
LOAD SAMPLER
BEFORENEXT DISP DILPGFA
#inc dilBtl
BEFORENEXT DISP DILPGFT
#dec dilbtl
#define wellVol 100
DISP PIPDIL
#inc dilBtl
#define wellVol 50
DISP PIPDIL
#inc dilBtl
SHAKE 3600 600
WASH WSH6
ENHDISP ESD1
SHAKE 300 300
#define plateType 2
COUNT CNT1
MOVETO SHAKE
/
```

Figure 2.5. AutoDELFIA 8-iso prostaglandin $F_{2\alpha}$ assay schedule. *(Taken from (Talbot and Butlin)).*

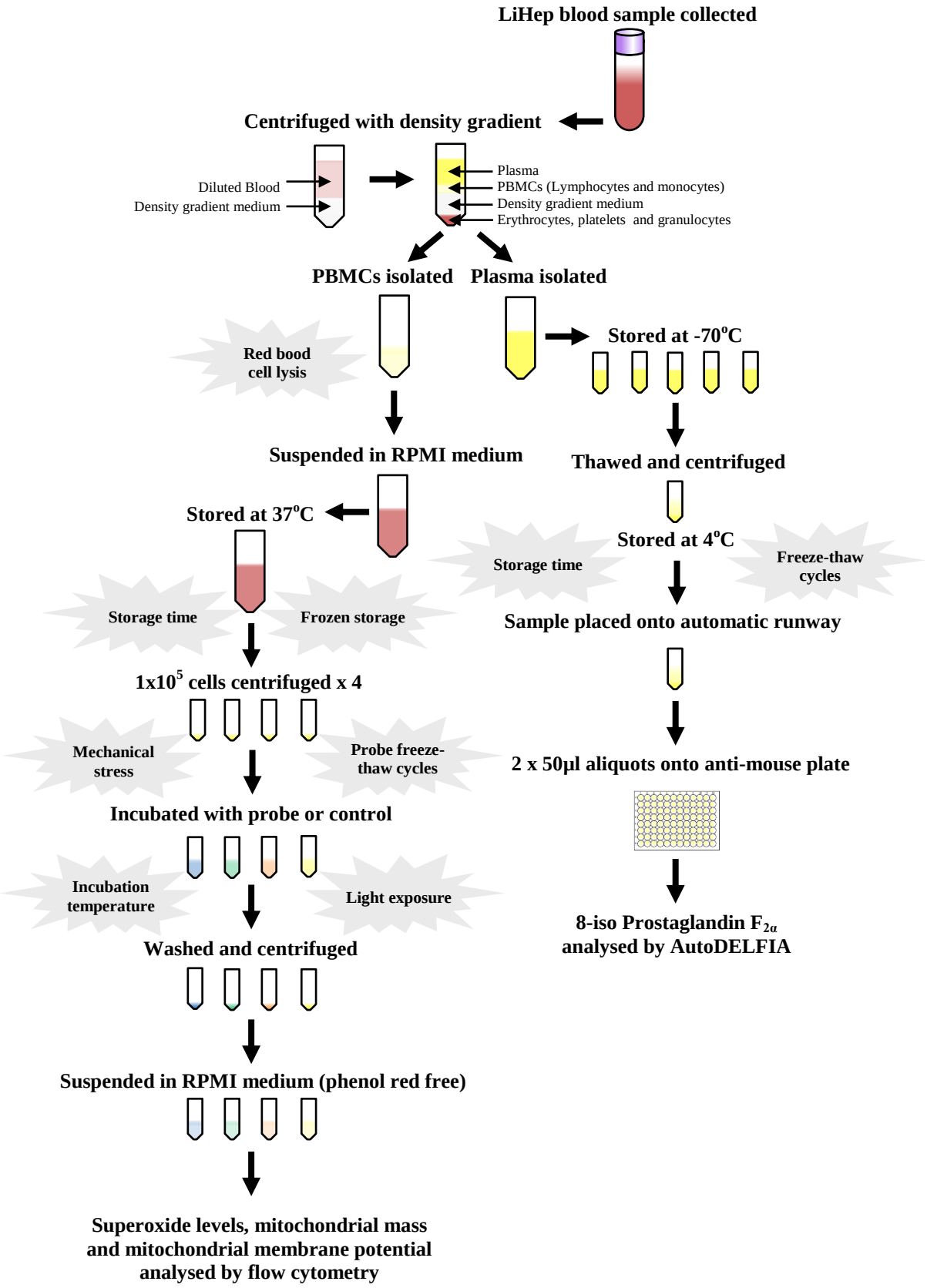


Figure 2.6. Workflow of flow cytometry analysis to measure ROS production and mitochondrial dysfunction, AutoDELFIA analysis to measure F_2-isoprostanes and handling factors that may affect the stability of the measurements.

Experimental variable	Method description
Short-term storage time	Isolated PBMCs were stored in RPMI at 37°C for 2 hours before analysis preparation and compared to 0 hour controls
Long-term storage time	Isolated PBMCs were stored in RPMI at 37°C for 24 hours before analysis preparation and compared to 0 hour controls
Frozen storage	Isolated PBMCs were stored in 90% FBS and 10% DMSO at -70°C before analysis preparation and compared to fresh controls
Cryogenic storage	Isolated PBMCs were stored in 90% FBS and 10% DMSO at -196°C before analysis preparation and compared to fresh controls
Red blood cell lysis	Red blood cells in isolated PBMC samples were lysed and compared to unlysed controls
Probe freeze-thaw cycles	PBMCs were stained with DHE, MitoTracker Green FM and JC-1 probes that had been frozen at -70°C and thawed for 5 or 10 cycles and compared to freshly prepared controls
Mechanical stress	Stained PBMCs before incubation were subjected to a 2000rpm or 3000rmp vortex compared to 1000rpm controls
Light exposure	Stained PBMCs that were unprotected from light were compared to light-protected (by foil) controls
Probe incubation temperature	Stained PBMCs were incubated for 30 minutes at 20°C or 4°C and compared to 37°C controls

Table 2.1. Description of experimental handling variables investigated specific to the measurement of ROS production and mitochondrial function by flow cytometry.

Experimental variable	Method description
Storage time	Plasma samples were thawed at room temperature and stored at 4°C for 24, 48 or 72 hours and compared to 0 hour controls
Plasma freeze-thaw cycles	Plasma sample were thawed at room temperature and refrozen at -80°C or 2, 3 or 4 cycles and compared to 1 freeze-thaw cycle controls

Table 2.2. Description of experimental handling variables investigated specific to the measurement of F_2-isoprostanes by AutoDELFIA.

2.6.2. Intra- and inter-assay precision analysis

After considering the above experimental variables the reliability of flow cytometry to measure PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential and AutoDELFIA to measure plasma 8-iso Prostaglandin $F_{2\alpha}$ was assessed by investigating the average intra- and inter-assay coefficient of variation (C) of three individual control samples. The individual intra- and inter-assay CV of the flow cytometry method was calculated from three repeats of the same sample tube and three individually prepared tubes respectively. The individual intra- and inter-assay CV of the AutoDELFIA method was calculated from ten repeats on the same assay plate and twenty repeats on separate assay plates respectively. A CV≤15% is considered acceptable for the above defined intra-assay and inter-assay precision (Litwin, 2001).

2.6.3. Day-day assay precision and intra-individual reproducibility analysis

To further assess the reliability of flow cytometry to measure PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential and AutoDELFIA to measure plasma 8-iso prostaglandin $F_{2\alpha}$ the day-day reproducibility was assessed, which also permitted the assessment of intra-individual stability. For the flow cytometry analysis six individual PBMC control samples were taken on two separate occasions with an interval of one week and measured independently. The average day-day CV was calculated to assess the short-term day-day reproducibility and the correlation between day one and day seven repeats was calculated to assess the short-term intra-individual stability of PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential. For the AutoDELFIA analysis twelve individual control samples were taken on two separate occasions with an interval of one week but were measured on the same occasion on the same plate. The short-term day-day reproducibility could therefore not be assessed by this method however the short-term intra-individual stability of plasma 8-iso prostaglandin $F_{2\alpha}$ was assessed by calculating the correlation between day one and day seven repeats. The long-term day-day reproducibility and thus long-term intra-individual stability was assessed by calculating the average day-day CV and correlation between the Newcastle 85+ samples at phase 1 and phase 2 (both measured on the same occasion) with phase 3 (measured independently of phase 1 and phase 2). A CV ≤20% is considered acceptable for day-day precision (Litwin, 2001).

2.6.4. Investigating the specificity of AutoDELFIA to the 8-iso Prostaglandin $F_{2\alpha}$ isomer

Four control plasma samples were spiked with 0, 5, 10, 20 and 40ng/ml of 8-iso Prostaglandin $F_{2\alpha}$ (16350, Cayman Chemical Ann Arbor, Michigan, US), 5-i$PF_{2\alpha}$ -V1 (16350, Cayman Chemical Ann Arbor, Michigan, US) or 8-iso Prostaglandin $F_{2\alpha}$ plus 5-i$PF_{2\alpha}$ -V1. The recovery of 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA was determined.

2.7. Structured literature reviews

2.7.1. Classical BoA and their ability to predictor age-related outcomes; specifically mortality, co-morbidity, disability and/or cognitive impairment

The literature search aimed to identify studies investigating various classical BoA as predictors of age-related outcomes, specifically mortality, co-morbidity, disability and/or cognitive impairment. The electronic database MEDLINE was searched via the database provider OvidSP up to November 2012 using specific search terms: [classical BoA* (*total of 62 using a combination of appropriate words/truncations)] AND [predict$] AND [survival OR death OR mortality OR $morbid$ OR disabil$ OR cognit$] restricted to abstracts, studies in humans and from 1946 onwards. Full details of the search terms are shown in Appendix A. The specific classical BoA (1-62 as described in Appendix A) were selected since these were available measures in the Newcastle 85+ study. Studies returned for each classical BoA were ordered by earliest date of publication and screened to find the first study that met a specific criterion I) participants were from a healthy population (i.e. not a disease study), II) participants were not institutionalised (i.e. to increase representativeness of the population groups), III) the study was not a review and IV) the study was a longitudinal follow up. Studies were divided into four age groups: I) young (<45 years), II) middle (>45 and <70 years), III) old (> 70 years) and VI) combined (combination of young, middle and/or old age groups). The age range of participants, number of participants, follow-up length, outcome predicted, association found, dataset used and study reference was recorded if available. Data from a previous publication of the Newcastle 85+ study cohort (Martin-Ruiz *et al.*, 2011b), which investigated the association between the classical BoA and

the four age-related outcomes investigated above, was compared to the structured review data to determine which classical BoA are valid within the very old population. This study used data collected at baseline (phase 1) of the Newcastle 85+ study (See Section 2.1.).

2.7.2. The effects of dietary restriction on potential oxidative stress and cellular senescence-related BoA in leukocytes

The literature search aimed to identify studies investigating the effect of dietary restriction on potential oxidative stress and cellular senescence-related BoA in leukocytes, specifically ROS production and mitochondrial dysfunction, telomere length, DNA damage, DNA repair, IL-6, TNF-α and lymphoid / myeloid ratio. The electronic database MEDLINE was searched via the database provider OvidSP and also PubMed during July 2012 using specific search terms: [calorie restriction OR dietary restriction] AND [peripheral blood OR white blood cells OR lymphocytes OR monocytes OR bone marrow OR lymphoid OR myeloid OR plasma* OR serum* (*cytokines only)] AND [Potential oxidative stress and cellular senescence-related BoA* (*total of 7 using a combination of appropriate words/truncations] restricted to abstracts and from 1946 onwards. Full details of the search terms including the stages of identifying the included articles are shown in Appendix B. ROS production and mitochondrial dysfunction was a combined search, however within the articles ROS production, mitochondrial mass and mitochondrial membrane potential were searched for. There was therefore a total of 9 potential oxidative stress and cellular senescence-related BoA searched. These specific potential oxidative stress and cellular senescence-related BoA were selected since these were available measures in the Newcastle 85+ study. Studies returned for each potential oxidative stress and cellular senescence-related BoA were screened to find all studies that met the search criteria. The diet group, species, gender, age at start of diet, age at end of diet, diet duration, number of participants, cell/tissue type, marker used where relevant, levels obtained, units, significance levels and conclusion were recorded if available.

2.8. Statistical analysis

For experimental data, the Student's t-Test was used to test differences between two samples, the coefficient of variation (CV, %) was used to assess assay precision and Pearson's correlation was used to test the strength of association between two continuous variables. All potential oxidative stress-related BoA had a non-normal distribution in the study population, assessed using the Kolmogorov-Smirnov test for normality ($p<0.05$) and also by viewing Q-Q plots (See Appendix C., Supplementary Table 7. and Supplementary Figure 1.). Attempts to normalise the data using logarithmic transformations (Lg10, Ln, Lngamma, Sqrt and Exp) were unsuccessful. Non-parametric statistical test were therefore employed including Spearman's correlation to test for the relationship between two continuous variables; Mann-Whitney U to test differences between two groups and Kruskal-Wallis to test differences between three or more groups of independent samples; and Wilcoxon Signed-Rank Test to test the differences between two groups and Friedman's Test to test differences between three or more groups of related samples. Chi-square test was used to compare the distribution of grouped variables between two population groups. To test associations with survival, participants were divided into quartiles of superoxide levels, mitochondrial mass and mitochondrial membrane potential with the middle two quartiles grouped together to form the reference category to represent medium levels (See Appendix C, Supplementary Table. 15. for cut-off values). Cox regression was used to test for the differences between grouped variables and survival. p-values <0.05 were considered statistically significant. Since many of the markers are expected to have strong correlations with each other, reflecting common biological mechanisms, formal statistical correction for multiple comparisons was not applied since this would have been over-conservative. Sensitivity analysis was carried out by removing extreme outliers which were values more than 3 times the interquartile range (IQR) below the 25^{th} or above the 75^{th} percentiles, which were identified by default in SPSS boxplots. Potential confounders investigated were gender, ethnicity, mitochondrial haplogroup, age of natural parents death, place of birth, has/had a sibling, had full-time higher education, number of years in main job/role, has offspring, marital status, living alone, housing type, alcohol status, smoking status, taking non-prescribed medicines, supplements and/or herbal remedies, taking prescribed medication, self-reported physical activity, use of aid(s)/appliance(s), social isolation, key event impact, sleep quality, body mass index, fasted blood sample and season of blood sample. Data analysis was performed using SPSS version 19.0.

Chapter 3. Structured literature review of classical BoA and their validation in the very old population

3.1. Abstract

Introduction To date, no biological parameter has been proven to meet the full criteria of a useful BoA. Although many studies investigating classical BoA have shown evidence for their ability to predict age-related outcomes there are many inconsistencies between studies. **Aims** This study aimed to investigate whether different age groups included in studies that investigate various classical BoA could be the reason for their conflicting conclusions. It then aimed to identify which classical BoA are valid in the very old population by comparing the literature and data from the very old population of the Newcastle 85+ study. **Methods** The literature was searched to identify studies that have investigated various classical BoA as predictors of age-related outcomes, specifically mortality, co-morbidity, disability and/or cognitive impairment, using specific search terms and criteria. Identified studies were divided into 4 age groups (young, middle, old and combined) and the type of association found, along with other study details, were recorded. Data from a previous study of the very old population in the Newcastle 85+ study was then compared to the literature review data. **Results** For 10 of the 62 classical BoA investigated the predictive value was found to change between age groups which were; body mass index, waist to hip ratio, fat free mass, diastolic blood pressure, systolic blood pressure, creatinine, glucose, triglycerides, cholesterol and low density lipoproteins (LDL). There were even disagreements between the 10 informative classical BoA identified in the very old population of the Newcastle 85+ study compared to the old population investigated in the literature which were; hand grip strength, timed up and go (TUG), systolic blood pressure, forced expiratory volume in 1 second (FEV_1), red blood cell count, haematocrit, haemoglobin, free triiodothyronine **(**Free T3), vitamin D and N-terminal pro b-type natriuretic peptide (NT-pro BNP). Changes in the predictive value for the remaining 52 classical BoA could not be concluded due to gaps in the literature. **Conclusion** The predictive value of many proposed classical BoA is different in different age groups, even in the old compared to the very old. This could be a reason why there are many inconsistencies between studies. Validating BoA should therefore be carried out in different age groups. There are 10 (and possibly 11) classical BoA that can be used to validate newer candidate cellular and molecular BoA in the very old population.

3.2. Introduction

Many candidates have been proposed as valid BoA including various anthropometric, physical, physiological, haematological and biochemical measurements, termed here as classical BoA, however there are many inconsistencies between studies (Chapter 1, Table 1.1.). None of these markers therefore meet the full criteria of a useful BoA which is being able to predict detrimental outcomes associated with ageing in the absence of disease better than chronological age and be applicable to all members of the population at any given age (Baker and Sprott, 1988; Sprott, 2010). Although classical BoA may not provide a mechanistic insight into biological ageing they can be informative by their association and thus construct validation of "newer" mechanistic cellular and molecular BoA. It is therefore important to understand why classical BoA may be valid in one population group however not in another. It is also important for this study to determine which classical BoA are valid within the very old population of the Newcastle 85+ study. This is so they can be used to validate the potential oxidative stress-related BoA investigated in the proceeding chapters. This will be useful since some of the potential oxidative stress-related BoA were only measured in phase 3 of the Newcastle 85+ study. Although it can be determined if the potential oxidative stress-related BoA are associated with various age-related outcomes at phase 3 and survival, validating them with classical BoA is important since we will be unable to determine if these markers are predictors of various age-related outcomes and thus healthy life expectancy, since these data beyond phase 3 are not available at the current time.

One reason for the conflicting conclusions between studies could be the differences in the age groups they have included since the predictive value of the marker investigated may vary by age group. For example, although there is an increased risk of mortality from high blood pressure in a younger population (Miura *et al.*, 2001) there is an increased risk of mortality due to low blood pressure in the very old population (Mattila *et al.*, 1988). Another example is cholesterol where again although there is an increased risk of mortality from high cholesterol in a younger population (Stamler *et al.*, 2000), there is an increased risk of mortality due to low cholesterol in the very old population (Forette *et al.*, 1989). This could be the case for other candidate BoA.

Differences in the prevalence of intra- and inter-individual factors that may alter the levels of candidate BoA could explain why their predictive value may be absent or reversed within different age groups. For example, the older population are more likely to be on antihypertensive medication than a younger population (Health and Social Care Information Centre (HSCIC, 2011) which could impact on the predictive value of blood pressure within this age group. Another reason could be because of different histories of specific diseases or disorders and thus different causes of mortality between a younger and older population. This can be explained by the various types of associations that may occur between a predictive variable and an age-related disease or disorder that increase the risk of mortality (Figure 3.1.). Many classical BoA follow a non-monotonic relationship in terms of their association with age-related outcomes. For example, high blood pressure (hypertension) is associated with certain cardiovascular diseases including stroke (Yu *et al.*, 2011), myocardial infarction (Pedrinelli *et al.*, 2012), aneurysms (Altay *et al.*, 2012) and also chronic kidney disease determined by a reduction in glomerular filtration or presence of albuminuria (Chia and Ching, 2012). However, low blood pressure (hypotension) is also associated with other cardiovascular diseases including atherosclerosis (Rose *et al.*, 2000) and neurological diseases (Qiu *et al.*, 2003). Another example is cholesterol where high levels of LDL cholesterol (hypercholesterolemia) is associated with various cardiovascular diseases including stroke (Imamura *et al.*, 2009), atherosclerosis (Kruth, 2001), coronary heart disease (Grundy, 1997) and myocardial infarction (Glazer *et al.*, 2005), and endocrine diseases including diabetes (von Eckardstein and Sibler, 2011). However, also low levels of LDL cholesterol (hypocholesterolemia) is associated with depression (Tedders *et al.*, 2011), cancer (Strohmaier *et al.*, 2013), haemorrhagic stroke (Wang *et al.*, 2013) and various respiratory diseases (Iribarren *et al.*, 1997). The prevalence of specific diseases or disorders and causes of mortality vary between age group hence the reason why the predictive value of a candidate BoA may also vary. For example, using data from the UK population, the prevalence of various age-related diseases varies between age group (Figure 3.2.) (Eastern Region Public Health Observatory (ERPHO), 2010). For both men and women below 55-59 years the most prevalent age-related disease is the endocrine disease diabetes mellitus (ERPHO, 2010). However in those above this age group the most prevalent age-related disease is coronary heart disease in men and chronic kidney disease in women (ERPHO, 2010). There is also a decline in the prevalence of coronary heart disease in men at 80-84 years where chronic kidney disease now becomes the most prevalent (ERPHO, 2010).

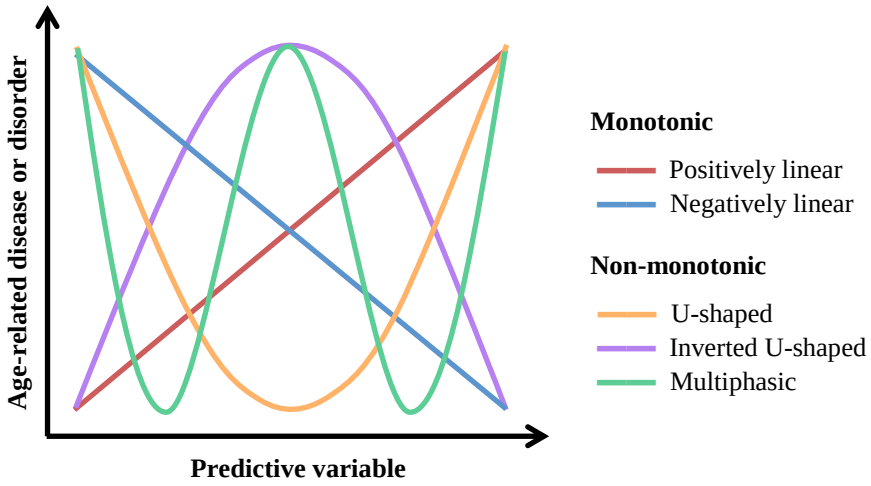


Figure 3.1. Types of associations that may occur between a predictive variable and an age-related disease or disorder.

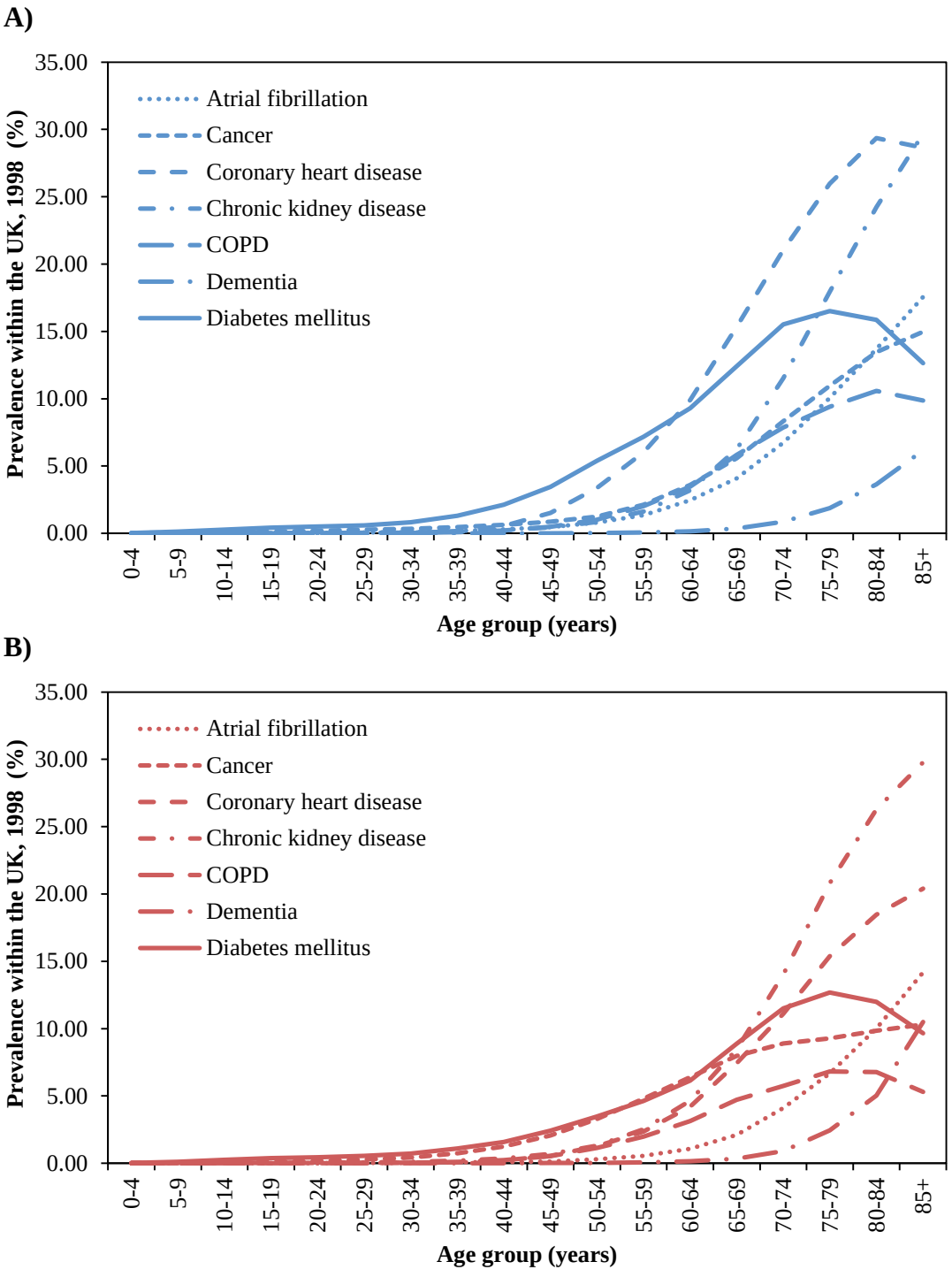


Figure 3.2. Chronic disease prevalence by age and gender in the UK in 1998. (ERPHO, 2010). A) Males, B) Females. (*Chart was created from data tables provided)*

There is also a decline in the prevalence of diabetes mellitus in men and women at 75-79 years and also in chronic obstructive pulmonary disease (COPD) at 80-84 years (ERPHO, 2010). Using data from the English and Welsh population demonstrates the causes of mortality also vary between age groups (Figure 3.3.) (ONS, 2006). In men aged between 50-54 and 70-74 years the leading cause of death is cancer however at ages above this the leading cause of death is cardiovascular disease (ONS, 2006). A similar pattern is shown for women however cancer is the leading cause of death between 1-4 and 70-74 years (ONS, 2006).

The inconsistencies in the validation of candidate BoA could be because studies do not employ appropriate statistical method to control for intra- and inter- individual factors such as medication use and history and/or presence of a disease or disorder within their population group. Also the type of age-related outcome or cause of mortality may vary or not be specific enough. However a study by Langer in 1991 ruled out the most obvious causes for the improved survival of the very old with higher blood pressure including the use of hypertensive medication, pulse pressure, history of hypertension, history of coronary heart disease, isolated systolic hypertension, interval change in diastolic pressure, cholesterol, triglycerides, fasting plasma glucose, smoking and body mass index (Langer *et al.*, 1991). They concluded that the paradoxical relation of improved all-cause and cardiovascular survival in men aged 80 years or older with higher diastolic pressure is not explained by a wide range of biological and historical factors (Langer *et al.*, 1991). It therefore needs to be determined whether the value of various classical BoA varies by age group and if so which are valid within the very old population.

This study therefore aimed to 1) carry out a structured literature review to determine whether different age groups included in studies investigating the predictive validity of various classical BoA is the reason for their opposing conclusions and 2) identify which classical BoA are valid within the very old population and determine the direction of association, which may be different to that of a younger age group, by investigating the predictive validity of various classical BoA in the very old population from the Newcastle 85+ study and comparing these results to the results of the literature review. It is hypothesised that the reason why there is disagreements between studies in the ability of classical BoA to predict age-related outcomes is because of the

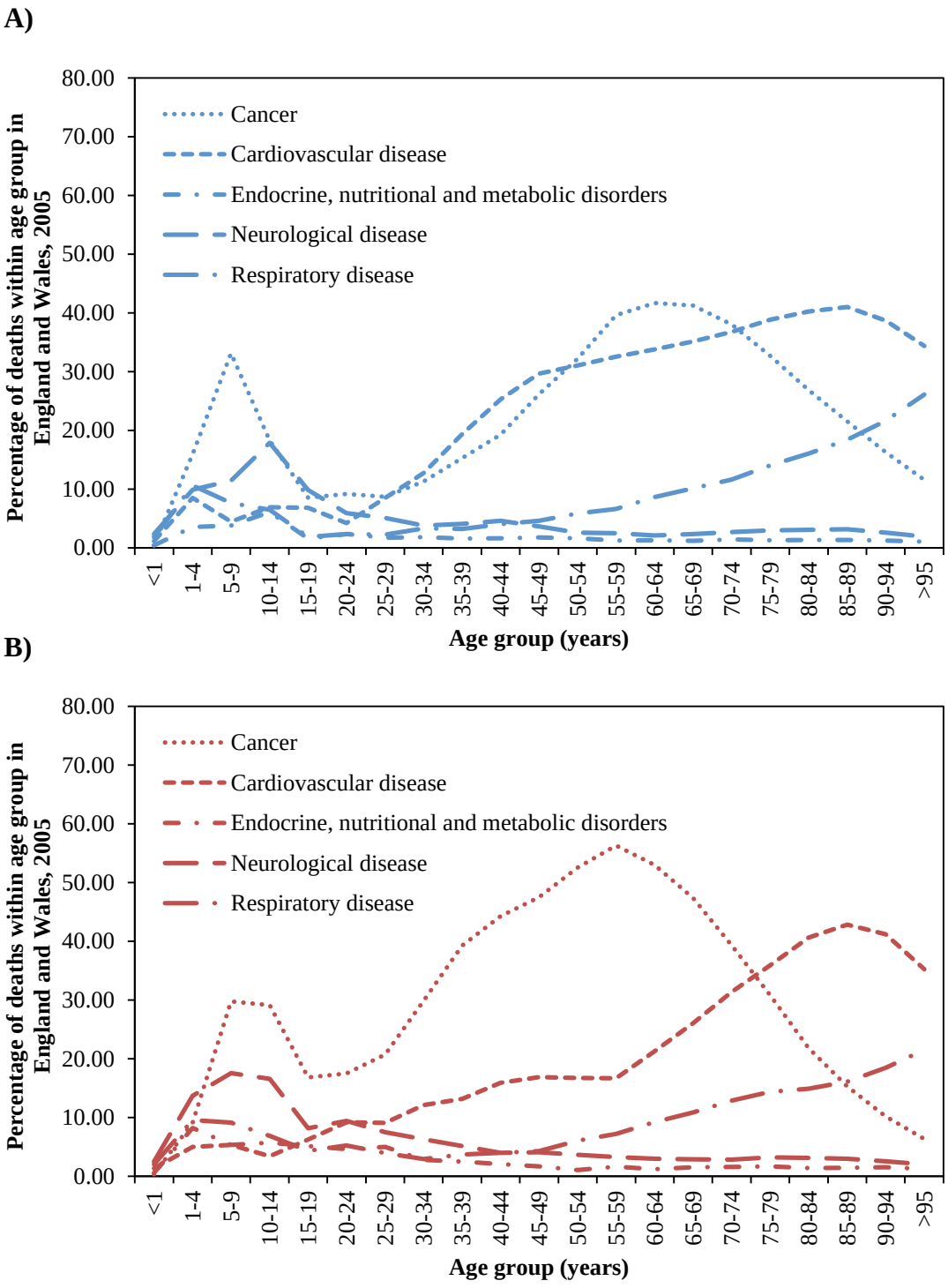


Figure 3.3. Causes of death by age and gender in England and Wales in 2005. (ONS, 2006)**.** A) Males, B) Females. *(Chart was created from data tables provided)*

different age groups within studies and that most classical BoA have different predictive values in an older population compared to a younger population.

3.3. Results

3.3.1. *The value of classical BoA as predictors of age-related outcomes in different age groups*

The first part of this study was to determine whether the predictive value of various classical BoA changes in different age groups and is therefore the reason why there are many inconsistencies between studies who investigate the validity of these measures as BoA. The literature was searched to identify longitudinal studies that have investigated the predictive ability of various classical BoA in a young (<45 years), middle (>45 and <70 years), old (> 70 years) and combined (combination of young, middle and/or old age groups) age group. The idea of the combined age group is to determine whether including a combination of young, middle and/or old age participants is the reason why no association is found, especially if different associations are found between the young, middle and old age groups.

The literature search revealed that 55 out of the 62 selected classical BoA including various anthropometric, physical, physiological, haematological and biochemical measures, according to the search terms, had been investigated as a predictor of age-related outcomes; specifically mortality, co-morbidity, disability and/or cognitive impairment (See Chapter 1, Figure 1.8.). After critically reviewing the literature using the specific criteria in Chapter 2. Section 2.7.1. data for 31 of the classical BoA was available for 1 or more of the age groups (Table 3.1.). A summary of these results is shown in Table 3.2.

For 4 of the 31 classical BoA there was data available for each of the young, middle and old age groups. The predictive value for all 4 of these classical BoA changed in different age groups. These were body mass index, systolic blood pressure, triglycerides and cholesterol. For 19 of the 31 classical BoA there was data available for 2 or 3 of the young, middle, old and combined age groups. For 6 out of these 19 classical BoA the predictive value changed in different age groups. These were waist to hip ratio, fat free

Classical BoA	Age group	Age (years)	n	Follow up (years)	Outcome predicted	Association	Study	Reference
Anthropometry								
Body mass index	Young	20-44	26977	6	Mortality	▲♀	The Italian RIFLE pooling project, Italy	(Seccareccia *et al.*, 1998)
	Middle	40-64	3452 ♂	18	Mortality	X	The Whitehall Study, England	(Strachan, 1992)
	Old	72 (Mean)	551	2.8	Mortality	▼	Elderly Program pilot project, USA	(Siegel *et al.*, 1987)
	Combined	25-79	17159 ♀	12	Mortality	~	Population-based based cohort study, Finland	(Rissanen *et al.*, 1991)
Waist to hip ratio	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	55-69	31702 ♀	11-12	Mortality	▲	Iowa Woman's Health Study, USA	(Folsom *et al.*, 2000)
	Old	70+	2032	3	Mortality	X	Population-based cohort study, Hong Kong	(Woo *et al.*, 2001)
	Combined	55-102	6296	5.4	Mortality	X	The Rotterdam study, England	(Visscher *et al.*, 2001)
Fat %	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	45-59	15609	5.7	Mortality	▲♀	Malmo Diet and Cancer Study, Sweden	(Lahmann *et al.*, 2002)
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	65-100	4809	3	Disability	▲	Cardiovascular Health Study, USA	(Visser *et al.*, 1998)
Fat free mass	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	69-80	1413	4	Mortality	▼	Community-based cohort study, Chile	(Bunout *et al.*, 2011)
	Combined	65-100	4809	3	Disability	X	Cardiovascular Health Study, USA	(Visser *et al.*, 1998)

Physical function

Physical activity	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	40-49	15000 ♂	4	Mortality	▼	The Oslo study, Sweden	(Holme *et al.*, 1981)
	Old	75+	545 ♀	7	Disability	▼	The EPIDOS study, France	(Carriere *et al.*, 2005)
	Combined	45-74	6109	7-13	Mortality	▼	NHANES I Epidemiologic follow-up study, USA	(Davis *et al.*, 1994)
Hand grip strength	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	45-68	6089 ♂	25	Disability	▼	The Honolulu Heart Program, USA	(Rantanen *et al.*, 1999)
	Old	71-91	140 ♂	4	Disability	▼	FINE study, Italy	(Giampaoli *et al.*, 1999)
	Combined	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Timed up and go (TUG)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	70+	144	1.5	Morbidity	x	Community-based cohort study, Italy	(Nikolaus *et al.*, 1996)
	Combined	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Physiological function

Diastolic blood pressure	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	45-68	7610 ♂	10	Mortality	▲	The Hoonolulu Heart Program, Hawaii	(Yano *et al.*, 1983)
	Old	78-92	331	2	Mortality	▼	PROTEGER study, France	(Vischer *et al.*, 2009)
	Combined	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Systolic blood pressure	Young	30-49	159	15	Mortality	▲	Community-based cohort study, England	(Bulpitt *et al.*, 1986)
	Middle	50-69	205	15	Mortality	▲	Community-based cohort study, England	(Bulpitt *et al.*, 1986)
	Old	70+	897	3-4	Mortality	▼♀	Community-based cohort study, Australia	(Korten *et al.*, 1999)
	Combined	50-79	1727	9	Mortality	▲	Community-based cohort study, USA	(Barrett-Connor and Khaw, 1985)
FEV_1	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	40-59	1712 ♂	25	Mortality	▼	Community-based cohort study, Italy	(Menotti *et al.*, 1987)
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	65+	1909	6	Mortality	▼	Community-based cohort study, Poland	(Jedrychowski *et al.*, 1994)
Forced vital capacity (FVC)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	40-59	1711 ♂	25	Mortality	▼	The East-West study, Finland	(Lammi *et al.*, 1990)
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	20-93	2710	8-10	Mortality	▼	The Copenhagen City Heart Study, Denmark	(Lange *et al.*, 1990)
Peak expiratory flow rate (PEFR)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	70+	754	5	Disability and mortality	▼	Community-based cohort study, USA	(Fragoso *et al.*, 2008)
	Combined	60+	2805	8	Disability	▼	Community-based cohort study, Australia	(Simons et al., 2000b)

Haematology

White blood cell count	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	47.5 (Mean)	2011 ♂	13.6	Mortality	▲	Normative aging study, England	(de Labry *et al.*, 1990)
Haematocrit	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	25-64	1238	13	Mortality	▲	MONICA survey, Scotland	(Woodward *et al.*, 2003)
Haemoglobin	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	20-49	6541 ♂	20	Mortality	~	The tromso study, Norway	(Skjelbakken *et al.*, 2006)

Biochemistry

Creatinine	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	75 (Mean)	4637	8	Mortality	~	Cardiovascular Health Study, USA	(Shlipak *et al.*, 2005)
	Combined	40-79	96664	5.2	Mortality	▲ ♀	Community-based cohort study, Japan	(Irie *et al.*, 2001)

Alanine transaminase	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	73-94	686	11	Mortality	x	Longitudinal Study of Aging Danish Twins, England	(Fraser *et al.*, 2009)
	Combined	50-75	1439	10	Mortality	x	The Hoorn study, Netherlands	(Schindhelm *et al.*, 2007)
Albumin	Young	n/a	n/a	n/a	n/a	n/a	n/a	
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	
	Old	71+	4116	3.7	Mortality	▼	Community-based cohort study, USA	(Corti *et al.*, 1994)
	Combined	50-89	2342	3	Mortality	▼	Community-based cohort study, USA	(Klonoff-Cohen *et al.*, 1992)
Alkaline phosphatase	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	38-65	1905644	12	Mortality	▲	Population-based study, USA	(Fulks *et al.*, 2008)
Glucose	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	45-64	10410	11.6	Mortality	▲	Community based Cohort study, Scotland	(Janghorbani *et al.*, 1993)
	Old	85+	558	5	Mortality	▼	Community-based cohort study, Finland	(Kaltiala *et al.*, 1987)
	Combined	60+	2419	9.4	Mortality	▲♀	The Dubbo study, Australia	(Simons *et al.*, 2000a)

Glycosylated haemoglobin **(HbA1c)**	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	70-89	314 ♂	8.1	Mortality	▲	The seven countries study, Finland	(Qiao *et al.*, 2004)
	Combined	49-77	1097 ♂	8.8	Mortality	▲	The hoorn study, Netherlands	(Qiao *et al.*, 2004)
Triglycerides	Young	38-46	803	24	Mortality	x	Population-based study, Sweden	(Lindquist *et al.*, 2002)
	Middle	50-60	659	24	Mortality	▲	Population-based study, Sweden	(Lindquist *et al.*, 2002)
	Old	70	u/k	10	Mortality	x	Community-based cohort study, Sweden	(Agner and Hansen, 1983)
	Combined	45-78	1729 ♂	5.7	Mortality	x	Population-based study, USA	(Rhoads and Feinleib, 1983)
Cholesterol	Young	40	u/k	u/k	Mortality	▲	Framington Heart Study, USA	(Kronmal *et al.*, 1993)
	Middle	50-70	u/k	u/k	Mortality	x	Framington Heart Study, USA	(Kronmal *et al.*, 1993)
	Old	80	u/k	u/k	Mortality	▼	Framington Heart Study, USA	(Kronmal *et al.*, 1993)
	Combined	n/a	n/a	n/a	n/a	n/a	n/a	n/a
High density lipoproteins (HDL)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	71-91	140 ♂	4	Disability	▼	FINE study, Italy	(Giampaoli *et al.*, 1999)
	Combined	40+	10059 ♂	15	Mortality	▼	Population-based cohort study, Israel	(Goldbourt *et al.*, 1985)

Low density lipoproteins (LDL)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	80	u/k	u/k	Mortality	▼	Framington Heart Study, USA	(Kronmal *et al.*, 1993)
	Combined	40-69	2541 ♂	10.1	Mortality	▲	Lipid Research Clinics Program Prevalence Study, USA	(Pekkanen *et al.*, 1990)
Cortisol	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	60-80	19	4	Cognition	▲	Community-based cohort study, Canada	(Lupien *et al.*, 1994)
Thyroid stimulating hormone	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	65+	660	4	Cognition	x	Population-based cohort study, Italy	(Forti *et al.*, 2012)
Rheumatoid factor (RhF)	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Combined	25+	2979	24	Mortality	▲	Community-based cohort study, USA	(Jacobsson *et al.*, 1993)

Vitamin D	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	77-100	998	3	Disability	▼	Cardiovascular Health Study All Stars, USA	(Houston *et al.*, 2011)
	Combined	43 -73	10899	5.8	Mortality	▼	Community-based cohort study, USA	(Vacek *et al.*, 2012)
Homocysteine	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	u/k	32	5	Cognitive impairment	▲	Community-based cohort study, Wales	(McCaddon *et al.*, 2001)
	Combined	50-85	321 ♂	3	Cognitive impairment	▲	Veterans Affairs Normative Aging Study, USA	(Tucker *et al.*, 2005)
NT-pro BNP	Young	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Middle	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	Old	75+	499	7.9	Mortality	▲	Kuopio 75+ health study, Finland	(Kerola *et al.*, 2011)
	Combined	52-74	13466	2.9	Mortality	▲♂	Iwate-KENCO study, Japan	(Nakamura *et al.*, 2010)

Table 3.1. Structured literature search results identifying studies that have investigated various classical BoA as predictors of age-related outcomes within various age groups. (Young: <45 years, Middle: ≥45-<70 years, Old: ≥70 years, Combined: Combination of young, middle and/or old groups, n: number of participants, ▲: positive association,▼: negative association, ~: non-linear association, **X**: no association, ♂: males only, ♀: females only, u/k: unknown, n/a: not available/no data identified)

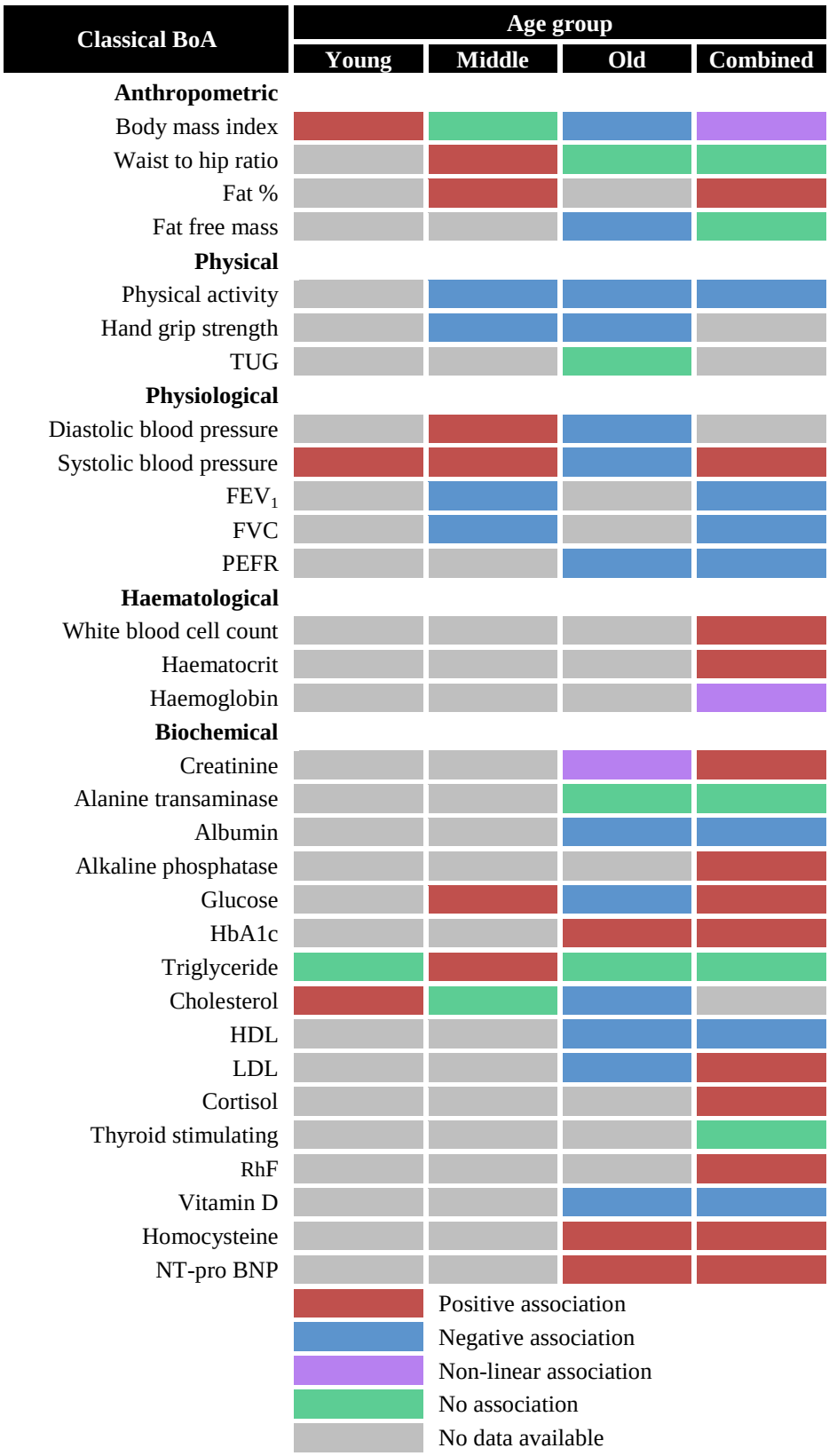


Table 3.2. Summary of the structured literature search results identifying studies that have investigated various classical BoA as predictors of age-related outcomes within various age groups. Only those classical BoA that were identified as being investigated in 1 or more of the 4 age groups are shown. (Young: <45 years, Middle: ≥45-<70 years, Old: ≥70 years, Combined: Combination of young, middle and/or old groups).

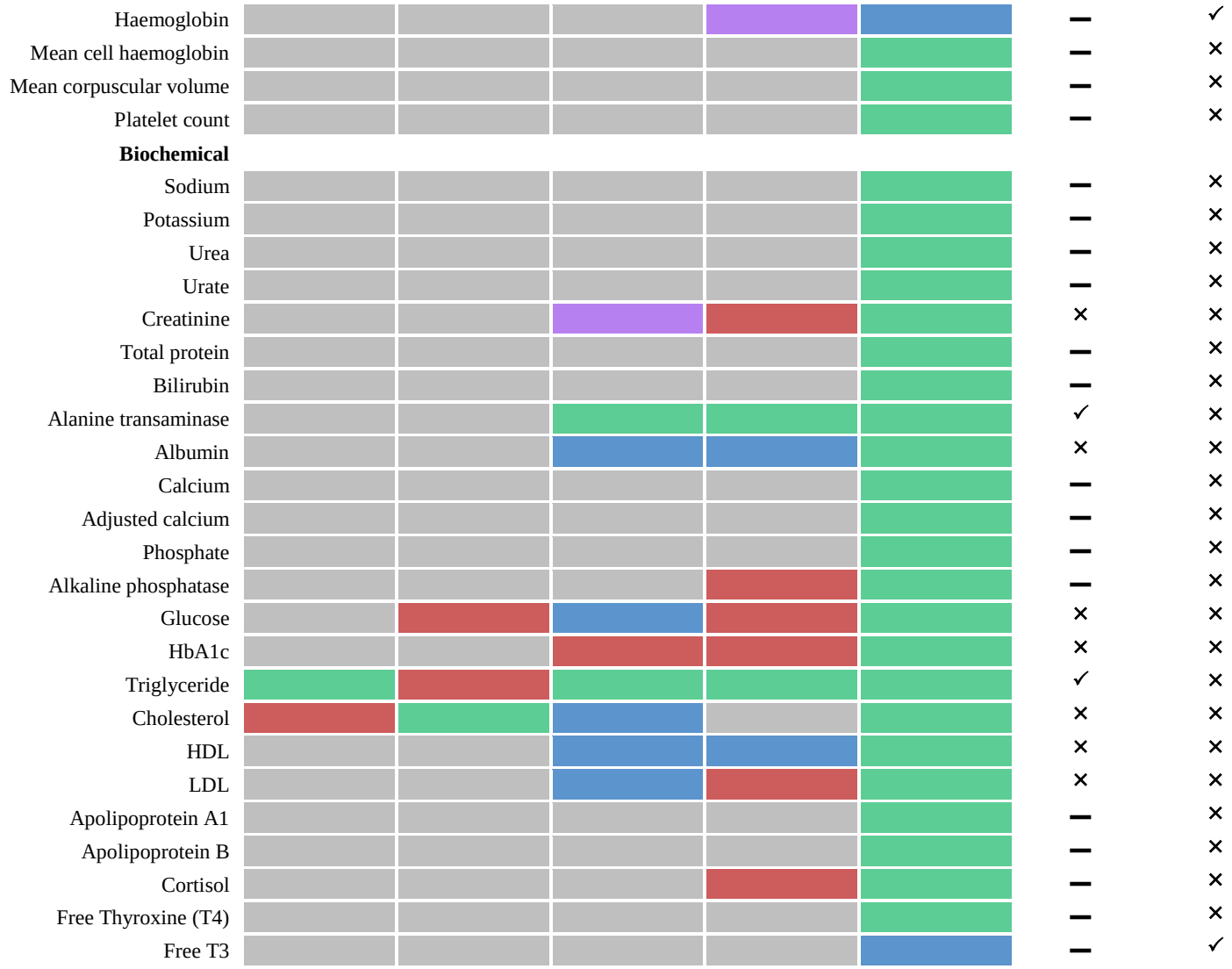
mass, diastolic blood pressure, creatinine, glucose and LDL. For 12 of these 19 classical BoA the predictive value is the same in at least 2 of the different age groups. These were fat %, physical activity, hand grip strength, FEV_1, forced vital capacity (FVC) , peak expiratory flow rate (PEFR), albumin, glycosylated haemoglobin (HbA1c), HDL, vitamin D, homocysteine and B-type naturiuretic peptide. However it can not be ruled out whether the predictive value of these 12 classical BoA changes or not in different age groups at this stage since data for each age group was not available. The 1 remaining classical BoA of these 19, alanine transaminase, was not found to be predictive of age-related outcomes. Again it can not be ruled out whether the predictive value of this classical BoA changes or not in different age groups at this stage since data for each age group was not available. For 8 of 31 classical BoA data for only 1 of the age groups was available. Therefore it can not be ruled out whether the predictive value of these classical BoA changes or not in different age group. 6 out of these 8 classical BoA were predictive of age-related outcomes. These were white blood cell count, haematocrit, haemoglobin, alkaline phosphatase, cortisol and rheumatoid factor (RhF). The remaining 2 of these 8 classical BoA were not predictive of age-related outcomes. These were TUG and thyroid stimulating hormone. Out of all 31 of the classical BoA within the literature search, 17 were valid predictors of age-related outcomes within the old population. These were body mass index, fat free mass, physical activity, hand grip strength, diastolic blood pressure, systolic blood pressure, PEFR, creatinine, albumin, glucose, HbA1c, cholesterol, HDL, LDL, vitamin D, homocysteine and NT-pro BNP. 4 classical BoA were not predictors of age-related outcomes within the old population. These were waist to hip ratio, TUG, alanine transaminase and triglycerides. The remaining 10 were not identified as being investigated as predictors of age-related outcomes in the old population. These were fat mass, FEV_1, FVC, white blood cell count, haematocrit, haemoglobin, alkaline phosphatase, cortisol, thyroid stimulating hormone and RhF. 31 of the 62 classical BoA searched in the literature were not identified as being investigated as predictors of age-related outcomes in any age group. Therefore in total there are 41 classical BoA that have not been investigated in the old population according to the literature search.

3.3.2. *Validated classical BoA in the very old population*

Next the results from the literature search were compared to data from a previous publication of the Newcastle 85+ study cohort (Martin-Ruiz *et al.*, 2011b) which investigated all but 2 (physical activity and oxygen saturation) of the classical BoA above and their association with the 4 age-related outcomes: mortality, co-morbidity, disability and/or cognitive impairment in the very old population. This study used ordinal polytomous regression to test for these associations. For each classical BoA gender-specific deciles were calculated and participants were allocated to 1 of 3 categories: <10th percentile, 10–90th percentile, >90th percentile. The age-related outcome measures were short-term (18 month) survival; disease count categorised as 1–2/3–6/7–18, disability score categorised as 0/1–6/7–12/13–17 and SMMSE categorised as 0–17/18–21/22–25/26–30. For associations between the classical BoA and short-term (18 month) survival, cox proportional hazards regression modelling was used for with proportional hazards verified via scaled Schoenfeld residuals. For associations between classical BoA and co-morbidity, disability and/or cognitive impairment, each classical BoA was first entered singly in regression models and then multivariable models were constructed using backwards elimination in both cases with adjustment for gender. The results for the classical BoA of the structured literature review were compared to classical BoA that were classified as informative BoA in the Newcastle 85+ study, which were those associating coherently with more than 1 age-related outcome in a multivariable analysis fitted with the 10/90th percentile (Figure 3.3.). The idea here was to determine if the results are comparable to the literature to support their use in the validation of potential oxidative stress-related BoA investigated in the proceeding chapters and also to fill in any gaps of the literature search if certain classical BoA were not identified as being investigated in the old population.

39 of the 41 classical BoA that were not identified as being investigated in the old population by the literature review were investigated in the very old of the Newcastle 85+ study. 5 of these were found to be informative BoA in the very old population. These were FEV_1, red blood cell count, haematocrit, haemoglobin and Free T3. Only 4 out of the 17 classical BoA that were found to be predictors of age-related outcomes of the old population were found as informative BoA

Classical BoA	Structured literature review				Very old of Newcastle 85+ study	Agreement with literature?	Informative BoA in very old?
	Age group						
	Young	Middle	Old	Combined			
Anthropometric							
Body mass index						×	×
Waist to hip ratio						✓	×
Fat %						–	×
Fat free mass						×	×
Total body water						–	×
Physical function							
Physical activity						–	✓
Hand grip strength						✓	✓
TUG						×	✓
Physiological							
Diastolic blood pressure						×	×
Systolic blood pressure						✓	✓
FEV_1						–	✓
FVC						–	×
PEFR						×	×
Oxygen saturation						–	–
Haematological							
Red blood cell count						–	✓
White blood cell count						–	×
Lymphocyte count						–	×
Monocyte count						–	×
Neutrophil count						–	×
Basophil count						–	×
Eosinophil count						–	×
Haematocrit						–	✓


Haemoglobin
Mean cell haemoglobin
Mean corpuscular volume
Platelet count
Biochemical
Sodium
Potassium
Urea
Urate
Creatinine
Total protein
Bilirubin
Alanine transaminase
Albumin
Calcium
Adjusted calcium
Phosphate
Alkaline phosphatase
Glucose
HbA1c
Triglyceride
Cholesterol
HDL
LDL
Apolipoprotein A1
Apolipoprotein B
Cortisol
Free Thyroxine (T4)
Free T3

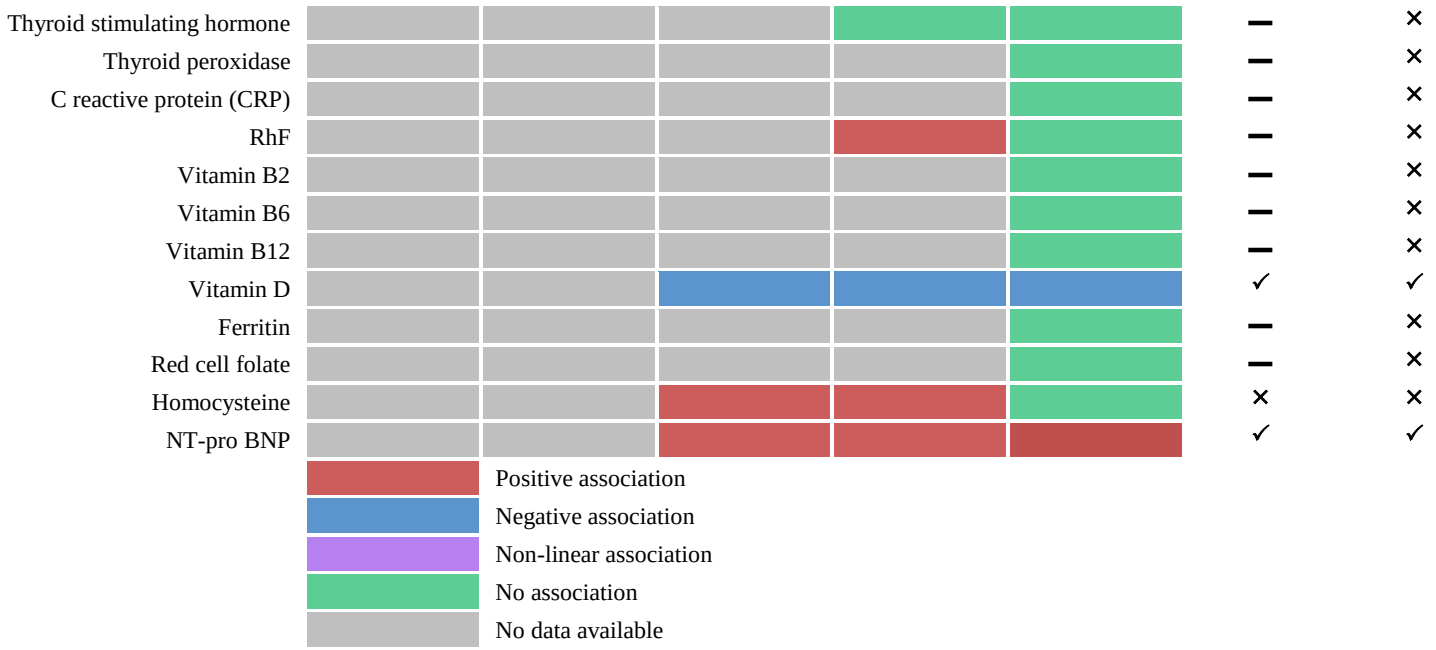


Table 3.3. Comparing the classical BoA in the structured literature search and Newcastle 85+ study. (Young: <45 years, Middle: ≥45-<70 years, Old: ≥70 years, Combined: Combination of young, middle and/or old groups).

in the very old population. These were hand grip strength, systolic blood pressure, vitamin D and NT-pro BNP. 3 out of the 4 classical BoA that were not found to be predictors of age related outcomes in the old population were also not found to be informative BoA in the very old population. 1 of these classical BoA that were not found to be predictors of age-related outcomes in the old population was found to be informative in the very old population. This was TUG. 1 of the classical BoA not investigated in the very old of the 85+ study but in the old population of literature review was predictive of age-related outcomes, this was physical activity. Since this is also predictive of age-related outcomes in the middle and combined age groups and in the same direction of association this is also likely to have predictive value in the very old population. 10 (and possibly 11) classical BoA can therefore be used to validate the potential oxidative-stress related BoA in the very old age group of the Newcastle 85+ study. These are: hand grip strength, TUG, systolic blood pressure, FEV_1, red blood cell count, haematocrit, haemoglobin, Free T3, vitamin D and NT-pro BNP and possibly physical activity.

3.4. Discussion

The first aim of this chapter was to determine whether different age groups included in studies that investigate various classical BoA could be the reason for their conflicting conclusions. Where data was available for more than 1 age group, which included 10 of the 31 classical BoA identified, the predictive value changed in different age groups. This supports the hypothesis that the reason why there are many inconsistencies between studies, who either claim the predictive validity of a classical BoA or not or even claim different directions of associations, is because classical BoA have different predictive values in different age groups. To further support this, although there are some gaps in the literature for specific age groups, it is clear that all the classical BoA identified as having a different predictive value in different age groups follow a positive association with age-related outcomes in the younger and/or middle age group but a negative association in the old age group. Where data was available for the young and/or middle age group and the old age group from the literature search this is true for: body mass index, which was predictive of mortality in a positive direction in the young (Seccareccia *et al.*, 1998) but in a negative direction in the old (Siegel *et al.*, 1987);

diastolic blood pressure, which was predictive of mortality in a positive direction in middle age (Yano *et al.*, 1983) but in a negative direction in the old (Vischer *et al.*, 2009); systolic blood pressure, which was predictive of mortality in a positive direction in the young and middle age (Bulpitt *et al.*, 1986) but in a negative direction in the old (Korten *et al.*, 1999); glucose, which was predictive if mortality in a positive direction in middle age (Janghorbani *et al.*, 1993) and in a combined age group (Simons *et al.*, 2000a) but in a negative direction in the old (Kaltiala *et al.*, 1987); and cholesterol, which was predictive of mortality in a positive direction in the young, not predictive in middle age, but predictive in a negative direction in the old (Kronmal *et al.*, 1993). To add to this; LDL, which was predictive of mortality in a positive direction in a combined age group aged 40-69 years, which thus can be classified in the young and middle age group (Pekkanen *et al.*, 1990), but in a negative direction in the old (Kronmal *et al.*, 1993). These markers could therefore potentially still be used as predictors of age-related outcomes in specific age groups they are identified as being valid, providing the direction of association is taken into consideration, however the difficulty would be where the cut-offs to define different age groups would be.

The second aim of this chapter was to identify which classical BoA are valid in the very old population by comparing the literature and data from the very old population of the Newcastle 85+ study. It was shown that are even different predictive values within the defined age groups when comparing the old population in the literature review to the very old population of the Newcastle 85+ study. For example, body mass index (Siegel *et al.*, 1987), fat free mass (Bunout *et al.*, 2011), diastolic blood pressure (Vischer *et al.*, 2009), PEFR (Fragoso *et al.*, 2008), creatinine (Irie *et al.*, 2001), albumin (Corti *et al.*, 1994), glucose (Kaltiala *et al.*, 1987), HbA1c (Qiao *et al.*, 2004), cholesterol (Kronmal *et al.*, 1993), HDL (Giampaoli *et al.*, 1999), LDL (Kronmal *et al.*, 1993) and homocysteine (McCaddon *et al.*, 2001) are predictors of age related outcomes in the old population but are not predictive in the very old population (Martin-Ruiz *et al.*, 2011a). There are also situations where some studies find that a classical BoA is valid in one age group but another study finds it is not valid in another age group. For example, waist to hip ratio, which was predictive of mortality in a positive direction in middle age (Folsom *et al.*, 2000) but was not predictive in the old (Woo *et al.*, 2001) or a combined age group aged 55-102 years (Visscher *et al.*, 2001); fat free mass, which was predictive of mortality in a negative direction in the old (Bunout *et al.*, 2011) but

not predictive of disability in a combined age group aged 65-100 years (Visser *et al.*, 1998) and triglycerides which was predictive of mortality in a positive direction in middle age (Lindquist *et al.*, 2002) but not in the young (Lindquist *et al.*, 2002) or old (Agner and Hansen, 1983). Could this therefore be due to overlapping age groups? Most studies don't look at the non-linear relationship a BoA may have with an age-related outcome; could this be useful? If so would this allow those validated classical BoA to be used across all age groups? There are two examples of classical BoA where a non-linear prediction is shown. The first is haemoglobin, which was predictive of mortality in a non-linear direction in the old population (Skjelbakken *et al.*, 2006), and creatinine which was predictive of mortality in a non-linear direction in the old (Shlipak *et al.*, 2005) but in a positive direction in a mixed age group aged 40-79 years (Irie *et al.*, 2001). If looking at non-linear predictions is not suitable however, the above classical BoA do not conform to the full criteria of useful BoA since they can not be applied to all members of the population at any given age.

The only classical BoAs that were not found not have different predictive values in different age groups (young, middle age and old) were 2 markers of physical function, physical activity and hand grip strength, which were shown to be predictive of age-related outcomes in a negative direction in middle and both old and very old age groups (physical activity not investigated in the very old) (Holme *et al.*, 1981; Davis *et al.*, 1994; Giampaoli *et al.*, 1999; Rantanen *et al.*, 1999; Carriere *et al.*, 2005; Martin-Ruiz *et al.*, 2011a). (Of course this could be the case for other classical BoA but these could not be identified in this study due to the gaps in the literature). One other marker of physical function, TUG, although was found to be predictive in the very old (Martin-Ruiz *et al.*, 2011a) was not found to be predictive in the old population of the literature search (Nikolaus *et al.*, 1996). The young age group however was not investigated according to the literature search. This information needs to be filled to determine if these potential BoA meet the full criteria of a BoA and be applicable to all the population.

For some classical BoA, inconsistencies between studies however could be explained by other differences between the two studies rather than difference between age groups included. One weakness of this study is that only one study per marker and age group were investigated. Comparisons of more than one study for the same marker and age group would allow the study to determine if differences in the predictive value between

studies were in fact due to age if similar studies show agreements. However, if they do not agree, then other factors could be the cause of inconsistences such as inter-cohort differences, numbers included, controlling of confounders and statistical analyses. Another weakness of this study is that the studies included were those that were older which may reduce the power of concluding that studies show true predictions since using newer studies may be more informative where they may employ more appropriate analysis due to advances over the years. It was assumed that it was more logical/fairer to describe the first study meeting the search criteria. It was not thought that this would effect the outcome of the study since the overall aim was to compare different age groups to see if there are different predictions in opposing directions, not to compare the quality of the studies. In fact, if the quality of the study was investigated, looking at older studies may be more informative of explaining that the disagreement between studies is due to differences in the quality of the studies and not because of different age groups investigated. To support this, it maybe useful to repeat the literature search looking at the more recent studies to see if there are agreements between older and newer studies to see if their associations and or analysis techniques are the same or different to further conclude that there are true differences in the predictive value of BoA between age groups.

3.5. Conclusion

The predictive value of many proposed classical BoA value is different in different age groups, even in the old compared to the very old. This could therefore be a reason why there are many inconsistencies between studies. Validating newer cellular and molecular BoA with classical BoA should therefore be carried out in different age groups. There are 10 (and possibly 11) classical BoA that can be used to validate newer candidate cellular and molecular BoA in the very old population.

Chapter 4. The experimental and intra-individual reliability of potential oxidative stress-related BoA

4.1. Abstract

Background It is critically important that the reliability of the methods used to measure candidate BoA are investigated before they are measured in large population studies in order to continue their validation as predictors of age-related outcomes. **Aims** This study therefore aimed to investigate the experimental and intra-individual reliability of measuring ROS production and mitochondrial function by flow cytometry and F_2-isoprostanes by AutoDELFIA. **Materials and Methods** PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential, and also plasma 8-iso Prostaglandin $F_{2\alpha}$ were measured in human controls to investigate various experimental handling, assay precision, intra-individual stability and method specificity. The Newcastle 85+ data was also used to investigate the longitudinal stability of 8-iso Prostaglandin $F_{2\alpha}$. **Results** Variables found to affect the stability of ROS production and mitochondrial function in PBMCs during preparation before flow cytometry analysis were: sample storage at 37^{o}C for 24 hours; frozen storage at -70^{o}C and -196^{o}C; removing red blood cells by lysis; and the staining temperature of cells. Storing plasma samples at 4^{o}C for more than 48 hours after thawing affected the stability of 8-iso Prostaglandin $F_{2\alpha}$. Intra- and inter-assay precision was acceptable for all parameters. There was good day-day repeatability of the flow cytometry analysis for superoxide and mitochondrial mass but not for mitochondrial membrane potential. Superoxide levels, mitochondrial mass and mitochondrial membrane potential were stable within the short-term in the PBMC and lymphocyte populations but not in the monocyte population. There was also good day-day repeatability of the AutoDELFIA analysis. F_2-isoprostanes were stable in the short-term and also in the long-term in the very old population. Good specificity of the flow cytometry quantification method was shown and also good specificity of the AutoDELFIA method to detect the 8-iso Prostaglandin $F_{2\alpha}$ isomer only. **Conclusion** This study identified variables that affect the stability of PBMC ROS production and mitochondrial function and plasma F_2-isoprostanes during experimental analysis which can therefore be controlled. It also provided evidence of their experimental reliability and intra-individual stability.

4.2. Introduction

The first step when investigating candidate BoA in large population studies should be to establish their degree of reliability. Reliability concerns measuring how much of the variability in a parameter can be attributed to genuine individual differences and not to measurement error (McClearn, 1992; McClearn, 1997). Although it is often thought that measurement error will be eliminated by repeating the measurement on two or more occasions this practice has its disadvantages. On one hand if there is no knowledge that certain handling factors can affect the stability of a candidate BoA then random systematic errors may occur during one of the multiple measurements thus making the combined measurements invalid. This would decrease the sensitivity and specificity of the method used and the power of obtaining statistical significance if this was the case when errors are not introduced. On the other hand it may only be appropriate to obtain one measurement due to cost, material and time constraints. Also when a new candidate BoA is investigated its measurement may be of value sometime after the start of a study, for example, in a life history cohort. One time measurements of newer more mechanistic cellular and molecular BoA can be validated with existing less mechanistic classical BoA, therefore one time measurements are not impractical. Determining factors that affect the reliability of BoA measurements is also valuable information when they are measured on different occasions by different investigators or in different laboratories which will prevent non-random systematic errors and thus experimental bias.

An important factor that may affect the reliability of candidate BoA is its experimental stability which could be eliminated or controlled if identified to prevent unwanted measurement error. It concerns various handling factors during preparation and analysis of the sample. Experimental stability could be tested directly by comparing altered handling factors to a control or indirectly by determining the intra- and inter-assay repeatability, agreements with similar parameters and agreements with alternative methods. The handling factors investigated will vary depending on the sample and method being used. For this study it will be necessary to identify and investigate specific handling factors before and during flow cytometry and AutoDELFIA analysis that could potentially have an effect on the potential oxidative stress-related BoA being investigated (See Chapter 2. Figure 2.6.). Once factors that have an effect are identified then a standardised procedure can be implemented for all measurements. The handling

of the PBMC and plasma samples and the effect it may have on the potential oxidative stress-related BoA is very different and therefore needs to be investigated independently.

Analysing ROS production and mitochondrial dysfunction in PBMCs is a direct way of measuring oxidative stress in cells and thus is still subject to variation if put under certain conditions *ex vivo.* Therefore conditions during sample preparation and analysis that might increase (or decrease) free radical reactions taking place within the cells needs to be investigated to prevent the measurement of artificial oxidative stress. This is particularly so when a large number of samples are to be compared and are measured on different occasions in large population studies.

One issue is sample storage time since the cells are not in their normal environment and thus the time spent in altered temperatures, movements and culture medium could have an effect on the induction of an oxidative stress response. It has previously been shown that storage time decreases PBMC mitochondrial membrane potential (Cassart *et al.*, 2007). This suggests that mitochondrial function may be affected by storage conditions which may also influence ROS production. Using fresh PBMCs in large population studies can be problematic since only a few participant samples are available per day and thus variations in handling factors that affect the stability of the measurements could occur. Also logistics might require cryopreservation in order to obtain large numbers of patient materials that can be analysed at a later time point (Jeurink *et al.*, 2008). One way around this is to freeze the PBMC samples on the day of collection and separation so a larger number of samples can be measured per day. Although this may not be feasible at present due to the time needed to analyse a large number of samples this would be useful if there was ever an automatic system for the described flow cytometry protocol (See Chapter 2. Section 2.4.1.).

A second issue is the separation of PBMCs (lymphocytes and monocytes) from other cells by density gradient centrifugation since this is not always perfect and sometimes red blood cells remain within the sample in different proportions which is dependant on the donor samples, the density gradient medium used and technical skills (Chan *et al.*, 2013). It would be ideal if red blood cells were eliminated from the PBMC samples since this will have an effect on the number of PBMCs counted by flow cytometry per individual if their red blood cell counts vary. Red blood cell presence may even have an

effect on PBMC ROS production and mitochondrial function in cells. It has been shown that lymphocytes in whole blood produce more cytokines when their production is stimulated than lymphocytes that have been separated by density gradient centrifugation (Suni *et al.*, 1998; Song *et al.*, 2002). This indicates that the presence of red blood cells in PBMC samples could have an effect on ROS production however this has not been investigated. Alternately red blood cell lysis buffer itself or factors released from lysed red blood cells could have an effect.

A third issue to consider is the handling of the fluorescent probes. It is often reported that fluorescent probes loose their effectiveness when repeatedly freeze-thawed, possibly due to altered solubilisation and thus uneven loading in different samples or due to increased light exposure (Dinnen *et al.*, 2013). A way around this is to store the probe in aliquots of the required volume. However the aliquots will need to be a larger volume than what is actually needed to stain the cells due to loss by pipetting which will lead to unnecessary probe wastage. It would therefore be more efficient to use larger aliquots thus the freeze-thaw stability of the probes should be investigated. Light exposure to the probe and to the stained cells is also an important factor to control since this may reduce the probes fluorescence known as photo-bleaching caused by the irreversible destruction of fluorophores due to prolonged exposure to light (Thermoscientific, 2013).

Since measuring ROS production directly can be difficult due to its high reactivity and short half-life an alternative indirect method of investigating oxidative stress is to measure the products of ROS reactions or damage. Since these are end-products of oxidative damage it is considered a better way of measuring oxidative stress *in vivo* due to its stability since they are less likely to be altered during the analysis process. The products of lipid peroxidation, F_2-isoprostanes, are thought to be the more accurate markers of oxidative stress *in vivo* (Milne *et al.*, 2005). Since samples used to measure F_2-isoprostanes including plasma require little preparation and can be frozen once collected they can be measured in large batches for which automation is possible, reducing the effects of day-day handling variations. Analysis of F_2-isoprostanes by EIA and TR-FIAs including AutoDELFIA also require less preparation steps than the alternative GCMS or LCMS/MS methods, again reducing the effects of handling. Storage time and the number of freeze-thaw cycles of plasma should be considered as factors that may influence F_2-isoprostane concentration.

The second factor that may affect the reliability of candidate BoA is their intra-individual variability which could also be eliminated or controlled if identified to prevent unwanted measurement error. Intra-individual variability concerns the day-day behaviours or environmental conditions of an individual that may change or fluctuate a candidate BoA and impact on its day-day repeatability, which could be eliminated or controlled before experimentation or by using statistical approaches during analysis (Hershberger and Moskowitz, 2001; Nesselroade and Ram, 2004). Intra-individual variability could be tested directly by comparing altered behaviour/environmental variables to a control or indirectly by determining short- and long-term stability. The elimination or control of intra-individual variables before experimentation would require the participants to refrain from a certain behaviour/environment known to affect the candidate BoA for a period of time before the sample collection. For example, variations in eating patterns are known to affect some blood measurements and therefore a 24 hour fasted blood sample is normally required. This however may not be feasible for many behaviours such as the taking of medication or environmental conditions such a seasonal changes. Therefore alternatively, information on participant's behaviours could be gathered by questionnaires and be controlled for through statistical analysis. Examples of intra-individual factors that may affect candidate BoA measurements include changes in diet, smoking, alcohol consumption, medication, sleep quality, physical activity, use of aids and appliances, social events and seasonal/environmental exposures. This chapter will investigate intra-individual variability indirectly by determining the day-day intra-individual reproducibility of potential oxidative stress-related BoA. The direct investigation of intra-individual variability by comparing altered behaviour or environmental variables to a control was not tested before this study. These variables, if any, were therefore not controlled by participants refraining from certain behaviours. This will therefore be investigated in chapter 5 through the examination of participant characteristics at the period the blood sample was taken. Variables found to have a significant impact on the parameters could then be controlled for in future analysis.

Since using fluorescent probes to measure ROS production and mitochondrial function by flow cytometry and F_2-isoprostanes by AutoDELFIA are rarely (if ever) measured in large population studies this information is therefore invaluable to continue their validation as potential BoA. This study therefore aimed to investigate the experimental

and intra-individual reliability of measuring ROS production and mitochondrial function by flow cytometry and F_2-isoprostanes by TR-FIA by AutoDELFIA.

4.3. Results

4.3.1. The effects of various experimental handling

To ensure that PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential by flow cytometry remained stable between sample handling and analysis, various handling factors that may affect their stability were investigated in human control samples (Table 4.1). There was no significant effect after 2 hours storage of samples at 37^{o}C prior to staining for any of the parameters but after 24 hours a significant increase in superoxide levels could be observed ($p<0.01$), independent of mitochondrial mass and mitochondrial membrane potential. All parameters were affected by frozen storage of cells in 90% FBS and 10% DMSO. At -70^{o}C superoxide levels increased ($p<0.01$) and mitochondrial membrane potential decreased ($p=0.02$), however there was no significant change in mitochondrial mass. At -196^{o}C superoxide levels and mitochondrial mass increased while mitochondrial membrane potential decreased ($p<0.01$). There was no significant effect of vortex-mediated mechanical stress for any of the parameters. Removing red blood cells by lysing them significantly stressed the remaining PBMCs as evidenced by increased superoxide levels and decreased mitochondrial membrane potential ($p=0.01$). There was no significant effect on repeatedly freeze-thawing DHE, MitoTracker Green FM or JC-1 stain solutions before cell staining on all parameters for up to 10 cycles. There was no effect of light exposure on stained cells but there was a significant effect of staining temperature on all parameters.

To ensure that plasma 8-iso Prostaglandin $F_{2\alpha}$ remained stable between sample handling and analysis by AutoDELFIA, various handling factors that may affect their stability were investigated in human control samples (Table 4.2). There was no significant effect of storing thawed plasma for 24 hours at 4^{o}C on 8-iso Prostaglandin $F_{2\alpha}$ concentration however it significantly deteriorated after 48 hours ($p=0.03$) and 72 hours ($p=0.01$). There was however no effect of three plasma freeze-thaw cycles.

Experimental variable		Superoxide levels (AU)	Mitochondrial mass (AU)	Mitochondrial membrane potential (AU)
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
Short-term storage time	0h (Control)	39.16 ± 5.53	28.53 ± 3.78	4.71 ± 0.58
	2h	32.42 ± 7.42	26.06 ± 2.45	4.52 ± 0.90
	p	0.28	0.40	0.77
Long-term storage time	0h (Control)	**28.01 ± 2.54**	50.83 ± 0.46	2.17 ± 0.17
	24h	**80.91 ± 2.02**	44.04 ± 4.50	2.26 ± 0.15
	p	**<0.01**	0.06	0.54
Frozen storage	Fresh (Control)	**41.03 ± 14.45**	42.62 ± 4.77	**5.52 ± 0.98**
	-70^{o}C	**85.9 ± 13.82**	46.00 ± 7.99	**3.28 ± 0.50**
	p	**<0.01**	0.56	**0.02**
Cryogenic storage	Fresh (Control)	**53.85 ± 4.56**	**41.47 ± 8.04**	**4.83 ± 0.30**
	-196^{o}C	**85.9 ± 13.82**	**101.63 ± 8.21**	**2.94 ± 0.27**
	p	**<0.01**	**<0.01**	**<0.01**
Mechanical stress	1000rpm (Control)	50.69 ± 12.84[1]	39.72 ± 2.55[1]	3.13 ± 1.59[1]
	2000rpm	49.63 ± 17.31[2]	41.92 ± 2.73[2]	3.49 ± 1.23[2]
	3000rpm	53.83 ± 19.79[3]	37.69 ± 0.89[3]	2.97 ± 1.16[3]
	p	0.94[1v2]	0.37[1v2]	0.77[1v2]
		0.83[1v3]	0.26[1v3]	0.90[1v3]
Red blood cell lysis	No lysis (Control)	**31.57 ± 1.68**	40.05 ± 8.17	**2.75 ± 0.40**
	Lysis	**126.31 ± 21.33**	48.90 ± 5.48	**1.56 ± 0.06**
	p	**0.01**	0.19	**0.01**

Probe freeze-thaw cycles	0 cycles (Control)	194.98 ± 15.21[1]	46.00 ± 7.99[1]	3.28 ± 0.50[1]
	5 cycles	195.65 ± 38.37[2]	48.22 ± 4.47[2]	3.31 ± 0.54[2]
	10 cycles	211.94 ± 44.08[3]	44.95 ± 6.45[3]	3.36 ± 0.60[3]
	p	0.98[1v2]	0.71[1v2]	0.94[1v2]
		0.56[1v3]	0.82[1v3]	0.86[1v3]
Light exposure	Unexposed (Control)	25.70 ± 5.05	60.12 ± 14.22	8.59 ± 0.62
	Exposed	25.40 ± 4.34	52.26 ± 3.59	6.41 ± 1.65
	p	0.94	0.41	0.10
Probe incubation temperature	37°C (Control)	**55.27 ± 11.18[1]**	**37.81 ± 12.25[1]**	**2.32 ± 0.20[1]**
	20°c	34.82 ± 5.54[2]	21.37 ± 6.11[2]	**5.82 ± 0.85[2]**
	4°C	**25.82 ± 6.33[3]**	**12.23 ± 1.57[3]**	3.47 ± 1.02[3]
	p	0.05[1v2]	0.11[1v2]	**<0.01[1v2]**
		0.02[1v3]	**0.02[1v3]**	0.13[1v3]

Table 4.1. Stability of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMCs during various experimental handling prior and during flow cytometry analysis. Student's t-Test was used to test for the difference between the experimental variable and control (n=3). Where more than one variable was tested the single experimental variable ([2] or [3]) was compared to the control ([1]).

Experimental variable		8-iso Prostaglandin $F_2\alpha$ (ng/ml)
		Mean ± SD
Storage time	0h (Control)	**39.66 ± 0.22**[1]
	24h	39.14 ± 0.37[2]
	48h	**38.66 ± 0.32**[3]
	72h	**37.68 ± 0.42**[4]
	p	0.11[1v2]
		0.03[1v3]
		0.01[1v2]
Plasma freeze-thaw cycles	1 (Control)	40.27 ± 0.26[1]
	2	39.33 ± 0.92[2]
	3	39.74 ± 1.90[3]
	4	39.49 ± 1.22[4]
	p	0.15[1v2]
		0.42[1v3]
		0.23[1v2]

Table 4.2. Stability of 8-iso Prostaglandin $F_{2\alpha}$ in plasma during various experimental handling prior to AutoDELFIA analysis. Student's t-Test was used to test for the difference between the experimental variable and control (n=2). Where more than one variable was tested the single experimental variable ([2] or [3] or [4]) was compared to the control ([1]).

4.3.2. Intra- and inter-assay precision

After considering the above experimental variables the reliability of the flow cytometry method to evaluate ROS production and mitochondrial function in PBMCs and subpopulations was assessed by calculating the average intra- and inter-assay CV of three individual control samples (Figures 4.1 and 4.2. respectively). The average intra- and inter-assay precision of all parameters in all cell subpopulations was acceptable (CV≤15%) (with the exception inter-assay precision of mitochondrial membrane potential in PBMCs and monocytes which was borderline acceptable (22.45% and 17.02% respectively)) (Litwin, 2001): superoxide levels measured by DHE showed an intra-assay CV of 4.77-6.84% and inter-assay CV of 5.22-6.81%, mitochondrial mass measured by MitoTracker Green FM showed intra-assay CV of 3.21-9.81% and inter-assay CV of 10.79-13.36% and mitochondrial membrane potential measured by JC-1 showed intra-assay CV of 6.43-12.23% and inter-assay CV (lymphocytes only) of 10.83%.

The reliability of the AutoDELFIA method to measure plasma 8-iso Prostaglandin $F_{2\alpha}$ concentration was also assessed by calculating the average intra- and inter-assay CV of ten and twenty repeats respectively of three individual control samples (Figure 4.3. and 4.4. respectively). The average intra- and inter-assay CV was 25.28% and 23.64% respectively which was above the acceptable CV≤15%. Considering the individual samples separately there was acceptable intra- and inter-assay precision for plasma 8-iso Prostaglandin $F_{2\alpha}$ concentrations above ≈6ng/ml with CVs of 2.51-6.00% and 8.20-4.99% respectively however at concentrations below ≈1ng/ml the intra- and inter-assay precision was unacceptable with CVs of 67.34% and 57.73% respectively. It is likely that the low 8-iso Prostaglandin $F_{2\alpha}$ concentration sample is below the limit of detection (LOD) for the AutoDELFIA assay which is 0.25ng/ml. The sensitivity or the LOD of the isoprostane AutoDELFIA assay is defined as the value which is 3 standard deviations below the mean of the zero standard measurement value (mean value–3SD) (n=26). Assay sensitivity was taken as the mean of the 3 values obtained from the 3 separate runs. A standardised adjustment to 0.25ng/ml are made for samples which are below the LOD.

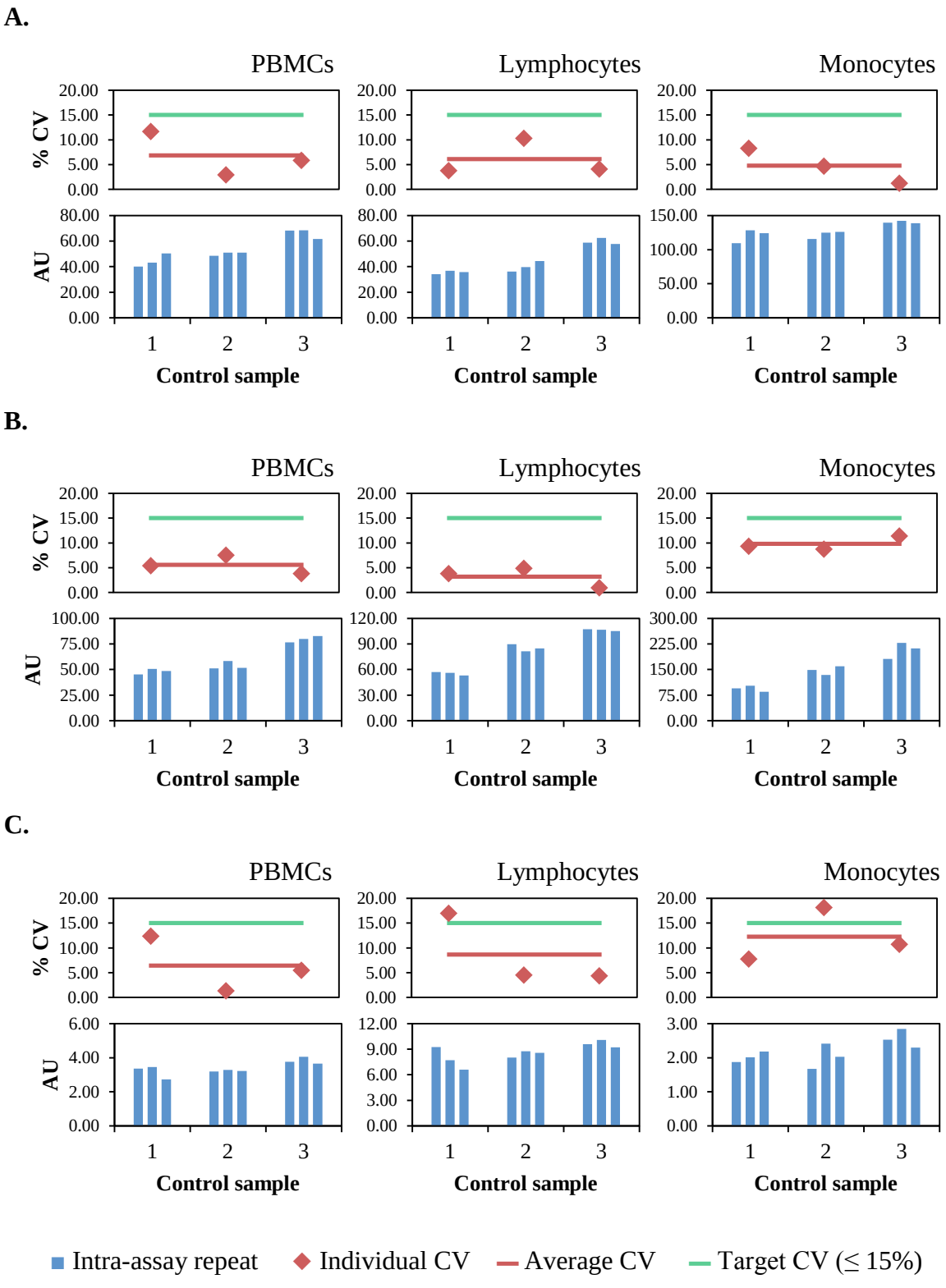


Figure 4.1. Intra-assay precision of A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis. (See Appendix C, Supplementary Table 1. for raw data).

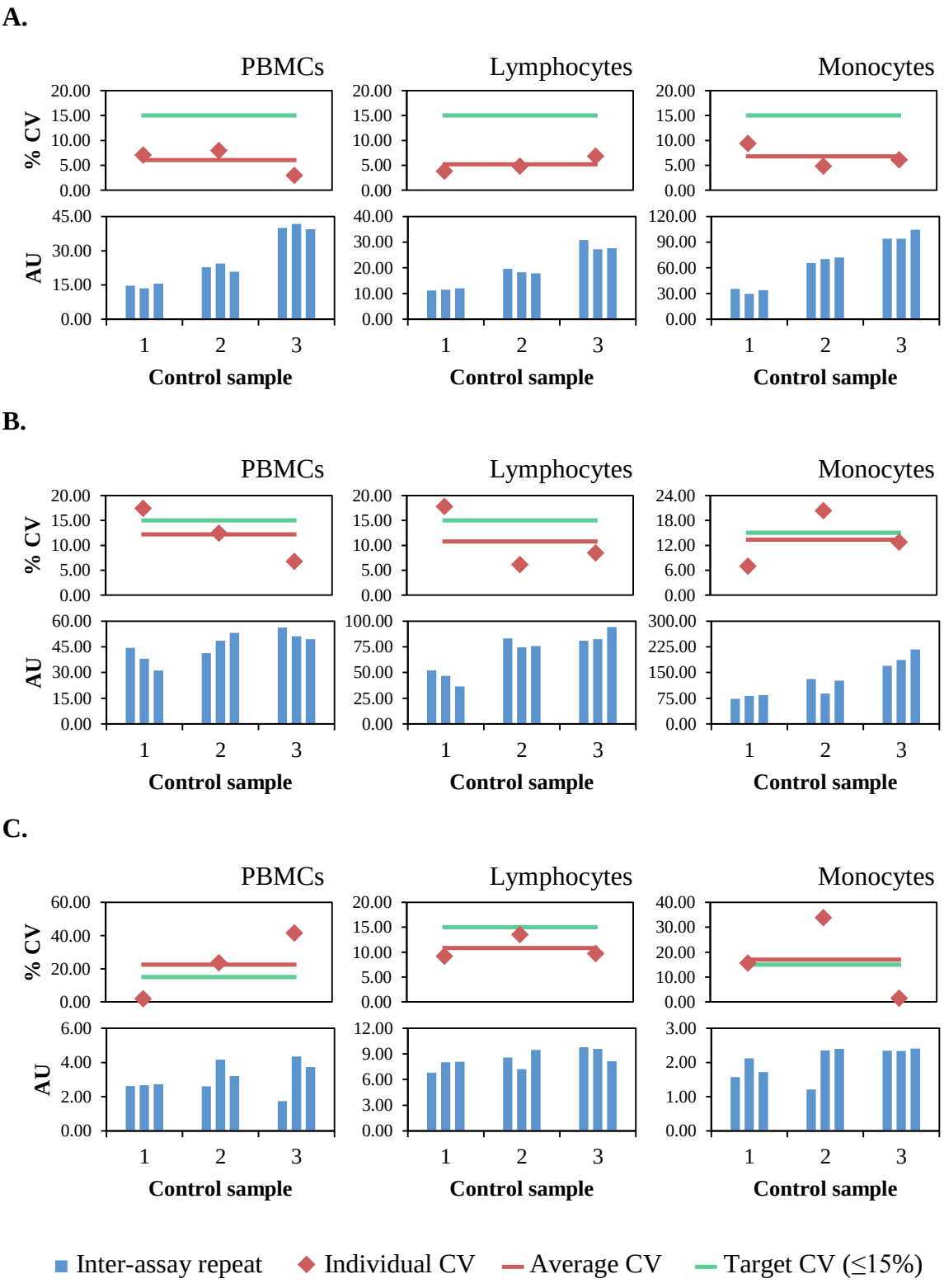


Figure 4.2. Inter-assay precision of A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis. (See Appendix C, Supplementary Table 2. for raw data).

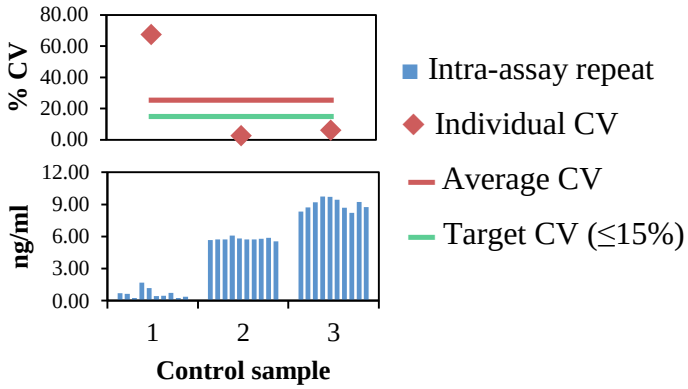


Figure 4.3. Intra-assay precision of 8-iso Prostaglandin $F_{2\alpha}$ in plasma by AutoDELFIA analysis. (See Appendix C, Supplementary Table 3. for raw data).

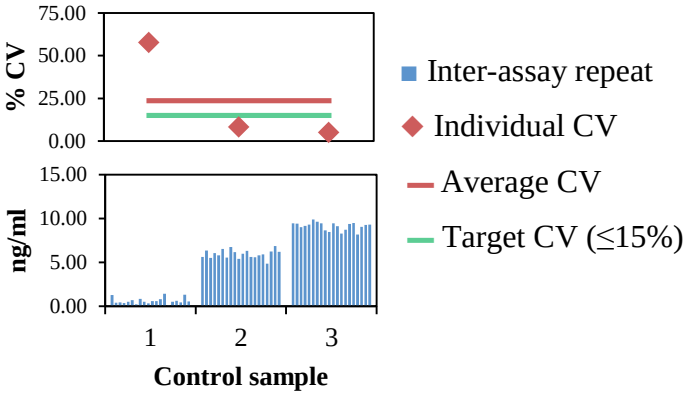


Figure 4.4. Inter-assay precision of 8-iso Prostaglandin $F_{2\alpha}$ in plasma by AutoDELFIA analysis. (See Appendix C, Supplementary Table 4. for raw data).

4.3.3. Day-day assay precision and intra-individual reproducibility

To evaluate the short-term day-day repeatability of the preparation and measurement by flow cytometry and also the short-term intra-individual stability of superoxide production, mitochondrial mass and mitochondrial membrane potential, six individual control blood samples were taken on two occasions with an interval of one week and samples were measured independently (On day 1 and day 7). Day-day assay precision of CV≤20% is considered acceptable. Measurements of superoxide levels and mitochondrial mass in total PBMCs and lymphocytes showed acceptable CVs (4.17%–8.31%) (Figure 4.5. A. and B.) and were strongly correlated at both time points (r≥0.66) (Figure 4.6. A. and B.), however measurements in monocytes, despite showing acceptable CVs (12.42% and 13.19%) (Figure 4.5. A. and B.), showed little correlation (r≤0.19) (Figure 4.6. A. and B.). Measurements of mitochondrial membrane potential in total PBMCs and lymphocytes showed large CVs between time points (39.02% and 23.92%) (Figure 4.5. C.) however strongly correlated (r≥0.64) (Figure 4.6. C.) while measurement in monocytes also had a large CV (56.97%) (Figure 4.5. C.) and little correlation between time points (r=0.25) (Figure 4.6 C).

To evaluate the short-term day-day repeatability of the preparation and storage (however not measurement by AutoDELFIA since all samples were measured on the same occasion on the same plate) and also the short-term intra-individual stability of plasma 8-iso Prostaglandin $F_{2\alpha}$, 16 individual control blood samples were taken on two occasions with an interval of one week and then samples were measured on the same occasion on the same plate. The average CV between time points was large (48.62%) (Figure 4.7.), however strongly correlated (r=0.94) (Figure 4.8.). Since CV varies between controls (10 had CV values below the 20% target and the remaining 6 were above 20%), the variation is more likely to be short-term intra-individual variation rather than preparation and storage variation. Also again the individual samples with CVs above 20% were of low 8-iso Prostaglandin $F_{2\alpha}$ concentration and thus it is likely that their concentration is below or close to the (LOD of 0.25ng/ml for the AutoDELFIA assay.

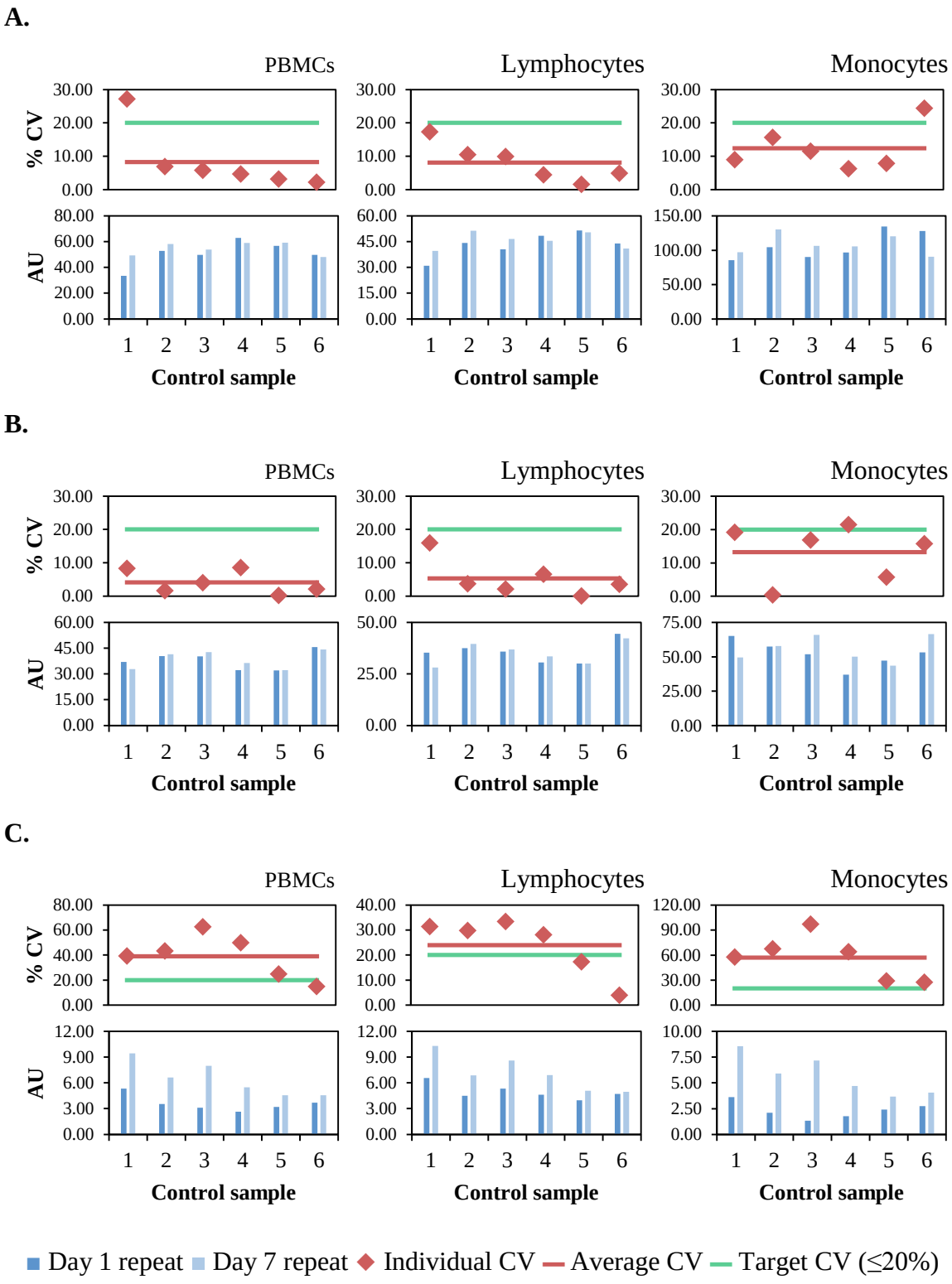
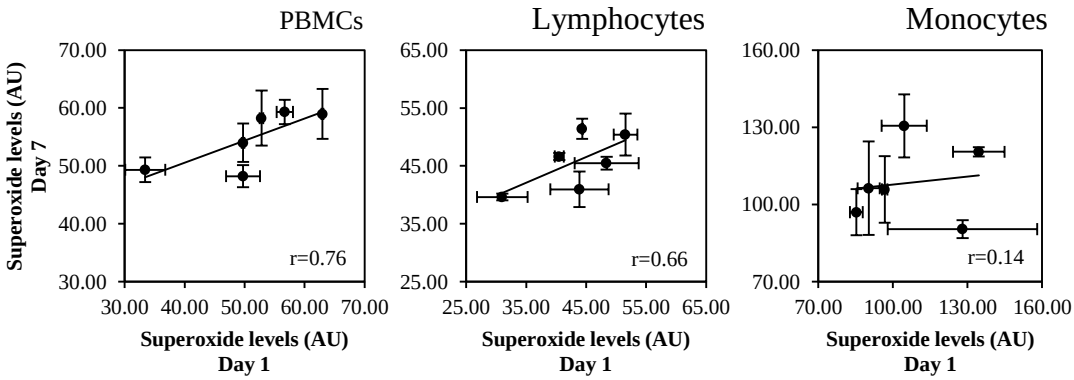
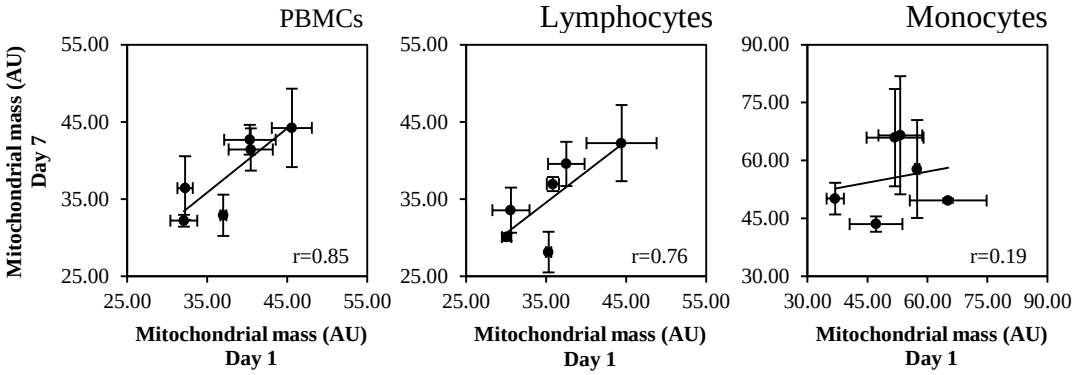


Figure 4.5. Short-term day-day precision of A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis. Day 1 and day 7 were measured independently. (See Appendix C, Supplementary Table 5. for raw data).

A.



B.



C.

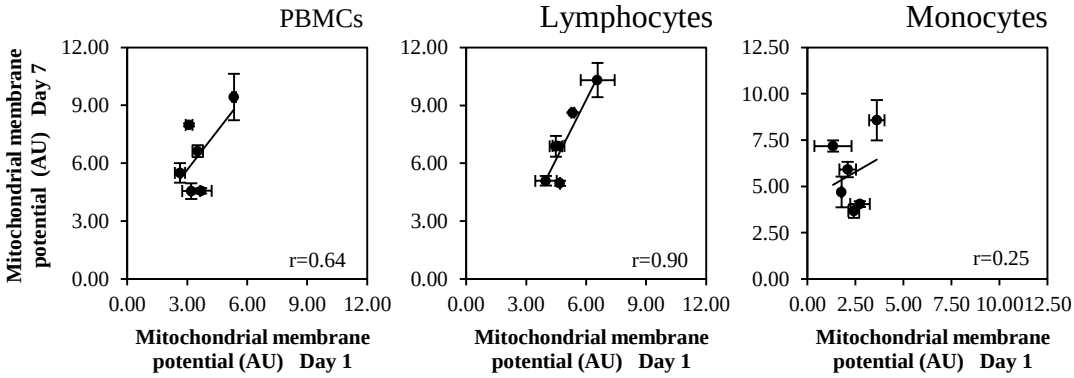


Figure 4.6. Short-term intra-individual stability of A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes. Pearson's correlation coefficient was used to test the strength of association between day 1 and day 7 intervals and thus *in vivo* stability (n=6). Error bars represent SD of two repeats.

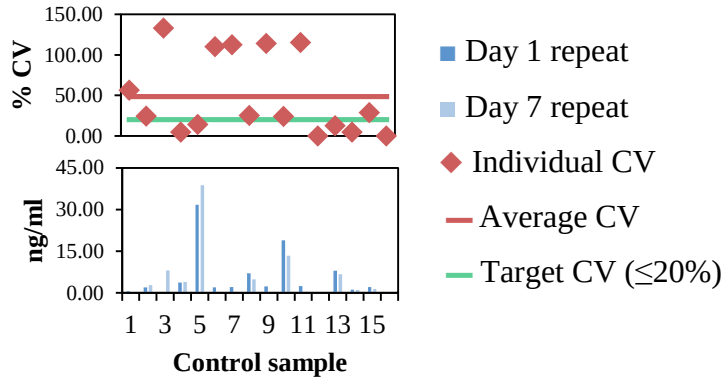


Figure 4.7. Short-term day-day precision of 8-iso Prostaglandin $F_{2\alpha}$ in plasma by AutoDELFIA analysis. Day 1 and day 7 were measured on the same occasion. (See Appendix C, Supplementary Table 6. for raw data).

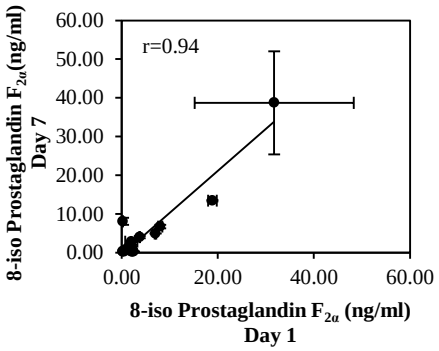


Figure 4.8. Short-term intra-individual stability of 8-iso Prostaglandin $F_{2\alpha}$ in plasma. Pearson's correlation coefficient was used to test the strength of association between day 1 and day 7 intervals and thus *in vivo* stability (n=16). Error bars represent SD of two repeats.

Since the short-term day-day reproducibility of the AutoDELFIA method to measure plasma 8-iso Prostaglandin $F_{2\alpha}$ could not be assessed by the previous method the long-term day-day reproducibility and thus long-term intra-individual stability was assessed by calculating the average day-day CV and correlation between the Newcastle 85+ samples at phase 1 and phase 2 (both measured on the same occasion) with phase 3 (measured independently of phase 1 and phase 2). The average CV between phase 1 and phase 3 was 29.11% and phase 2 and phase 3 was 30.22%, slightly above the target of 20% (Figure 4.9.). There is however a wide variation in individual CVs between phases with many above and below 20%. Thus the higher than target long-term day-day CV is more likely to be long-term intra-individual variation for some individuals rather than AutoDELFIA day-day variation. There are also many individuals with low 8-iso Prostaglandin $F_{2\alpha}$ concentration and thus it is likely that their concentration is below or close to the LOD of 0.25ng/ml for the AutoDELFIA assay contributing to high CV. There was however a high correlation between phase 1 vs. phase 2, phase 1 vs. phase 3 and phase 2 vs. phase 3 ($r=0.86$, $r=0.75$ and $r=0.78$ respectively, $p<0.01$ for all) demonstrating overall good long-term intra-individual stability (Figure 4.10.).

4.3.4. The comparison and agreement of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMC subpopulations, lymphocytes and monocytes

Since the density gradient centrifugation method used isolates PBMC subpopulations lymphocytes and monocytes only we identified these subpopulations within the PBMC sample by flow cytometry which was confirmed by CD45 and CD14 antibody staining (See Chapter 2. Section 2.4. Figure 2.2.). In the very old population, superoxide levels and mitochondrial mass in monocytes were significantly higher than in lymphocytes whereas membrane potential was significantly lower ($p<0.01$) (Figure 4.11. A.). There was however a high positive correlation between superoxide levels and mitochondria mass and in both subpopulations of PBMCs ($r=0.78$ and $r=0.84$ respectively, $p<0.01$ for both) demonstrating high specificity of the flow cytometry quantification method and also cell type independency of these parameters (Figure 4.11. B.). The correlation between mitochondrial membrane potential in both subpopulations of PBMCs was also positive however of lower magnitude ($r=0.40$, $p<0.01$), which could be because of less specificity of the quantification method or cell type independency of this parameter (Figure 4.11. B.).

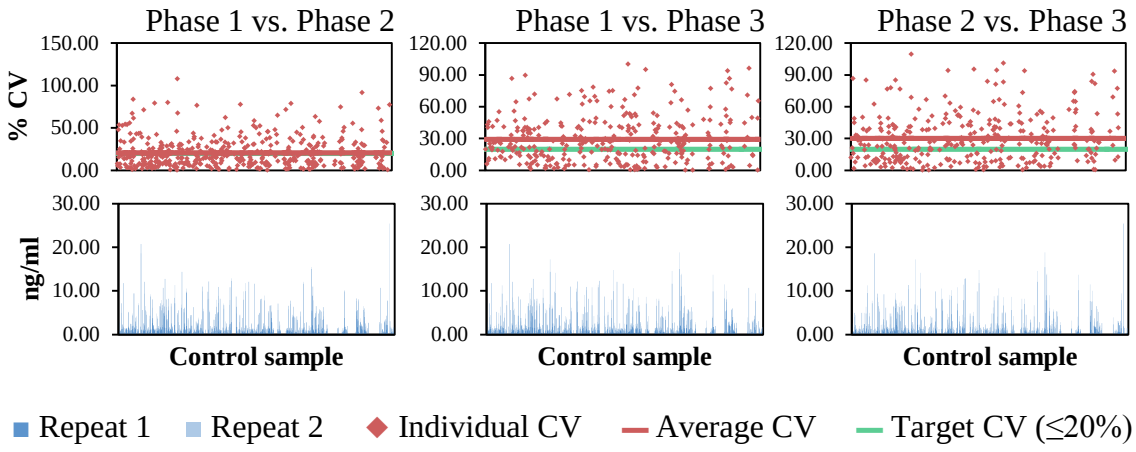


Figure 4.9. Long-term day-day precision of 8-iso Prostaglandin $F_{2\alpha}$ in plasma by AutoDELFIA analysis. Average CV for phase 1 vs. phase 2 was 20.70 %, phase 1 vs. phase 3 was 29.11% and phase 2 vs. phase 3 was 30.22%. Phase 1 and phase 2 were measured on the same occasion, phase 3 was measured independently.

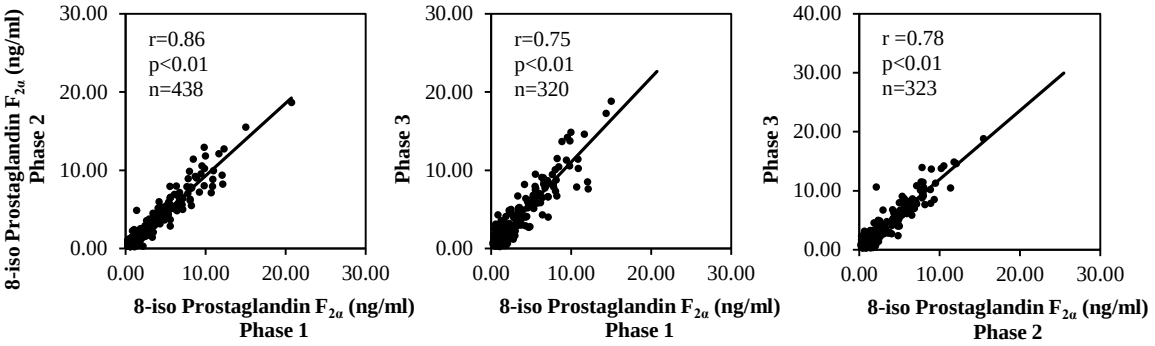


Figure 4.10. Long-term intra-individual stability of 8-iso Prostaglandin $F_{2\alpha}$ in plasma of the very old population. Spearman's correlation coefficient was used to test the strength of association between phase 1, phase 2 and phase 3 intervals and thus *in vivo* stability.

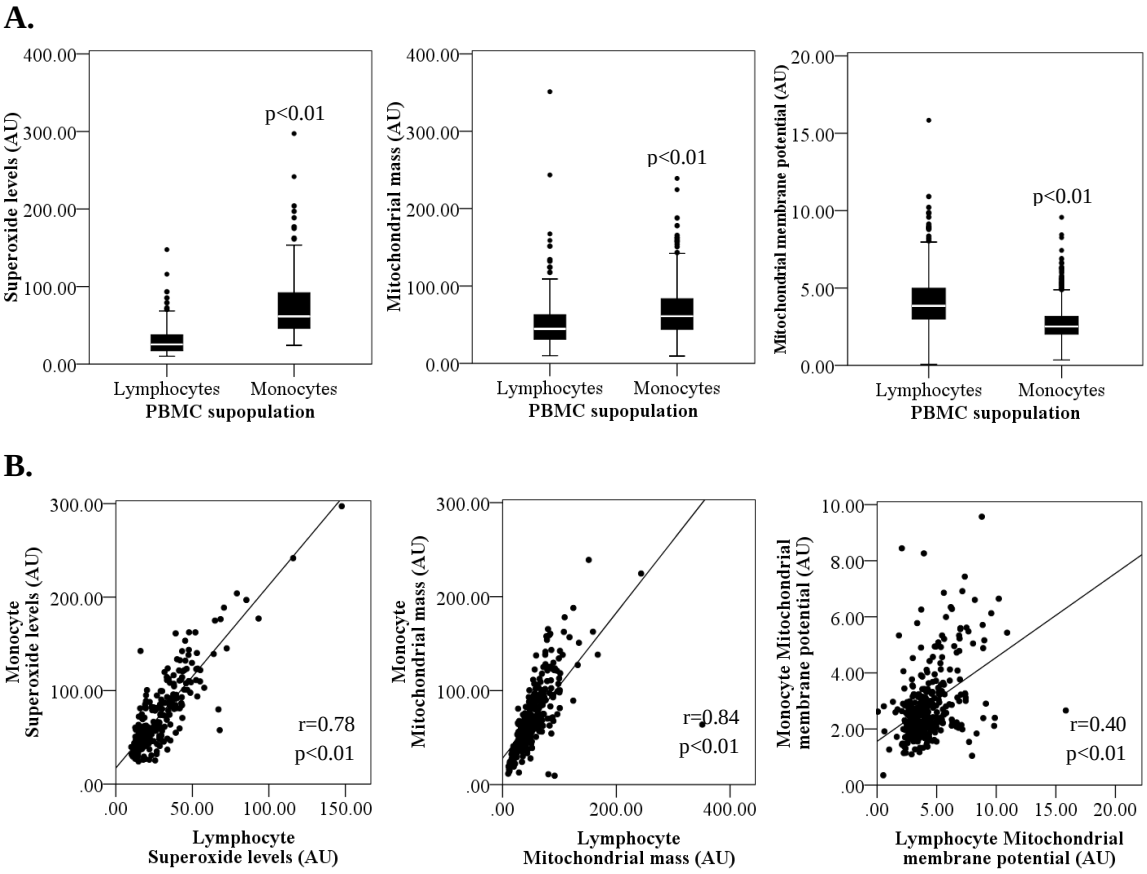


Figure 4.11. Comparing ROS production and mitochondrial function in PBMC subpopulations, lymphocytes and monocytes, of the very old population. A. Comparison of medians and B. correlations of superoxide levels (n=248), mitochondrial mass (n=341) and mitochondrial membrane potential (n=347). Wilcoxon Signed-Rank Test was used to test differences between medians and Spearman's correlation coefficient was used to test the strength of associations.

4.3.5. The agreement between the AutoDELFIA and the LCMS/MS method to measure plasma F_2-isoprostanes

Since there are many different isomers of F_2-isoprostanes generated from the action of ROS on arachidonic acid *in vivo* the effectiveness of AutoDELFIA is based on the specificity of the antibody to the 8-iso Prostaglandin $F_{2\alpha}$ isomer. Individual isoforms may be a result of different oxidative stress pathways and may have different biological roles. Using an alternative LCMS/MS method to measure F_2-isoprostane isomers has advantages over AutoDELFIA including a high resolution of isomer separation, high specificity and sensitivity and determines multiple F_2-isoprostane isomers at a time. However it is not the method of choice when analysing a large number of samples since it is laboriously intensive, requires expensive equipment, skilled operators and is time consuming. To further investigate the reliability of the AutoDELFIA method and its specificity to the 8-iso Prostaglandin $F_{2\alpha}$ isomer it was compared to the 8-iso Prostaglandin $F_{2\alpha}$ and 5-i$PF_{2\alpha}$-V1 isomers measured by LCMS/MS in phase 1 samples of the Newcastle 85+ study. Concentrations of 8-iso Prostaglandin $F_{2\alpha}$ measured by AutoDELFIA were not significantly different compared with the 8-iso Prostaglandin $F_{2\alpha}$ isomer by LCMS/MS ($p=0.14$) however was significantly lower compared with 5-$IPF_{2\alpha}$ V1 isomer by LCMS/MS ($p<0.01$) (Figure 4.12. A.). There was however no correlation between 8-iso Prostaglandin $F_{2\alpha}$ measured by AutoDELFIA with the 8-iso Prostaglandin $F_{2\alpha}$ isomer or 5-$IPF_{2\alpha}$ V1 isomer measured by LCMS/MS in the same participants ($r=-0.04$, $p=0.26$ and $r=-0.01$, $p=0.81$ respectively) but there was an agreement between both F_2-isoprostane isomers measured by LCMS/MS ($r=0.75$, $p<0.01$) (Figure 4.12. B.). Since samples for the LCMS/MS assay were measured in batches and the batch-batch precision is unknown, this may have introduced experimental variability within the samples due to different experimental conditions. This could explain the disagreement between the AutoDELFIA and LCMS/MS methods. There were significant differences between 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA for both 8-iso Prostaglandin $F_{2\alpha}$ and 5-$IPF_{2\alpha}$ V1 isomers for most LCMS/MS assay runs and there was no correlation between 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA with either of the F_2-isoprostane isomers measured by LCMS/MS (Figure 4.12. C.) confirming no agreement between AutoDELFIA and LCMS/MS.

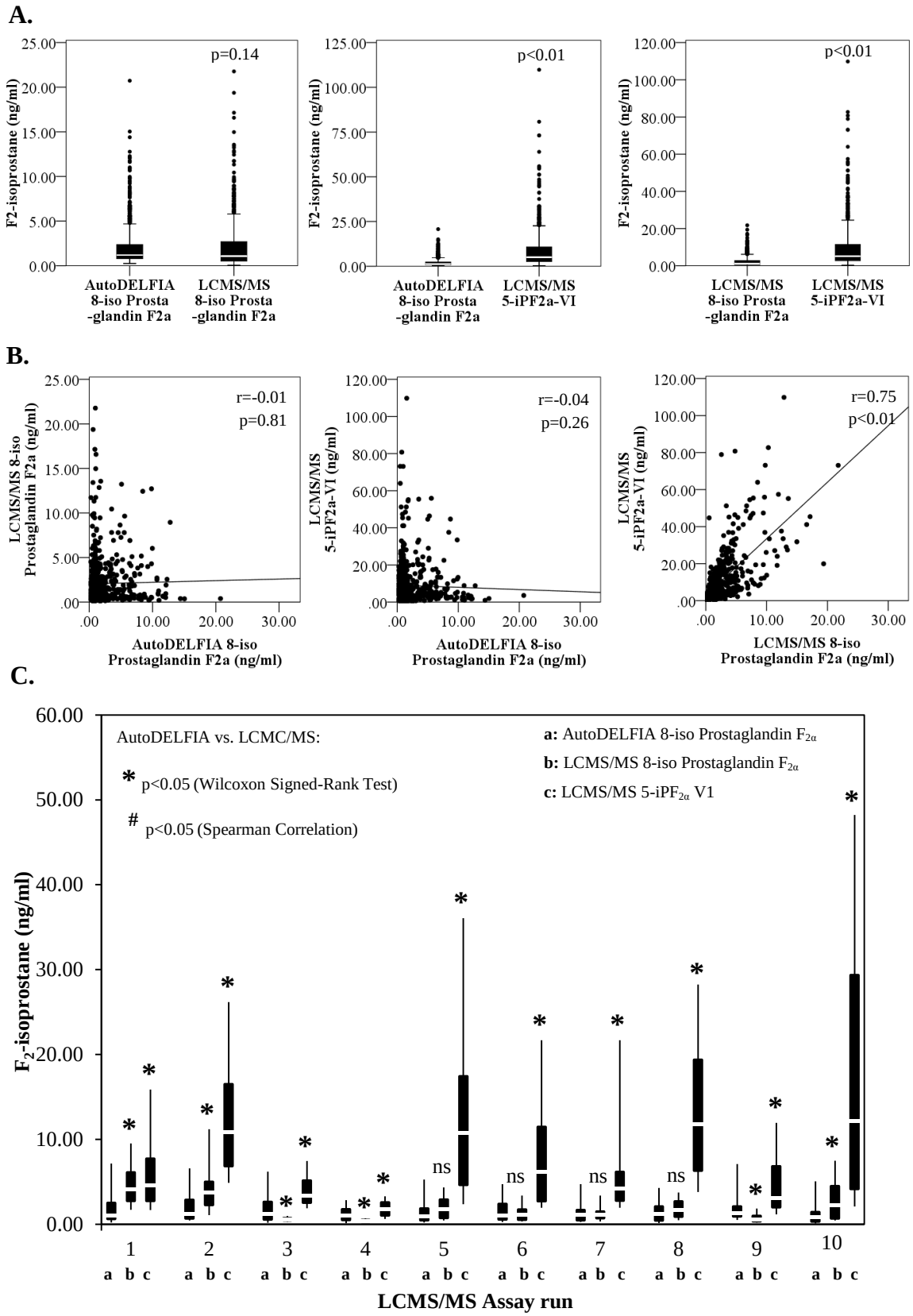


Figure 4.12. Agreement between 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA with 8-iso Prostaglandin $F_{2\alpha}$ and 5-$IPF_{2\alpha}$ V1 isomers by LCMS/MS A. Comparison of medians B. correlations and C. comparison of medians and correlation by LCMS/MS assay number (AutoDELFIA 8-iso Prostaglandin $F_{2\alpha}$ vs. LCMS/MS 8-iso Prostaglandin $F_{2\alpha}$ or 5-$IPF_{2\alpha}$ V1: n=645; LCMS/MS 8-iso Prostaglandin $F_{2\alpha}$ vs. LCMS/MS 5-$IPF_{2\alpha}$ V1: n=762). Wilcoxon Signed-Rank Test was used to test differences between medians and Spearman's correlation coefficient was used to test the strength of associations.

4.3.6. The specificity of AutoDELFIA to the 8-iso Prostaglandin $F_{2\alpha}$ isomer

The reason for the disagreement between the AutoDELFIA and LCMS/MS methods to measure 8-iso Prostaglandin $F_{2\alpha}$ is uncertain. It could be due to difference in the specificity of the methods to detect the 8-iso Prostaglandin $F_{2\alpha}$ isomer. To further explore the ability of AutoDELFIA do detect the 8-iso Prostaglandin $F_{2\alpha}$ isomer, control plasma samples were spiked with varying concentrations of 8-iso Prostaglandin $F_{2\alpha}$ and 5-IP$F_{2\alpha}$ V1 isomers, alone or in combination, and the recovery of 8-iso Prostaglandin $F_{2\alpha}$ was determined (Table 4.3.). AutoDELFIA detected increasing concentration of the 8-iso Prostaglandin $F_{2\alpha}$ isomer however not for the 5-i$PF_{2\alpha}$-V1 demonstrating its specificity for the 8-iso Prostaglandin $F_{2\alpha}$ isomer only.

4.4. Discussion

Assessing the reliability of potential BoA is critically important in population studies to ensure that variability reflects genuine inter-individual differences and not methodological error. This should be carried out before further validation analyses of potential BoA since controlling for these factors will increase the power of obtaining true results in future analysis. This is increasingly important when samples are handled on a daily basis where day-day experimental variables may vary. Since there is limited information in the literature on the reliability of measuring PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential by flow cytometry or plasma 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA it was important that this was investigated for the future analysis of these parameters within this study. The overall aim of this chapter was to evaluate measurement error by investigating variables that may affect the experimental stability of these potential oxidative stress-related BoA .

The first step was to identify experimental handing variables that may introduce error during the measurement of the potential oxidative stress-related BoA in question. A significant experimental handling variable that had an effect on both ROS production and mitochondrial function in PBMCs and plasma 8-iso Prostaglandin $F_{2\alpha}$ was the storage conditions. It was shown that the superoxide production in PBMCs was increased by sample storage at 37^{o}C between 2 and 24 hours however there was no change in mitochondrial mass or membrane potential. This indicates that the increase in

Control plasma	F_2-isoprostane spike	Plasma + 8-iso Prostaglandin $F_{2\alpha}$ spike			Plasma + 5-iP$F_{2\alpha}$-V1 spike			Plasma + 8-iso Prostaglandin $F_{2\alpha}$ + 5-iP$F_{2\alpha}$–V1 spikes		
	(ng/ml)	Raw (ng/ml)	Recovered (ng/ml)	% Recovery	Raw (ng/ml)	Recovered (ng/ml)	% Recovery	Raw (ng/ml)	Recovered (ng/ml)	% Recovery
1	0	13.78	0.00	-	13.78	0.00	-	13.78	0.00	-
	5	17.15	3.37	67.37	13.05	-0.73	-14.67	15.88	2.10	42.02
	10	21.09	7.31	73.09	14.04	0.26	2.62	20.32	6.54	65.40
	20	30.08	16.30	81.49	13.14	-0.64	-3.21	30.65	16.86	84.32
	40	51.57	37.79	94.47	14.30	0.52	1.30	47.61	33.83	84.58
2	0	6.14	0.00	-	6.14	0.00	-	6.14	0.00	-
	5	10.31	4.17	83.42	4.64	-1.50	-29.95	8.19	2.05	41.09
	10	14.85	8.71	87.08	5.09	-1.05	-10.50	12.77	6.63	66.33
	20	19.66	13.52	67.58	4.57	-1.57	-7.84	22.73	16.59	82.96
	40	42.63	36.49	91.22	4.59	-1.55	-3.86	45.43	39.29	98.22
1	0	1.14	0.00	-	1.14	0.00	-	1.14	0.00	-
	5	4.11	2.97	59.31	0.80	-0.34	-6.88	2.89	1.75	34.92
	10	5.17	4.03	40.26	0.25	-0.89	-8.92	5.93	4.78	47.85
	20	12.36	11.22	56.08	2.22	1.08	5.38	13.14	12.00	59.98
	40	25.06	23.91	59.79	0.85	-0.29	-0.72	25.90	24.76	61.89
4	0	6.45	0.00	-	6.45	0.00	-	6.45	0.00	-
	5	10.14	3.69	73.76	6.07	-0.39	-7.75	10.35	3.89	77.84
	10	14.69	8.24	82.41	6.72	0.26	2.63	14.62	8.16	81.61
	20	23.20	16.75	83.74	6.24	-0.21	-1.05	24.26	17.80	89.01
	40	40.45	34.00	84.99	6.54	0.09	0.22	39.44	32.98	82.45

Table 4.3. The recovery of 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA in plasma samples spiked with 8-iso Prostaglandin $F_{2\alpha}$ and/or 5-iP$F_{2\alpha}$–V1.

superoxide levels was independent of mitochondrial function. It is therefore unclear by what mechanism superoxide production is increased since DHE is thought to reflect mitochondrial superoxide production. However these results suggest that this may not be the case and independent mechanisms of superoxide production may be involved. This study contradicts an earlier study that found storage time decreases PBMC mitochondrial membrane potential between 5 and 24 hours (Cassart *et al.*, 2007). However differences between this study was that they investigated the storage time of the whole blood sample and not the isolated PBMCs and also their storage was at room temperature and not 37^{o}C. Their cells, although stored in the natural medium unlike the samples of this study, were therefore not at their normal *in vivo* temperature which could explain the decrease in mitochondrial membrane potential. Interestingly, from the experiment of this study investigating the incubation temperature of cells stained with the JC-1 probe, mitochondrial membrane potential was significantly increased at room temperature (20^{o}C) compared to 37^{o}C, contradicting the study by Cassart *et al,* 2007. It seems however that PBMC mitochondrial function may be affected by temperature. If temperature is affecting mitochondrial function it would be interesting to know if it is also affected by temperature changes *in vivo,* which could be due to an illness, a medication or from the seasonal environment. It would not be so surprising if mitochondria are affected by temperature changes *in vivo* due to their dynamic nature in response to changes in physiological conditions, which serves to maintain their function (Seo *et al.*, 2010). This could have implication in the second part of this chapter that investigated intra-individual reproducibility. Could differences in mitochondrial membrane potential between or within individuals reflect varying body temperatures? Unfortunately body temperature was not assessed within participants of the Newcastle 85+ study; however, an indirect way of investigating this could be to look at seasonal variations which will be investigated in Chapter 5. The overall conclusion of this experiment for this chapter however is that PBMC samples that are to be used to measure ROS production and mitochondrial function should be analysed within 2 hours of storage. This can be problematic if there are a large number of samples to process and analyse. This study therefore investigated whether PBMC sample could be frozen so a larger number of samples could be measured per day. This could also be useful for future automatic measurement by flow cytometry. However, it was found that freezing (-70^{o}C) and cryopreservation (-196^{o}C) increased PBMC superoxide production and mitochondrial dysfunction and therefore frozen storage will not be possible.

Plasma 8-iso Prostaglandin $F_{2\alpha}$ was also affected by storage conditions. Once plasma is thawed for experimental analysis, sample are stored at 4^{o}C whilst material is being prepared for analysis by AutoDELFIA. This could also be done overnight so samples are ready for analysis the next day to save time. Also, sometimes the plasma sample are re-analysed, especially when their first measurement CV value was above 10%, and are therefore stored at 4^{o}C until the next analysis. This study found that there was a significant deterioration in 8-iso Prostaglandin $F_{2\alpha}$ between 24 and 48 hours storage at 4^{o}C. An explanation for this could be the presence of a metabolic enzyme in plasma that works efficiently at this temperature to metabolise 8-iso prostaglandin $F_{2\alpha.}$ Thawed plasma samples should therefore not be stored above -70^{o}C for longer than 24 hours. Although storing plasma in several vials will minimise the need for multiple freezing and thawing, it is sometimes necessary to use plasma that has undergone more than one freeze-thaw cycle. There was however no effect of three repeated freeze-thaw cycles compared to the control. Plasma samples are therefore reliable up to a maximum of three freeze/thaw cycles.

The remaining experimental handling variables investigated were specific to the analysis ROS production, mitochondrial mass and mitochondrial membrane potential by flow cytometry. This was because there are many more sample handling steps that were unknown to influence these parameters and also the fact that this was a direct way of measuring oxidative stress in cells, which are much more susceptible to changes from the *in vivo* environment than the indirect method of measuring endpoints of oxidative stress in the plasma, potentially generating artificial oxidative stress.

Since it was originally thought that it would be more ideal that red blood cells were removed from the PBMC samples before analysing superoxide levels, mitochondrial mass and mitochondrial membrane potential for the reasons discussed previously in Section 4.1., red blood cells in PBMC samples were lysed and compared to unlysed controls. The idea was that if this method of removing red blood cells did not influence ROS production or mitochondrial function, then the sensitivity of the analysis may be improved allowing a more accurate counting of PBMC cells only, which may increase the power of obtaining true results in future analysis. However lysing red blood cells from the PBMC samples significantly stressed the remaining cells evidenced by increased superoxide levels which was dependent on mitochondrial dysfunction, evidenced by a significant decrease in mitochondrial membrane potential. Many studies

employ this method of removing red blood cells when PBMCs are being investigated. It could be argued that as long as the same conditions of red blood cell lysis are used for all the samples (i.e. the same concentration and length of time in lysis buffer) then this may not be a problem. However, different individuals may have difference sensitivities to the toxic effect of the lysis buffer, if this was involved in the induction of oxidative stress, and therefore this routine would not be ideal. Alternatively this study could support the findings of two earlier studies who found that PBMCs in the presence of red blood cells produce more cytokines than when red blood cells are removed by density gradient centrifugation (Suni *et al.*, 1998; Song *et al.*, 2002). If this was the case then red blood cell lysis buffer itself may not be the inducer of oxidative stress but the removal of red blood cells themselves. Could factors released by red blood cells reduce oxidative stress in PBMCs? Interestingly this is addressed in a study by Fonseca *et al*, in 2001 who show that the presence of red blood cells in cultures of stimulated human peripheral blood lymphocytes strengthens their proliferation and survival by inhibiting apoptosis through the reduction of ROS (Fonseca *et al.*, 2001). This suggests that red blood cells play a role in maintaining PBMC homeostasis. This study supports this and also suggests that mitochondrial function is involved in the maintenance of PBMCs by red blood cells.

The remaining experimental handling factor to influence superoxide levels, mitochondrial mass and mitochondrial membrane potential was the staining temperature of PBMCs. This is not so much surprising since it is expected that the probes are more likely to penetrate the cells at higher temperatures because of higher membrane permeability and thus increasing the effectiveness of the staining, evident by higher superoxide levels, mitochondrial mass and lower mitochondrial membrane potential at 37^{o}C compared to 20^{o}C and 40^{o}C. The overall message of this experiment is the importance of maintaining a strict staining time at the same temperature for each sample. It could be more interesting to investigate the storage temperature of the cells before staining which would indicate whether oxidative stress is induced due to changes from the normal physiological temperature of the cells at 37^{o}C. This could be more informative on the effects experimental handling, since preparation of the cells is carried out at room temperature and thus the length of time in this temperature could vary for samples, especially when there are numerous samples to measure. Although it is suggested that experiments are carried out when cells are on ice, this also could introduce experimental error since individuals could have difference susceptibilities to

cellular stress at this temperature. The measurement would therefore reflect a cellular stress response rather than basal oxidative stress.

One other factor investigated that was unknown to induce a stress response in PBMCs was putting the cells under a brief mechanical stress. The force of mechanical stress investigated was reflected in the force that the cells might be put under during the preparation of samples for flow cytometry analysis. This is when the cells are briefly vortex after staining. The strength of the vortex could vary between samples or between investigators therefore it is interesting to know if this does induce a stress response and at what force. There was however no effect of vortex mechanical stress at 1000, 2000 and 3000rpm compared to the control concluding that this factor doe not need to be controlled for at this level of mechanical stress.

Issues relating to the handling of the fluorescent probes were also investigated including the effects of freeze thawing probe aliquots since it is thought that this may reduce their effectiveness as discussed in Section 4.1. The reason why this is important to know is because this could not only have experimental implications but also financial. Storing aliquots in smaller volumes for one time use is more costly due to probe wastage by pipetting losses than being able to re-freeze the probes in larger aliquots and thawed for further experiments. It also requires more material for storing the probes. This study however found that freeze-thawing the DHE, MitoTracker Green and JC-1 probes for up to 10 cycles does not significantly reduce their effectiveness, supporting the use of larger probe aliquots and being able to freeze-thaw them without introducing error to the measurements. This study also found that the effectiveness DHE, MitoTracker Green and JC-1 probes are not reduced by unprotecting stained PBMCs from light.

After considering the above handling variables, it was important to provide evidence of the assay precision of both the quantification of PBMCs superoxide levels, mitochondrial mass and mitochondrial membrane potential and also plasma 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA. This was to determine whether handling variables, other than the ones investigated above, could be influencing and thus introducing errors within the measurements. There were three types of assay precision analyses investigated: 1) intra-assay precision, by repeating the same sample preparation (i.e. within the same tube or on the same assay plate) which would determine whether errors are introduced during the instrumental measurement itself (i.e. by the flow cytometer or

AutoDELFIA equipment); 2) inter-assay precision, by repeating the same sample however from a different preparation (i.e. between different tubes or different assay plates) which would determine whether errors are introduced prior to the instrumental measurement during sample handling, which mainly reflects pipetting accuracy since other variables were kept the constant (See Chapter 2. Figure 2.6.); and 3) day-day assay precision, by repeating samples from the same individual at a seven day interval and thus the repeatability of all the experimental variables. This also permitted the analysis of intra-individual reproducibility.

Concerning the measurement of ROS production and mitochondrial dysfunction by flow cytometry, there was good within and between-assay precision for PBMC and cell subpopulation superoxide levels, mitochondrial mass and membrane potential. This confirms that individual samples measured on the same day are comparable. However, although all markers were stable within individuals during a seven day interval in the PBMC and lymphocyte populations, this was not the case in the monocyte population. It is therefore unlikely that intra-individual factors will contribute to measurement error in the short term within the two former cell populations however this could be the case within the monocyte population. This could either reflect method precision or intra-individual variability within the monocyte subpopulation. In support of the latter, monocyte populations have been shown to have a weaker diurnal rhythm than lymphocytes and other PBMC subpopulations, which demonstrates that they may be more sensitive to intra-individual factors (Ackermann *et al.*, 2012), which could thus affect ROS production and mitochondrial function. One potential intra-individual factor is sleep quality since different PBMC subpopulation levels have different sensitivities to sleep deprivation (Ackermann *et al.*, 2012). Other potential causes of intra-individual variation that could be explored in future analysis include diet, smoking, alcohol consumption, medications, illnesses and other key events. The seven day interval experiment also demonstrated good day-day repeatability of the flow cytometry analysis for superoxide and mitochondrial mass but not for mitochondrial membrane potential. A potential problem with the JC-1 dye for measuring mitochondrial membrane potential is that it is poorly soluble in aqueous solutions (Schwartz, 2001) and thus variation in the preparation of the stain on different occasions could be the reason for its day-day variability.

Concerning the measurement of F_2-isoprostanes by AutoDELFIA, there was good precision for plasma 8-iso Prostaglandin $F_{2\alpha}$ concentrations above ≈6ng/ml within and between assays and also day-day, however poor precision in samples at lower concentration. We can therefore be confident in the repeatability of plasma 8-iso Prostaglandin $F_{2\alpha}$ measurements above 6ng/ml however we are less confident on the repeatability of samples under ≈1 ng/ml. It is likely that the lowest 8-iso Prostaglandin $F_{2\alpha}$ concentration sample is below the LOD for the AutoDELFIA assay which is 0.25ng/ml. Adjustments will be made for samples which are below LOD in future analysis (i.e. to 0.25ng/ml). Further analysis could be to calculate the CV for a range of samples between 0.25ng/ml to 6ng/ml to determine which concentrations produce an acceptable CV, and thus further adjustments can be made accordingly. Although plasma 8-iso Prostaglandin $F_{2\alpha}$ in most individual (younger control and the very old population) was stable in the short (days) and long term (years), this was not the case for all individuals, demonstrated by a high CV between the two different occasions of blood sample withdrawals. It would therefore be worth investigating potential confounders as discussed above.

Since PBMCs are used in this study as surrogate tissues to investigate potential oxidative stress-related BoA, specifically ROS production and mitochondrial function, the next part of this study was to determine if these parameters can be specifically measured and compared in different PBMC subpopulations. Measurement of these parameters specifically within lymphocyte and monocyte subpopulations was confirmed using antibodies that are specifically associated with these subpopulations (See Chapter 2, Section 2.4.6.). Although the levels of superoxide and mitochondrial parameters varied between subpopulations, there was a correlation between them, providing evidence of good specificity of the flow cytometry quantification method and also suggesting that ROS production and mitochondrial function is independent, at least, of white blood cell type. This could be indicative that ROS production and mitochondrial function are also independent of tissue type. Since the overall goal of using a surrogate tissue for the measurement of a BoA is that they should be reflective of ageing in other tissues, further experiments investigating these parameters in different tissues would be invaluable to their future validation as candidate BoA. This however has impracticality issues for most tissues, however could, for example, be investigated in buccal cells, hair follicles and skin. To support this, various studies have also found different levels but

agreements between various markers implicated in the oxidative stress-driven pathway to cellular senescence in peripheral blood and other tissues, suggesting they are tissue independent (Godschalk *et al.*, 1998; Friedrich *et al.*, 2000; Herrera *et al.*, 2009). However, the effects of the different levels of oxidative stress on driving cellular senescence and thus ageing in each cell type/tissue could vary.

Alternatively, an overall measure of oxidative stress that is reflective of a combination of tissues, including F_2-isoprostanes in plasma, would be more practical. Although this may not permit the prediction of a specific age-related outcome, it could be indicative of an overall age-related decline. The last part of the study was to provide further evidence of the reliability of plasma 8-iso Prostaglandin $F_{2\alpha}$ by AutoDELFIA. This was to determine its agreement with an alternative method of measuring plasma 8-iso Prostaglandin $F_{2\alpha}$ and also to determine whether there are agreements with an alternative F_2-isoprostane isomer, 5-IP$F_{2\alpha}$ V1. There are many different F_2-isoprostane isomers present in plasma that could reflect different tissues, extent of oxidative damage or even different biological roles, therefore it was interesting to determine if there are agreements between different isomers. The comparison with LCMS/MS permitted both these investigations, since it determines multiple F_2-isoprostanes isomers at a time. There was agreement in the overall concentration of the 8-iso Prostaglandin $F_{2\alpha}$ isomer, but the concentration of the 5-IP$F_{2\alpha}$ V1 isomer was higher, concluding that different F_2-isoprostanes isomers are present in plasma in different concentrations. However, the disagreement between AutoDELFIA and LCMS/MS was confirmed when no correlation between the 8-iso Prostaglandin $F_{2\alpha}$ isomer measured by both methods was shown. There was however an agreement between F_2-isoprostane isomers, indicated by a correlation between the 8-iso Prostaglandin $F_{2\alpha}$ and 5-IP$F_{2\alpha}$ V1 isomers measured by LCMS/MS. One issue in the disagreement between methods could be the specificity of the AutoDELFIA to the 8-iso Prostaglandin $F_{2\alpha}$ isomer. This was further tested in a spiking experiment which provided evidence that AutoDELFIA was specific to 8-iso Prostaglandin $F_{2\alpha}$ and did not detect the 5-IPF2α V1 isomer. However, cross-reactivity with other isomers could also occur. The unavailability of many isomers and pure material to fully characterise antibodies against specific isomers makes the assessment of cross-reactivity difficult. However, there are also disadvantages to LCMS/MS that could be the reason for the disagreement between methods including the fact that there are potential losses due to the required purification and derivatisation steps, where as

AutoDELFIA is performed directly on the sample. Further investigation as to why there are disagreements between methods is therefore required.

4.5. Conclusion

This study identified experimental variables that may affect the reliability of various potential oxidative stress-related BoA. These therefore should be controlled for in future analysis. When controlled there was evidence of experimental reliability and intra-individual stability permitting their further validation as potential BoA.

Chapter 5. The construct validity of potential oxidative stress-related BoA in the very old population

5.1. Abstract

Background An important step when validating candidate BoA is to confirm that they reflect known phenomena of biological ageing. **Aims** This study therefore aimed to provide evidence of construct validity for potential oxidative stress-related BoA. **Materials and Methods** These were measured in the very old participants from the Newcastle 85+ study and also in younger controls to investigate associations with chronological age and other known biomarkers that reflect biological ageing including associations between the potential oxidative stress-related BoA themselves, biomarkers of known biological mechanisms of ageing, biomarkers of immune function and validated classical BoA in the very old population. **Results** It was surprising that superoxide levels were lower in the very old compared to the younger controls ($p<0.01$), however mitochondrial mass was higher ($p=0.04$) and mitochondrial membrane potential was lower ($p<0.01$). 8-iso Prostaglandin $F_{2\alpha}$ was also lower in the very old population however did not reach significance ($p=0.69$). The correlations with chronological age was in the same direction as in the comparison between the young and very old, however only reached significance for mitochondrial membrane potential ($r=-0.72$, $p<0.01$) and nearly mitochondrial mass ($r=0.51$, $p=0.08$). There was significant longitudinal change in 8-iso Prostaglandin $F_{2\alpha}$ at age 85, 86.5 and 88 years however these changes were not linear with age. There was an association between superoxide levels and mitochondrial mass (positive in lymphocytes and monocytes, $p\leq0.01$) and superoxide levels and mitochondrial membrane potential (negative in PBMCs, $p=0.01$; positive in lymphocytes and monocytes, $p=0.05$ and $p=<0.01$ respectively). There were no associations between ROS production and mitochondrial parameters with 8-iso Prostaglandin $F_{2\alpha}$. There were some significant associations between potential oxidative stress-related BoA and other biomarkers implicated in biological ageing, however not all were in the same direction as expected. An interesting further finding was that all potential oxidative stress-related BoA were associated with seasonal variation. **Conclusion** Some evidence of construct validity was shown for potential oxidative stress-related BoA and thus should be investigated for their predictive potential. Seasonal variation is a potential confounder and should be controlled for in future predictive validation analyses.

5.2. Introduction

After establishing the methodological reliability of a candidate BoA the second step should be to provide evidence of its validity. There are various methods of evaluating the validity of a biomarker which can be complex depending on what type of biomarker is being investigated (Mayeux, 2004). However evaluating the validity of candidate BoA can be split into two major groups: construct validity and predictive validity (Ingram *et al.*, 2001). Construct validity is defined here as the ability of the candidate BoA to reflect known phenomena of biological ageing (as the construct) which will be investigated in this chapter and also chapter 6. Predictive validity is defined as the ability of a candidate BoA to correlate with and predict various age-related outcomes which will be investigated in chapter 7.

The first and most obvious assessment to provide evidence of the construct validity of a candidate BoA is that it should to some degree show a correlation with chronological age since it is well acknowledge that this is associated with age-related decline and an increased risk of mortality. It should also be shown that the direction of correlation with age should be the same direction in a cross sectional analysis of different age groups (Ingram *et al.*, 2001). However, although physiological decline is an inevitable and inescapable consequence of advancing age, this decline is not uniform and there are often considerable differences between and within individuals reflecting biological age (Ekonomov *et al.*, 1989; Fozard *et al.*, 1990; Ueno *et al.*, 2003). Therefore the rate of change of a BoA should not be dependant on chronological age, after all the value of a BoA is that it can measure biological ageing better than chronological age.

A further assessment of the construct validity of a candidate BoA should be to evaluate their correlation with other known biomarkers that reflect biological ageing. In terms of the potential oxidative stress-related BoA investigated in this study these could be 1) parallel biomarkers, for example those that claim to measure oxidative stress; 2) biomarkers of known biological mechanisms of ageing, for example biomarkers of cellular senescence; 3) biomarkers of age-related functional decline, for example immunosenescence phenotypes; and 4) validated classical BoA.

Providing evidence of the correlation between other potential oxidative stress-related BoA with an association in the same direction would not only confirm that the biomarkers are likely to be measuring oxidative stress but also provide a further wealth of information. Although correlations may be seen, these may not be perfect suggesting that one marker may be more experimentally reliable than the other. If the oxidative stress-related biomarkers are from different tissues/sample mediums then it could be confirmed whether oxidative stress is dependant on tissue type or not. For example, a correlation between mitochondrial ROS production and dysfunction in PBMCs and plasma F_2-isoprostanes would provide evidence that oxidative stress is tissue independent. This information is also valuable when searching for the most suitable biomarkers in large population studies where time, cost and the practicality of biomarker measurement and sample type are important.

Investigating the association between other biomarkers of known cellular and molecular mechanisms of ageing would also add strength to the construct validation of candidate BoA. One of the most important models for investigating the mechanism of biological ageing is cellular senescence. There are other various markers that are claimed to be involved in oxidative stress-induced cellular senescence including telomere length shortening, increased DNA damage, decrease DNA repair and increased inflammatory cytokines (von Zglinicki *et al.*, 1995; von Zglinicki, 2002; Passos *et al.*, 2007; Wang *et al.*, 2009; Passos *et al.*, 2010; Nelson *et al.*, 2012). Robust associations between these markers and increased mitochondrial ROS production and dysfunction are therefore expected. Since much evidence for the involvement of these markers in cellular senescence comes from cell culture studies this information would confirm that these associations also occur *in vivo.* Since this study will be investigating the very old population it is therefore more likely that these associations will be found than if the study was in a younger population, since it is expected that the elderly have a higher proportion of cellular senescent cells. This information would also aid in the validation of these cellular and molecular mechanisms, identify potential targets for and evaluate possible interventions.

A functional aspect that could be utilized to investigate the construct validation of potential oxidative stress-related BoA in blood is the correlation with immunosenescence phenotypes. Immunosenescence refers to the age-related decline of immune function increasing the susceptibility to infectious diseases (Gavazzi and

Krause, 2002; Goronzy and Weyand, 2013). There are various phenotypical changes that have been shown to occur in the immune system with age that reflect immunosenescence (Figure 5.1.). These major changes include the decline in the ratio of various cell subpopulations including the lymphocyte to monocyte ratio, the CD4 to CD8 T lymphocyte ratio and the naïve to memory B and T lymphocyte ratio (Lerner *et al.*, 1989; Lehtonen *et al.*, 1990; Callahan *et al.*, 1993; Herndler-Brandstetter *et al.*, 2013). These are thought to occur because of the decreased generative capacity of hematopoietic stem cells and the decreased maturation of hematopoietic progenitor cells within the thymus with chronological age (Rossi *et al.*, 2007; Aw *et al.*, 2008). Another important change occurring in the immune system with age is an increased fraction of CD27- memory cells (Nijhuis *et al.*, 1994).

The final set of biomarker that could be used to validate potential oxidative stress-related BoA are those that are described in Chapter 1. Section 1.2.3 as classical BoA including various anthropometric, physical, physiological, haematological (particularly in relation to immunology and biochemical measurements. Since Chapter 3 revealed that the predictive validity of these classical BoA can change with age it is important to investigate only those that are valid predictors of age-related outcomes in the very old population. These have been identified as hand grip strength, TUG, systolic blood pressure, FEV_1, red blood cell count, haematocrit, haemoglobin, free T3, vitamin D and NT-pro BNP (Martin-Ruiz *et al.*, 2011). Physical activity is also a suggested classical BoA in the elderly population (>70 years) however this was not previously investigated in the very old population of the Newcastle 85+ study. Physical activity could also be a potential confounding variable since it may not only measure the physical fitness of individual, but also the voluntary amount of physical activity an individual has carried out pre blood sample withdrawal. It will therefore be investigated in the final part of this chapter as a confounder, as discussed below.

Another important aspect of the construct validation of candidate BoA is the identification of potential confounding variables. These are extraneous factors that may be associated with both the candidate BoA and the outcome which may or may not be association with biological ageing. There are two main types of confounding assessments. The first was addressed in chapter 4, assessing the reliability of candidate BoA, which involves the identification of experimental handling variables that alter the

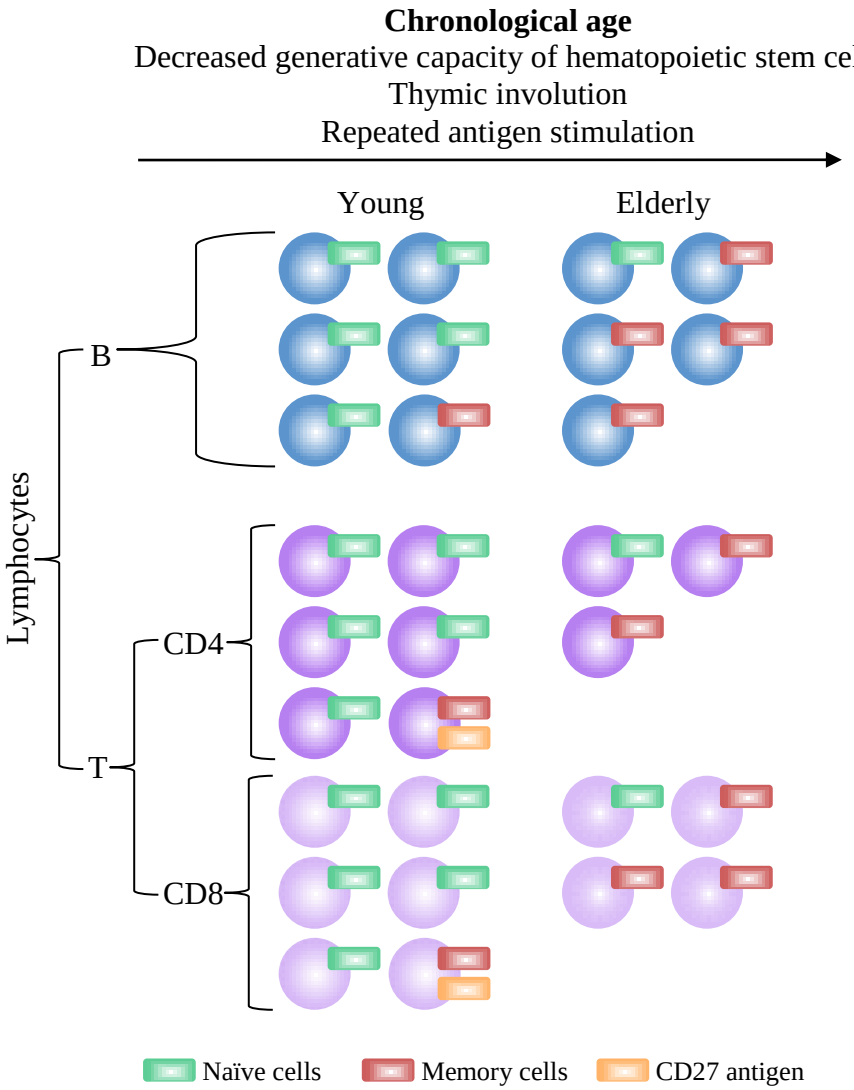


Figure 5.1. Phenotypic changes occurring in immunosenescence. (Lerner *et al.*, 1989; Lehtonen *et al.*, 1990; Callahan *et al.*, 1993; Herndler-Brandstetter *et al.*, 2013). (Nijhuis *et al.*, 1994).

candidate BoA and therefore should be controlled before the experimental measurement. The second assessment is the identification of individual variables that may confound future analysis but may be associated with biological mechanisms of ageing. The analysis of individual confounders can therefore get complex since this could form part of the reliability assessment but also provide construct validation of candidate BoA. This is because individual variables that alter candidate BoA could be controlled for before experimental analysis, for example participants are asked to refrain from a certain behaviour. However that behaviour, which may be associated with biological ageing, may be a regular occurrence of the individual and controlling it for all individual may reduce the power of the study, i.e. you would only want to control it if the behaviour is not a regular occurrence of the individual. It is difficult to know which variables are associated with both the candidate BoA and biological ageing and therefore various models of controlling for confounders should be investigated.

5.3. Results

5.3.1. Descriptive statistics

Descriptives of potential oxidative stress-related BoA, potential confounders and age-related construct variables of the very old from the Newcastle 85+ study are shown in Appendix C. Supplementary Table. 11., 12., and 13. respectively, stratified by all participants and also by participants with superoxide level, mitochondrial mass, mitochondria membrane potential and/or 8-iso Prostaglandin measurements to determine the representativeness of the whole population compared to those participants with oxidative-stress related measures. Possible selection bias occurred for: 1) differences in alcohol status, where a higher proportion of current drinkers and a lower proportion of previous drinkers were present in those with oxidative-stress related measures (significant for mitochondrial mass, mitochondrial membrane potential and 8-iso Prostaglandin) compared to the whole population; 2) differences in taking non-prescribed medicines, supplements and/or herbal remedies, where a higher proportion of those who take non-prescribed medicines, supplements and/or herbal remedies were present in those with oxidative-stress related measures (significant for 8-iso Prostaglandin) compared to the whole population; 3) differences in the use of aid(s)/appliance(s), where a lower proportion of those who use aid(s)/appliance(s) were present in those with oxidative-stress related measures (significant for 8-iso

Prostaglandin) compared to the whole population; and 4) differences in FEV_1, which was higher in those with oxidative-stress related measures (significant for mitochondrial membrane potential and 8-iso Prostaglandin) compared to the whole population. An explanation for these differences is that they most likely reflect age-related the changes in these variables since they were measured in phase 1 of the study. A summary of the younger control participants is shown in Appendix C. Supplementary Table. 19.

5.3.2. Association with chronological age

To investigate the association between potential oxidative stress-related BoA and chronological age cross-sectionally, the younger controls (with an age range of 22 to 55 years) were compared with the very old population of the Newcastle 85+ study. Surprisingly, superoxide levels were significantly lower in the very old population compared to the younger controls ($p<0.01$), however mitochondrial mass was significantly higher ($p=0.04$) and mitochondrial membrane potential was significantly lower ($p<0.01$) (Figure 5.2. A.). This direction of association was supported by the direction of correlation with chronological age: superoxide levels decreased, although this was of low correlation and did not reach statistical significance ($r=-0.33$, $p=0.26$); mitochondrial mass increased, which was of moderate correlation but again not quite reaching statistical significance ($r=0.51$, $p=0.08$); and mitochondrial membrane potential significantly decreased, which was of high correlation and statistically significant ($r=-0.72$, $p<0.01$) (Figure 5.2. B.). There was however no significant difference in 8-iso Prostaglandin $F_{2\alpha}$ concentration between the young controls and the very old population ($p=0.69$) (Figure 5.2. A.). There was also no significant correlation between in 8-iso Prostaglandin $F_{2\alpha}$ and chronological age ($r=-0.22$, $p=0.47$) (Figure 5.2. B.).

Longitudinal data was available for 8-iso Prostaglandin $F_{2\alpha}$ in the very old population of the Newcastle 85+ study, which was measured at phase 1 (≈85 years), phase 2 (≈86.5 years) and phase 3 (≈88 years). Despite extremely high intra-individual stability between all phases (See Chapter 4. Section 4.3.3.), 8-iso Prostaglandin $F_{2\alpha}$ concentration did not follow a linear association with age in this population, where its concentration significantly decreased between phase 1 and 2 ($p<0.01$), however increased between phase 2 and 3 ($p>0.01$) (Figure 5.3.).

5.3.3. Association between potential oxidative stress-related BoA in the very old population

The next construct validation assessment was to determine the agreement between the potential oxidative stress-related BoA. Superoxide levels were significantly associated with mitochondrial mass (positive in PBMCs: $p=0.04$; lymphocytes: $p=0.01$; and monocytes: $p<0.01$) and mitochondrial membrane potential (negative in PBMCs: $p=0.01$; but positive in lymphocytes: $p=0.04$ and monocytes: $p<0.01$), however the magnitude of correlations for all parameters was very low ($r \geq -0.18$ and ≤ 0.22) (Table 5.1.).

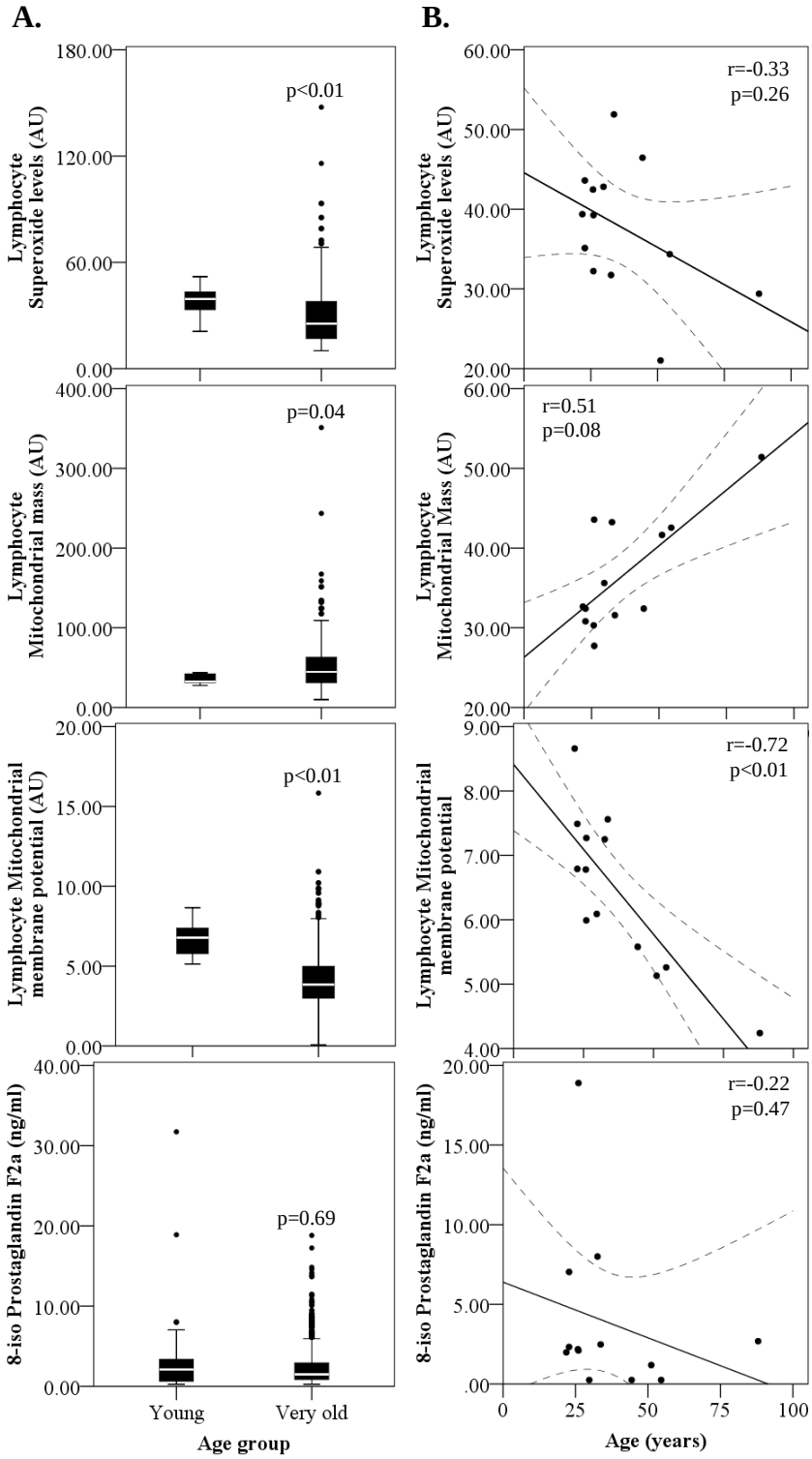


Figure 5.2. The association between potential oxidative stress-related BoA and chronological age. A. Median comparison between younger controls and the very old population and B. correlation with chronological age. Mann-Whitney U Test was used to test differences between the medians of two groups and Spearman's correlation coefficient was used to test the strength of associations **(**For numbers see Appendix C. Supplementary Table. 11 and Table 18). *(Age 88 years in the correlation analysis was the mean of the very old population, the rest were single repeats) (Numbers are different for 8-iso Prostaglandin in A. and B, since date of birth was known for 12 control, however 19 in total were used for median comparison).*

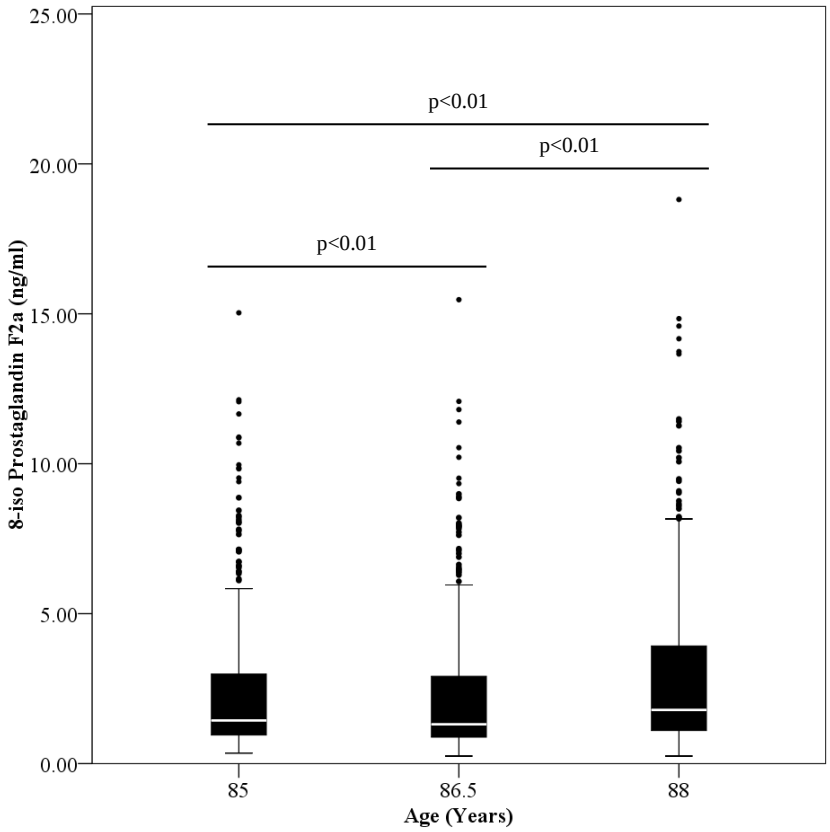


Figure 5.3. Longitudinal changes of 8-iso Prostaglandin $F_{2\alpha}$ in the very old population. Wilcoxon Signed-Rank Test was used to test differences between the medians of two groups and Friedman'sTest was used to test differences between the medians of three groups (n=287). *(Longitudinal intra-individual stability was addressed in chapter 4. Section 4.3.4).*

Potential oxidative stress-related BoA		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential		
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes
Superoxide levels PBMCs	r	–	–	–	–	–	–	–	–	–
	p	–	–	–	–	–	–	–	–	–
	n	–	–	–	–	–	–	–	–	–
Superoxide levels Lymphocytes	r	**0.88**	–	–	–	–	–	–	–	–
	p	**0.00**	–	–	–	–	–	–	–	–
	n	**248**	–	–	–	–	–	–	–	–
Superoxide levels Monocytes	r	**0.84**	**0.78**	–	–	–	–	–	–	–
	p	**0.00**	**0.00**	–	–	–	–	–	–	–
	n	**248**	**248**	–	–	–	–	–	–	–
Mitochondrial mass PBMCs	r	**0.13**	**0.16**	0.12	–	–	–	–	–	–
	p	**0.04**	**0.01**	0.07	–	–	–	–	–	–
	n	**243**	**243**	243	–	–	–	–	–	–
Mitochondrial mass Lymphocytes	r	**0.13**	**0.17**	**0.15**	**0.84**	–	–	–	–	–
	p	**0.04**	**0.01**	**0.02**	**0.00**	–	–	–	–	–
	n	**243**	**243**	**243**	**341**	–	–	–	–	–
Mitochondrial mass Monocytes	r	**0.15**	**0.19**	**0.22**	**0.80**	**0.84**	–	–	–	–
	p	**0.02**	**0.00**	**0.00**	**0.00**	**0.00**	–	–	–	–
	n	**243**	**243**	**243**	**341**	**341**	–	–	–	–
Mitochondrial membrane Potential PBMCs	r	**-0.18**	-0.03	0.03	0.00	0.04	0.11	–	–	–
	p	**0.01**	0.62	0.60	0.96	0.52	0.05	–	–	–
	n	**239**	239	239	332	332	332	–	–	–
Mitochondrial membrane Potential Lymphocytes	r	0.09	**0.14**	**0.18**	-0.04	0.03	0.08	**0.55**	–	–
	p	0.18	**0.04**	**0.01**	0.44	0.58	0.15	**0.00**	–	–
	n	239	**239**	**239**	332	332	332	**347**	–	–
Mitochondrial membrane Potential Monocytes	r	0.06	**0.18**	**0.22**	-0.08	-0.06	0.06	**0.66**	**0.40**	–
	p	0.37	**0.00**	**0.00**	0.15	0.26	0.29	**0.00**	**0.00**	–
	n	239	**239**	**239**	332	332	332	**347**	**347**	–
8-iso Prostaglandin $F_{2\alpha}$	r	-0.05	-0.03	-0.04	0.06	0.03	0.01	0.03	0.02	0.00
	p	0.45	0.65	0.58	0.34	0.64	0.93	0.62	0.77	0.95
	n	216	216	216	302	302	302	304	304	304

Table 5.1. Agreements between potential oxidative stress-related BoA (All data). (r: Spearman's correlation coefficient, p: probability, n: number of participants, significant positive association, significant negative association).

Sensitivity analysis removed the significance of the association between superoxide levels and mitochondrial mass in the PBMC population (p=0.13), however the significance still remained in the lymphocyte (p=0.01) and monocyte (p<0.01) subpopulations (Table 5.2.). The sensitivity analysis slightly removed the significance of the association between superoxide levels and mitochondrial membrane potential in the lymphocyte population (p=0.05), however the significance still remained in the PBMC (p=0.01) and monocyte (p<0.01) subpopulations (Table 5.2.). Superoxide levels, mitochondrial mass and mitochondrial membrane potential were not associated with 8-iso Prostaglandin $F_{2\alpha}$ ($p \geq 0.34$), even when extreme outliers were removed in the sensitivity analysis ($p \geq 0.42$). The very low magnitude of correlations for significant associations still remained after sensitivity analysis for all parameters ($r \geq -0.18$ and ≤ 0.21) giving little evidence that they are suggestive of biological significance.

5.3.4. Association with biomarkers of oxidative stress-induced cellular senescence in the very old population

The association between the potential oxidative stress-related BoA and other biomarkers of oxidative stress-induced cellular senescence was next investigated. There were consistent significant associations (i.e. for more than one cell population) between superoxide levels and DNA repair activity (negative, $p \leq 0.02$) and levels of the pro-inflammatory cytokines IL-6 and TNF-α (negative, $p<0.01$) and also between mitochondrial mass and telomere length (positive, $p \leq 0.04$) (Table 5.3.). The magnitude of these correlations was however very low ($r \geq -0.32$ and ≤ 0.30). Sensitivity analysis by removing extreme outliers did not affect the significance or the direction of these association and also revealed a consistent association between mitochondrial membrane potential and C-reactive protein (CRP) (negative, $p<0.01$) (Table 5.4.). There was no association between 8-iso Prostaglandin $F_{2\alpha}$ or any other biomarker of oxidative stress-induced cellular senescence in the raw data analysis ($p \geq 0.11$) (Table 5.3.), however sensitivity analysis, after removal of extreme outliers, revealed an association between 8-iso Prostaglandin $F_{2\alpha}$ and inflammatory cytokines, IL-6 and TNF-α (positive, $p \leq 0.04$). The very low magnitude of correlations for significant associations still remained after sensitivity analysis for all parameters ($r \geq -0.30$ and ≤ 0.13) giving little evidence that they are suggestive of biological significance.

Potential oxidative stress-related BoA		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential		
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes
Superoxide levels PBMCs	r	-	-	-	-	-	-	-	-	-
	p	-	-	-	-	-	-	-	-	-
	n	-	-	-	-	-	-	-	-	-
Superoxide levels Lymphocytes	r	**0.87**	-	-	-	-	-	-	-	-
	p	**0.00**	-	-	-	-	-	-	-	-
	n	**245**	-	-	-	-	-	-	-	-
Superoxide levels Monocytes	r	**0.83**	**0.78**	-	-	-	-	-	-	-
	p	**0.00**	**0.00**	-	-	-	-	-	-	-
	n	**245**	**246**	-	-	-	-	-	-	-
Mitochondrial mass PBMCs	r	0.10	**0.14**	0.09	-	-	-	-	-	-
	p	0.13	**0.03**	0.15	-	-	-	-	-	-
	n	240	**241**	241	-	-	-	-	-	-
Mitochondrial mass Lymphocytes	r	0.13	**0.17**	**0.16**	**0.83**	-	-	-	-	-
	p	0.05	**0.01**	**0.01**	**0.00**	-	-	-	-	-
	n	236	**237**	**237**	**337**	-	-	-	-	-
Mitochondrial mass Monocytes	r	0.12	**0.17**	**0.21**	**0.80**	**0.83**	-	-	-	-
	p	0.07	**0.01**	**0.00**	**0.00**	**0.00**	-	-	-	-
	n	239	**240**	**240**	**339**	**336**	-	-	-	-
Mitochondrial membrane Potential PBMCs	r	**-0.18**	-0.04	0.04	0.01	0.06	**0.11**	-	-	-
	p	**0.01**	0.53	0.58	0.88	0.27	**0.04**	-	-	-
	n	**234**	235	235	328	324	**327**	-	-	-
Mitochondrial membrane Potential Lymphocytes	r	0.08	0.13	**0.18**	-0.04	0.06	0.09	**0.54**	-	-
	p	0.21	0.05	**0.01**	0.52	0.29	0.09	**0.00**	-	-
	n	237	238	**238**	331	327	330	**343**	-	-
Mitochondrial membrane Potential Monocytes	r	0.06	**0.17**	**0.21**	-0.09	-0.04	0.06	**0.66**	**0.39**	-
	p	0.39	**0.01**	**0.00**	0.13	0.45	0.31	**0.00**	**0.00**	-
	n	232	**233**	**233**	325	321	324	**338**	**339**	-
8-iso Prostaglandin $F_{2\alpha}$	r	-0.01	-0.01	0.01	0.05	0.03	0.01	0.02	0.05	0.00
	p	0.87	0.88	0.89	0.42	0.56	0.85	0.71	0.43	0.95
	n	201	202	202	286	282	284	284	287	281

Table 5.2. Agreements between potential oxidative stress-related BoA (Sensitivity analysis – extreme outliers removed). (r: Spearman's correlation coefficient, p: probability, n: number of participants, significant positive association, significant negative association).

Other biomarkers of oxidative stress -induced cellular senescence		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Telomere length	r	0.11	0.08	**0.17**	**0.11**	**0.13**	0.07	**0.13**	0.04	0.00	-0.01
	p	0.08	0.23	**0.01**	**0.04**	**0.02**	0.17	**0.01**	0.41	0.97	0.86
	n	248	248	**248**	**340**	**340**	340	**345**	345	345	376
DNA damage	r	-0.06	-0.09	-0.07	0.05	-0.03	0.01	0.03	0.03	0.07	0.08
	p	0.33	0.16	0.25	0.38	0.53	0.82	0.63	0.64	0.23	0.12
	n	248	248	248	341	341	341	347	347	347	379
DNA repair	r	**-0.16**	**-0.15**	-0.07	-0.06	-0.07	-0.01	0.06	-0.03	0.02	0.01
	p	**0.01**	**0.02**	0.29	0.26	0.21	0.79	0.24	0.59	0.69	0.92
	n	**248**	**248**	248	341	341	341	347	347	347	379
IL-6	r	**-0.31**	**-0.32**	**-0.30**	-0.02	-0.05	-0.05	-0.07	**-0.15**	-0.10	0.08
	p	**0.00**	**0.00**	**0.00**	0.72	0.31	0.35	0.23	**0.00**	0.06	0.11
	n	**247**	**247**	**247**	340	340	340	345	**345**	345	377
TNF-α	r	**-0.23**	**-0.23**	**-0.24**	-0.03	-0.05	-0.05	-0.03	**-0.11**	-0.09	0.05
	p	**0.00**	**0.00**	**0.00**	0.60	0.33	0.33	0.58	**0.04**	0.08	0.30
	n	**247**	**247**	**247**	340	340	340	345	**345**	345	377
HbA1c	r	0.04	0.06	0.04	0.02	0.02	0.01	0.05	0.02	0.06	-0.02
	p	0.49	0.32	0.53	0.71	0.77	0.84	0.34	0.72	0.25	0.64
	n	247	247	247	339	339	339	344	344	344	375
CRP	r	0.08	0.11	0.04	0.03	0.00	-0.03	-0.10	-0.01	**-0.13**	0.03
	p	0.19	0.08	0.55	0.56	0.94	0.64	0.05	0.81	**0.02**	0.58
	n	248	248	248	341	341	341	347	347	**347**	379
Albumin	r	0.05	0.06	-0.01	-0.03	-0.03	0.01	-0.04	-0.07	0.03	-0.08
	p	0.41	0.33	0.86	0.54	0.59	0.86	0.41	0.17	0.62	0.11
	n	248	248	248	341	341	341	347	347	347	379
RhF^{P1}	r	0.11	0.10	0.03	**0.13**	0.08	0.07	-0.04	0.03	-0.03	0.02
	p	0.08	0.12	0.61	**0.02**	0.14	0.18	0.50	0.53	0.54	0.65
	n	239	239	239	**328**	328	328	335	335	335	367

Table 5.3. Potential oxidative stress-related BoA in relation to other biomarkers of oxidative stress-induced cellular senescence (All data). (r: Spearman's correlation coefficient, p: probability, n: number of participants, $^{P1:}$ Phase 1 data, significant positive association, significant negative association).

Other biomarkers of oxidative stress -induced cellular senescence		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Telomere length	r	0.08	0.06	**0.15**	**0.11**	**0.12**	0.07	**0.12**	0.04	0.00	0.02
	p	0.19	0.36	**0.02**	**0.04**	**0.03**	0.18	**0.03**	0.41	0.95	0.67
	n	245	246	**246**	**340**	**336**	338	**342**	345	338	356
DNA damage	r	-0.05	-0.09	-0.07	0.05	-0.04	0.01	0.03	0.03	0.06	0.07
	p	0.39	0.17	0.27	0.38	0.51	0.88	0.63	0.62	0.27	0.21
	n	245	246	246	341	337	339	343	346	340	359
DNA repair	r	**-0.17**	**-0.15**	-0.07	-0.06	-0.05	-0.01	0.07	-0.02	0.02	0.03
	p	**0.01**	**0.02**	0.29	0.26	0.33	0.90	0.19	0.65	0.71	0.60
	n	**245**	**246**	246	341	337	339	343	346	340	359
IL-6	r	**-0.30**	**-0.30**	**-0.26**	0.02	-0.06	-0.04	-0.07	**-0.18**	-0.05	**0.12**
	p	**0.00**	**0.00**	**0.00**	0.73	0.29	0.53	0.25	**0.00**	0.42	**0.04**
	n	**207**	**208**	**208**	296	292	294	299	**302**	295	**308**
TNF-α	r	**-0.20**	**-0.20**	**-0.17**	-0.04	-0.10	-0.08	-0.04	**-0.12**	-0.04	**0.13**
	p	**0.00**	**0.00**	**0.01**	0.54	0.07	0.15	0.53	**0.03**	0.47	**0.02**
	n	**215**	**216**	**216**	310	306	308	312	**315**	308	**325**
HbA1c	r	0.02	0.04	0.01	0.02	0.03	0.02	0.05	0.02	0.07	0.01
	p	0.78	0.50	0.87	0.67	0.63	0.68	0.34	0.75	0.19	0.85
	n	242	243	243	336	332	334	338	341	334	352
CRP	r	0.03	0.06	-0.02	0.07	0.03	-0.04	**-0.15**	-0.09	**-0.15**	0.06
	p	0.65	0.38	0.77	0.27	0.63	0.49	**0.01**	0.15	**0.02**	0.28
	n	201	202	202	274	270	273	**277**	280	**274**	289
Albumin	r	0.06	0.08	0.01	-0.04	-0.06	0.00	-0.03	-0.06	0.02	-0.06
	p	0.39	0.22	0.93	0.49	0.30	0.95	0.53	0.27	0.73	0.22
	n	244	245	245	340	336	338	342	345	339	358
RhF^{P1}	r	0.09	0.08	0.01	**0.13**	0.09	0.06	-0.03	0.04	-0.04	0.05
	p	0.15	0.22	0.88	**0.02**	0.10	0.26	0.59	0.51	0.48	0.38
	n	236	237	237	**328**	324	326	331	334	328	348

Table 5.4. Potential oxidative stress-related BoA in relation to other biomarkers of oxidative stress-induced cellular senescence (Sensitivity analysis – extreme outliers removed). (r: Spearman's correlation coefficient, p: probability, n: number of participants, $^{P1:}$ Phase 1 data, significant positive association, significant negative association).

5.3.5. Association with markers of immunosenescence in the very old population and in separated immunosenescent cells

To provide further construct validity with a more functional aspect of ageing, the association between potential oxidative stress-related BoA and immunosenescenc phenotypes in the very old population was investigated. There were consistent associations (i.e. for more than one cell population) between: superoxide levels and lymphocyte/monocyte ratio (negative, $p \leq 0.01$) and memory/naïve B lymphocyte ratio (positive, $p \leq 0.03$); mitochondrial mass and memory/naïve CD4 T lymphocyte ratio (positive, $p \leq 0.01$); and mitochondrial membrane potential and lymphocyte/monocyte ratio (positive, $p \leq 0.03$) (Table 5.5.). The magnitude of these correlations was however very low ($r \geq -0.30$ and ≤ 0.21). Sensitivity analysis removed the significance of the association between superoxide levels and memory/naïve B lymphocyte ratio, however all other significant association remained (Table 5.6.). There was no association between 8-iso Prostaglandin $F_{2\alpha}$ concentration and immunosenescence phenotypes, before or after removal of extreme outliers in sensitivity analysis ($p \geq 0.12$) (Table 5.5. and Table 5.6. respectively) The very low magnitude of correlations for significant associations remained for all parameters ($r \geq -0.29$ and ≤ 0.21) giving little evidence that they are suggestive of biological significance.

Next, an experiment was conducted to investigate the association between ROS production and phenotypically separated PBMC subpopulations. It was shown that superoxide levels were increased in Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27-) compared to non-senescent cells including; Helper T lymphocytes (CD8-), Cytotoxic T lymphocytes (CD3+/CD8+), Natural Killer T lymphocytes (CD8+CD56+) and Naïve and Non-Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+) (Figure 5.4.). This experiment was only carried out once, however a similar experiment was conducted by Thomas von Zglincki, who also investigated mitochondrial function. This experiment also found that superoxide levels were significantly increased in Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27-) compared to Natural Killer T lymphocytes (CD8+CD56+) ($p<0.01$) and Naïve and Non-Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+) ($p<0.01$) (Figure 5.5.). There was no significant difference in superoxide levels in Natural Killer T lymphocytes (CD8+CD56+) and Naïve and Non-

Immunosenescence phenotypes		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Lymphocyte / Monocyte ratio	r	**-0.30**	**-0.19**	**-0.17**	0.05	0.03	0.00	**0.21**	0.08	**0.12**	0.06
	p	**0.00**	**0.00**	**0.01**	0.36	0.56	0.98	**0.00**	0.14	**0.03**	0.22
	n	**246**	**246**	**246**	338	338	338	**343**	343	**343**	374
CD4 / CD8 T lymphocyte ratio	r	0.00	-0.09	0.05	0.11	**0.11**	-0.03	0.02	0.01	0.01	0.06
	p	0.96	0.16	0.48	0.05	**0.04**	0.58	0.75	0.85	0.82	0.29
	n	239	239	239	319	**319**	319	325	325	325	355
Memory / Naive B lymphocyte ratio	r	**0.14**	**0.14**	**0.15**	0.05	**0.11**	0.11	**0.11**	0.02	0.09	0.00
	p	**0.03**	**0.03**	**0.02**	0.35	**0.04**	0.05	**0.04**	0.71	0.10	0.97
	n	**246**	**246**	**246**	339	**339**	339	**344**	344	344	376
Memory / Naive CD4 T lymphocyte ratio	r	-0.01	0.04	-0.05	**0.15**	**0.16**	0.09	-0.06	-0.01	-0.07	-0.06
	p	0.90	0.56	0.45	**0.01**	**0.00**	0.09	0.29	0.89	0.21	0.27
	n	245	245	245	**338**	**338**	338	343	343	343	375
Memory / Naive CD8 T lymphocyte ratio	r	-0.11	-0.01	-0.12	0.10	0.09	0.08	-0.02	0.00	-0.02	-0.04
	p	0.10	0.85	0.07	0.08	0.11	0.17	0.74	0.95	0.74	0.43
	n	236	236	236	316	316	316	322	322	322	352
Senescent (CD27-/RO-) CD4 T lymphocytes (%)	r	-0.11	-0.07	-0.10	0.08	0.08	0.07	0.04	-0.01	-0.04	-0.05
	p	0.09	0.31	0.13	0.15	0.13	0.21	0.42	0.91	0.51	0.29
	n	246	246	246	339	339	339	344	344	344	376
Senescent (CD27-/RO-) CD8 T lymphocytes (%)	r	-0.09	-0.04	-0.05	0.09	0.08	0.00	0.03	-0.02	-0.02	0.08
	p	0.17	0.57	0.47	0.12	0.14	0.97	0.62	0.75	0.78	0.15
	n	237	237	237	317	317	317	323	323	323	353

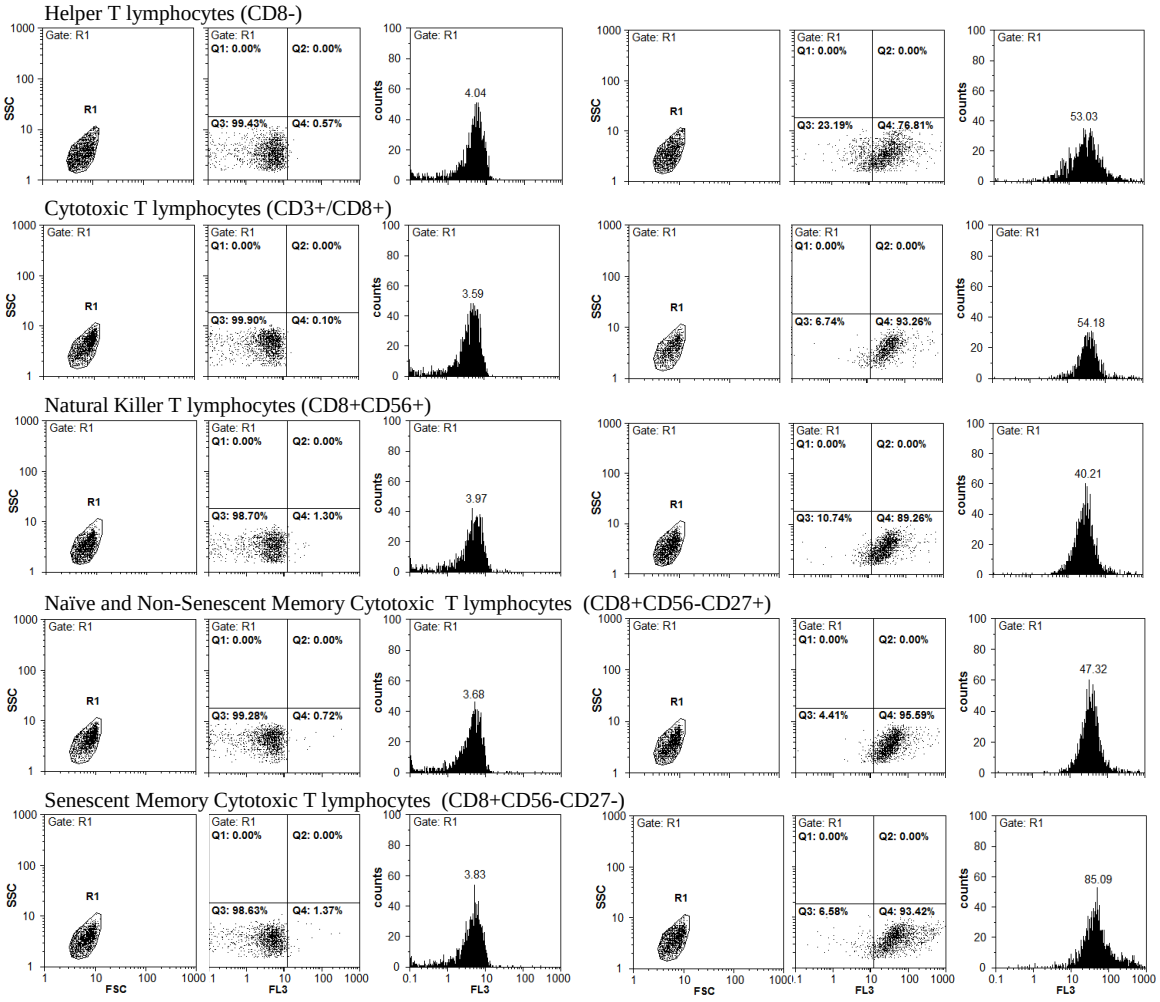
Table 5.5. Potential oxidative stress-related BoA in relation to immunosenescent phenotypes (All data). (r: Spearman's correlation coefficient, p: probability, n: number of participants, significant positive association, significant negative association).

Immunosenescence phenotypes		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Lymphocyte / Monocyte ratio	r	**-0.29**	**-0.19**	**-0.15**	0.05	0.04	0.01	**0.21**	0.09	**0.12**	0.08
	p	**0.00**	**0.00**	**0.02**	0.35	0.48	0.85	**0.00**	0.10	**0.03**	0.12
	n	**242**	**243**	**243**	336	332	334	**338**	341	**334**	352
CD4 / CD8 T lymphocyte ratio	r	0.01	-0.08	0.07	-0.11	-0.09	-0.01	0.05	0.05	0.05	0.06
	p	0.83	0.23	0.31	0.05	0.13	0.83	0.40	0.42	0.41	0.24
	n	231	232	232	309	305	307	314	316	309	326
Memory / Naive B lymphocyte ratio	r	0.12	0.11	**0.15**	0.03	0.08	0.08	**0.12**	0.02	0.09	0.01
	p	0.07	0.08	**0.02**	0.65	0.16	0.15	**0.03**	0.67	0.10	0.82
	n	235	235	**235**	324	320	322	**326**	327	321	336
Memory / Naive CD4 T lymphocyte ratio	r	0.02	0.05	-0.01	**0.15**	**0.15**	0.11	-0.07	0.02	-0.09	-0.08
	p	0.72	0.49	0.83	**0.01**	**0.01**	0.05	0.22	0.73	0.13	0.13
	n	228	229	229	**313**	**309**	311	315	317	311	333
Memory / Naive CD8 T lymphocyte ratio	r	-0.11	-0.03	-0.12	0.10	0.09	0.08	-0.03	0.02	-0.04	0.00
	p	0.11	0.64	0.07	0.08	0.14	0.16	0.65	0.70	0.53	0.97
	n	225	226	226	302	298	300	306	308	302	322
Senescent (CD27-/RO-) CD4 T lymphocytes (%)	r	-0.09	-0.04	-0.08	0.06	0.08	0.06	0.06	0.01	-0.02	-0.07
	p	0.19	0.53	0.23	0.29	0.16	0.31	0.32	0.90	0.71	0.21
	n	228	229	229	314	311	312	316	319	313	332
Senescent (CD27-/RO-) CD8 T lymphocytes (%)	r	-0.07	-0.03	-0.02	0.09	0.10	0.01	0.03	-0.02	-0.01	0.04
	p	0.27	0.70	0.71	0.12	0.09	0.85	0.58	0.75	0.90	0.52
	n	234	235	235	317	313	315	321	323	316	336

Table 5.6. Potential oxidative stress-related BoA in relation to immunosenescent phenotypes (Sensitivity analysis – extreme outliers removed). (r: Spearman's correlation coefficient, p: probability, n: number of participants, significant positive association, significant negative association).

A.

i) Unstained **ii) DHE stained**



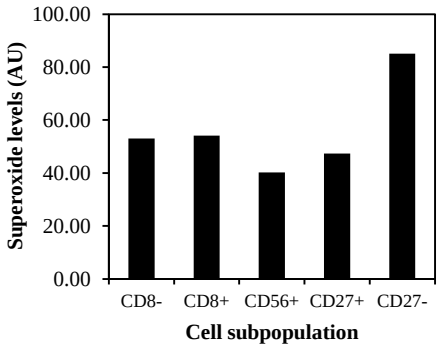


Figure 5.4. Comparison of superoxide levels in separated lymphocytes. A. flow cytometry graphs for quantification and B. comparison of superoxide levels in non-senescent and senescent lymphocyte populations (n=1). (CD8- (Helper T lymphocytes (CD8-)); CD8+ (Cytotoxic T lymphocytes (CD3+/CD8+)); CD56+ (Natural Killer T lymphocytes (CD8+CD56+)); CD27+: (Naïve and Non-Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+)); CD27- (Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27-)).

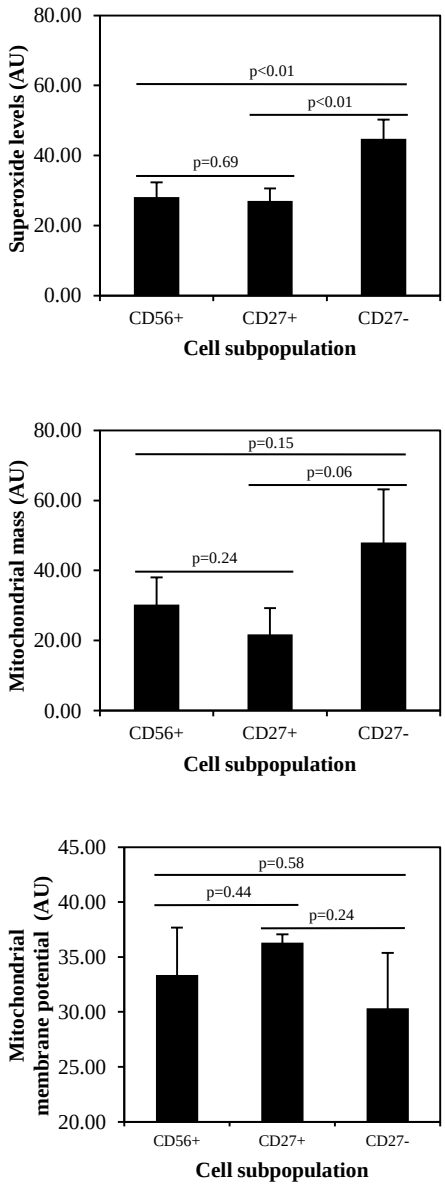


Figure 5.5. Comparison of superoxide levels, mitochondrial mass and mitochondrial membrane potential in separated lymphocytes. (This data was obtained and supplied by Thomas von Zglinicki) Superoxide levels (n=4), mitochondrial mass (n=3), Mitochondrial membrane potential (n=2). Student T-test was used to compare the means of two groups. Values are normalised to 100%. *(*CD56+ (Natural Killer T lymphocytes (CD8+CD56+)); CD27+: (Naïve and Non-Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+)); CD27- (Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27-))).

Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+) (p=0.69) (Figure 5.5.). There was a trend for mitochondrial function to be decreased in Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27-) compared to Natural Killer T lymphocytes (CD8+CD56+) and Naïve and Non-Senescent Memory Cytotoxic T lymphocytes (CD8+CD56-CD27+), indicated by an increase in mitochondrial mass and decrease in mitochondrial membrane potential, however these did not reach statistical significance ($p \geq 0.06$) (Figure 5.5.).

5.3.6. Association with informative BoA of the very old population

Previously, 10 out of a list of 72 candidate markers were confirmed as informative BoA in the Newcastle 85+ study because they were associated with two or more of the following age-related outcomes: cognitive impairment, disability score, disease count and survival as discussed in Chapter 3 (Martin-Ruiz *et al.*, 2011). The association between potential oxidative stress-related BoA of this study and the 10 informative BoA was investigated. Consistent associations (i.e. for more than one cell population) were found between superoxide levels and mitochondrial membrane potential with baseline vitamin D levels (negative for both, $p \leq 0.04$) (Table 5.7.). The magnitude of these correlations was however very low ($r \geq -0.18$). Sensitivity analysis by removing extreme outliers however reduced the significance of the association between superoxide levels and vitamin D in the lymphocyte subpopulation (p=0.08); the other significant associations remained (Table 5.8.). There was an association between 8-iso Prostaglandin $F_{2\alpha}$ and free T3, both before (positive, p=0.01); and after sensitivity analysis (positive, p=0.01) (Table 5.7. and Table 5.8. respectively). Sensitivity analysis also revealed an association between 8-iso Prostaglandin $F_{2\alpha}$ and FEV1 at baseline (negative, p=0.04) (Table 5.8.). The very low magnitude of correlations for significant associations remained for all parameters ($r \geq -0.18$ and ≤ 0.14) giving little evidence that they are suggestive of biological significance.

5.3.7. Confounding factors in the very old population

The final construct validation assessment was to identify potential confounders of the potential oxidative stress-related BoA in the very old population. It was not relevant to test the association between ethnicity and place of birth since there was too little variance within the population (See Appendix C. Supplementary Table 12.). There were consistent significant associations (i.e. for more than one cell population) between:

superoxide levels and taking non-prescribed medicines, supplements and/or herbal remedies ($p=0.01$) and season of blood withdraw ($p<0.01$) (Table 5.9. A.); and mitochondrial membrane potential and the number of years in main job/role ($p\leq0.02$) and season of blood withdrawal ($p<0.01$) (Table 5.9. C.) Sensitivity analysis removed the consistent association between mitochondrial membrane potential and the number of years in main job/role: all other significant association however remained (data not shown). There was an association between 8-iso Prostaglandin $F_{2\alpha}$ and gender ($p=0.02$) and also season of blood withdrawal ($p=0.01$) (Table 5.9. D.). These association remained after removal of extreme outliers by sensitivity analysis (data not shown).

5.4. Discussion

The first assessment of this chapter was to provide evidence that potential oxidative stress-related BoA to some degree show a correlation with chronological age. This is a required criteria for a valid BoA (Baker and Sprott, 1988), since chronological age is a major risk factor associated with age-related decline and an increased risk of mortality. It was therefore expected that the potential oxidative stress-related BoA investigated in this study would increase with chronological age, since oxidative stress is hypothesised to be the cause of the progressive loss of homeostatic regulation of biological function over time due to the damage caused by them at the cellular and molecular level, increasing vulnerability to detrimental health outcomes and thus mortality (Harman, 1956). This was true for mitochondrial dysfunction, indicated by a significant increase in mitochondrial mass and decrease in membrane potential with chronological age, supporting their role as valid BoA; however, what was surprising was that superoxide levels actually decreased with chronological age when comparing the young controls to the very old population. These findings therefore do not support the theory that oxidative stress is the cause of the decline in mitochondrial and thus biological function with age, in leukocytes at least. There could be various explanations for this including the fact that ROS released during the oxidative burst of leukocytes play an important role in the immune system to kill invading microorganisms. Therefore this could be the reason for the reduced superoxide levels with age in leukocytes, which could reflect the decline in immune function and thus increased susceptibility to infection with age (Haddy, 1988). Some studies agree with this and find a decline in ROS production with chronologic age in leukocytes (Braga *et al.*, 1998a; Braga *et al.*, 1998b). This however

Informative BoA		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	Plasma	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Hand grip strength	r	0.01	0.00	0.01	-0.02	-0.05	-0.02	-0.06	-0.01	-0.08	-0.05
	p	0.85	0.95	0.93	0.71	0.35	0.76	0.29	0.87	0.13	0.34
	n	242	242	242	336	336	336	342	342	342	374
TUG	r	-0.01	-0.01	0.01	0.03	0.05	0.06	0.06	0.07	0.05	0.03
	p	0.94	0.91	0.93	0.64	0.37	0.32	0.30	0.23	0.38	0.62
	n	218	218	218	304	304	304	312	312	312	340
FEV1[P1]	r	0.07	0.06	0.08	0.02	-0.04	0.01	0.01	-0.01	0.05	-0.10
	p	0.29	0.32	0.23	0.77	0.49	0.84	0.85	0.83	0.39	0.07
	n	244	244	244	335	335	335	341	341	341	372
Systolic blood pressure	r	0.02	-0.02	0.07	0.07	0.09	0.08	0.01	0.01	0.02	0.00
	p	0.77	0.79	0.27	0.22	0.09	0.16	0.92	0.87	0.65	1.00
	n	245	245	245	338	338	338	343	343	343	376
Haematocrit	r	0.06	0.07	0.03	0.05	-0.03	0.03	-0.04	-0.05	-0.09	0.10
	p	0.37	0.26	0.61	0.35	0.53	0.56	0.49	0.36	0.10	0.06
	n	246	246	246	338	338	338	343	343	343	374
Haemoglobin	r	0.05	0.07	0.02	0.08	-0.01	0.04	0.01	-0.06	-0.05	0.05
	p	0.44	0.25	0.78	0.16	0.81	0.44	0.82	0.29	0.36	0.33
	n	246	246	246	338	338	338	343	343	343	374
Red blood cells	r	0.08	0.12	0.05	0.10	0.05	0.09	-0.03	-0.08	-0.08	0.08
	p	0.20	0.07	0.41	0.08	0.36	0.11	0.60	0.13	0.16	0.14
	n	246	246	246	338	338	338	343	343	343	374
Free T3	r	-0.09	-0.11	-0.08	0.03	-0.04	-0.03	-0.07	**-0.12**	-0.04	**0.13**
	p	0.16	0.08	0.22	0.61	0.51	0.60	0.19	**0.03**	0.45	**0.01**
	n	246	246	246	338	338	338	343	**343**	343	**374**
Vitamin D [P1]	r	-0.12	**-0.13**	**-0.16**	-0.01	-0.05	-0.07	-0.10	**-0.18**	**-0.15**	0.01
	p	0.06	**0.04**	**0.01**	0.89	0.35	0.19	0.06	**0.00**	**0.01**	0.83
	n	240	**240**	**240**	331	331	331	338	**338**	**338**	371
NT-pro BNP	r	-0.10	-0.19	-0.25	-0.09	-0.17	-0.12	-0.10	0.11	-0.09	0.02
	p	0.50	0.19	0.09	0.39	0.12	0.26	0.33	0.30	0.38	0.82
	n	46	46	46	85	85	85	95	95	95	108

Table 5.7. Potential oxidative stress-related BoA in relation to informative BoA (All data). (r: Spearman's correlation coefficient, p: probability, n: number of participants, [P1:] Phase 1 data, significant positive association, significant negative association).

Informative BoA		Superoxide levels			Mitochondrial mass			Mitochondrial membrane potential			8-iso Prostaglandin F2α
		PBMCs	Lymphocytes	Monocytes	Plasma	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Hand grip strength	r	0.01	0.00	0.01	-0.02	-0.05	-0.02	-0.04	0.00	-0.06	-0.07
	p	0.91	0.99	0.89	0.71	0.34	0.78	0.42	0.98	0.24	0.19
	n	240	241	241	336	332	335	338	341	335	354
TUG	r	0.00	-0.02	0.00	0.04	0.04	0.05	0.06	0.08	0.03	0.00
	p	0.95	0.80	0.98	0.49	0.44	0.36	0.32	0.17	0.64	0.94
	n	213	214	214	299	295	298	304	306	301	316
FEV1[P1]	r	0.05	0.06	0.07	0.02	-0.04	0.01	0.02	0.00	0.05	**0.11**
	p	0.41	0.35	0.25	0.77	0.45	0.90	0.72	0.95	0.40	**0.04**
	n	241	242	242	335	331	333	337	340	334	**352**
Systolic blood pressure	r	0.01	-0.02	0.07	0.07	0.08	0.07	0.01	0.01	0.03	-0.03
	p	0.90	0.78	0.27	0.22	0.14	0.19	0.82	0.78	0.56	0.51
	n	242	243	243	338	334	336	339	342	336	356
Haematocrit	r	0.06	0.07	0.04	0.05	-0.04	0.03	-0.04	-0.05	-0.06	0.09
	p	0.36	0.24	0.58	0.35	0.42	0.53	0.50	0.36	0.27	0.08
	n	243	244	244	338	334	336	340	343	336	354
Haemoglobin	r	0.06	0.08	0.02	0.08	-0.02	0.05	0.01	-0.06	-0.03	0.05
	p	0.39	0.20	0.70	0.16	0.66	0.40	0.89	0.29	0.61	0.33
	n	243	244	244	338	334	336	340	343	336	354
Red blood cells	r	0.09	0.12	0.06	0.10	0.04	0.09	-0.03	-0.08	-0.04	0.06
	p	0.17	0.06	0.39	0.08	0.48	0.10	0.62	0.13	0.47	0.29
	n	243	244	244	338	334	336	340	343	336	354
Free T3	r	-0.08	-0.10	-0.07	0.03	-0.03	-0.02	-0.07	**-0.12**	-0.05	**0.14**
	p	0.20	0.11	0.30	0.61	0.62	0.72	0.19	**0.03**	0.40	**0.01**
	n	243	244	244	338	334	336	340	**343**	336	**354**
Vitamin D [P1]	r	-0.11	-0.11	**-0.14**	-0.01	-0.07	-0.07	-0.10	**-0.18**	**-0.13**	0.00
	p	0.08	0.08	**0.03**	0.89	0.18	0.18	0.06	**0.00**	**0.02**	0.97
	n	237	238	**238**	331	327	329	334	**337**	**331**	352
NT-pro BNP	r	-0.16	-0.18	-0.28	-0.09	-0.13	-0.12	-0.09	0.12	-0.09	-0.11
	p	0.31	0.25	0.07	0.44	0.24	0.30	0.40	0.26	0.39	0.30
	n	44	44	44	81	80	81	90	91	91	95

Table 5.8. Potential oxidative stress-related BoA in relation to informative BoA (Sensitivity analysis – extreme outliers removed). (r: Spearman's correlation coefficient, p: probability, n: number of participants, [P1:] Phase 1 data, significant positive association, significant negative association).

A.

Potential confounders	Superoxide levels (AU)											
	PBMCs				Lymphocytes				Monocytes			
Gender [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Male	35.09	20.47	91	0.43	26.89	18.72	91	0.20	61.32	46.90	91	0.94
Female	32.44	20.00	157		23.74	21.27	157		61.66	46.04	157	
Mitochondrial haplogroup [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
H	30.69	21.69	89		23.22	21.24	89		58.13	41.79	89	
T	34.33	19.05	25		24.66	19.41	25		65.89	57.06	25	
J	33.78	18.92	22		24.22	17.32	22		60.91	42.91	22	
U	39.79	19.40	36		34.39	18.88	36		84.80	49.23	36	
K	25.55	13.19	15	0.13	19.77	9.42	15	0.21	53.99	37.28	15	0.64
X	36.10	14.46	4		30.65	17.45	4		86.57	55.53	4	
W	35.20	15.34	8		30.24	20.26	8		72.84	51.79	8	
I	26.00	-	3		17.11	-	3		55.14	-	3	
V	31.86	31.78	7		19.23	16.92	7		74.62	63.17	7	
Age of natural parents death (years) [P1]	r		n	p	r		n	p	r		n	p
Mother	0.05		239	0.43	0.08		239	0.20	0.10		239	0.13
Father	0.03		232	0.64	0.00		232	0.97	0.01		232	0.82
Has/had a sibling(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	33.07	19.89	221	0.64	25.60	20.76	221	0.69	60.73	45.69	221	0.37
No	34.68	30.97	26		24.20	27.44	26		73.14	57.37	26	
Had full-time higher education [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	30.69	29.50	33	0.66	21.31	21.41	33	0.36	57.18	45.51	33	0.72
No	34.33	19.81	215		26.05	20.82	215		61.99	46.20	215	
Number of years in main job/role [P1]	r		n	p	r		n	p	r		n	p
	0.06		197	0.37	-0.02		197	0.77	0.00		197	0.98
Has offspring [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	33.94	20.10	206	0.91	24.95	19.81	206	0.66	61.04	45.12	206	0.86
No	32.77	20.82	41		27.37	23.78	41		61.99	59.84	41	

Marital status [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Single	31.95	15.37	19		18.98	11.82	19		59.37	26.97	19	
Married	37.16	25.15	54		29.79	23.76	54		62.52	59.75	54	
Re-married	41.68	-	3	0.57	35.44	-	3	0.57	91.72	-	3	0.19
Separated	-	-	0		-	-	0		-	-	0	
Divorced	36.90	33.71	7		34.55	28.37	7		92.87	56.11	7	
Widowed	32.64	19.90	164		23.31	17.01	164		60.55	42.58	164	
Lives alone [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	**31.75**	**20.54**	**141**	**0.04**	23.22	18.48	141	0.21	59.69	42.21	141	0.17
No	**37.02**	**26.85**	**79**		28.77	22.37	79		70.56	58.66	79	
Housing type [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Standard housing	32.78	19.34	186		24.95	20.77	186		61.49	44.75	186	
Sheltered housing with warden	33.07	28.99	33	0.77	26.05	24.92	33	0.62	62.57	71.89	33	0.51
Institution	35.17	15.62	27		24.98	14.91	27		59.53	37.04	27	
Alcohol status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current drinker	35.11	21.08	164		26.75	20.30	164		66.15	46.70	164	
Occasional drinker	29.91	17.53	41	0.12	20.12	16.84	41	0.34	49.90	43.36	41	0.12
Previous drinker	31.26	18.38	11		22.82	17.21	11		56.56	26.03	11	
Never drinker	31.44	29.00	32		20.15	27.85	32		61.20	54.43	32	
Smoking status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current smoker	32.59	18.65	10		24.74	14.75	10		60.90	30.31	10	
Past smoker	32.78	20.53	154	0.86	25.87	20.79	154	0.50	59.56	46.42	154	0.47
Never smoker	34.48	21.85	84		22.79	22.07	84		64.69	47.54	84	
Takes non-prescribed medicines, supplements and/or herbal remedies [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	**30.96**	**15.40**	**118**	**0.01**	**22.02**	**14.71**	**118**	**0.01**	**57.06**	**35.68**	**118**	**0.01**
No	**36.70**	**25.94**	**130**		**28.53**	**24.51**	**130**		**77.39**	**55.92**	**130**	
Takes prescribed medication [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	34.24	20.18	238	0.97	25.87	20.83	238	0.46	61.49	46.27	238	0.95
No	33.10	29.17	8		17.78	33.54	8		69.04	60.87	8	
Self-reported physical activity score [P3]	r		n	p	r		n	p	r		n	p
	0.09		248	0.14	0.13		248	0.05	0.13		248	0.05
Uses aid(s)/appliance(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	33.07	20.31	211	0.73	24.66	20.95	211	0.76	62.07	44.43	211	0.32
No	34.55	19.86	37		26.89	15.90	37		53.19	51.57	37	
Social isolation score [P3]	r		n	p	r		n	p	r		n	p
	-0.01		246	0.82	0.00		246	0.96	0.05		246	0.43

Key event impact [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Positive key event(s)	30.70	19.36	49		19.93	15.80	49		53.99	36.64	49	
Negative key event(s)	34.29	16.74	65		26.07	20.18	65		72.93	48.36	65	
Positive and negative key event(s)	32.23	21.89	35	0.58	24.59	21.22	35	0.44	61.32	38.98	35	0.32
Key event(s) but impact unknown	31.86	32.31	27		26.85	26.24	27		57.59	68.01	27	
No key event	36.62	23.94	72		28.44	22.76	72		66.11	47.05	72	
Has problems sleeping [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	31.90	23.08	67	0.33	20.18	21.97	67	0.22	56.79	43.76	67	0.43
No	34.37	19.65	181		26.66	20.54	181		62.96	45.35	181	
Body mass index [P3]	r		n	p	r		n	p	r		n	p
	-0.08		222	0.22	-0.04		222	0.54	-0.11		222	0.11
Fasted blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	33.94	19.99	242	0.73	25.65	20.74	242	0.52	61.49	45.90	242	0.55
No	38.36	33.50	6		17.94	32.50	6		73.48	68.19	6	
Season of blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Spring	**40.52**	**17.59**	**87**		**33.66**	**15.77**	**87**		**84.58**	**46.41**	**87**	
Summer	**28.27**	**11.07**	**75**	**0.00**	**18.62**	**7.48**	**75**	**0.00**	**48.86**	**14.41**	**75**	**0.00**
Autumn	**23.41**	**10.04**	**34**		**15.06**	**5.09**	**34**		**42.89**	**27.64**	**34**	
Winter	**44.49**	**33.96**	**52**		**34.07**	**26.80**	**52**		**80.92**	**67.74**	**52**	

B.

Potential confounders	**Mitochondrial mass (AU)**											
	PBMCs				Lymphocytes				Monocytes			
Gender [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Male	41.51	17.69	124	0.90	43.06	32.59	124	0.56	59.37	30.00	124	0.48
Female	41.90	22.05	217		44.96	32.20	217		62.34	46.30	217	
Mitochondrial haplogroup [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
H	42.16	21.40	127		42.85	30.68	127		60.82	41.48	127	
T	42.33	16.37	34		44.65	33.37	34		51.47	35.57	34	
J	41.07	18.76	34		46.50	27.99	34		51.47	36.86	34	
U	42.76	17.40	50		49.44	29.26	50		63.96	47.70	50	
K	42.53	29.42	22	0.46	47.35	36.18	22	0.13	67.88	45.56	22	0.33
X	31.45	22.78	5		32.43	26.96	5		65.38	28.00	5	
W	47.68	38.19	10		46.13	66.08	10		67.89	79.15	10	
I	46.46	19.90	4		57.60	26.23	4		92.05	43.05	4	
V	34.16	15.85	10		30.84	12.37	10		49.81	19.05	10	

Age of natural parents death (years) [P1]	r		n	p	r		n	p	r		n	p
Mother	0.95		332	0.95	0.03		332	0.55	0.05		332	0.34
Father	0.08		318	0.14	0.02		318	0.76	0.08		318	0.18
Has/had a sibling(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	**42.33**	**20.19**	**310**	**0.04**	44.93	33.06	310	0.14	62.27	42.08	310	0.12
No	**38.56**	**20.73**	**30**		40.79	27.78	30		55.76	33.07	30	
Had full-time higher education [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	40.86	14.46	51	0.53	44.48	28.25	51	0.78	60.71	32.63	51	0.85
No	41.86	20.61	290		44.85	34.89	290		61.05	41.06	290	
Number of years in main job/role [P1]	r		n	p	r		n	p	r		n	p
	0.01		264	0.94	-0.05		264	0.42	-0.04		264	0.54
Has offspring [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.51	19.99	284	1.00	44.98	32.45	284	0.94	60.93	39.01	284	0.56
No	42.10	18.06	56		44.21	28.18	56		61.30	47.07	56	
Marital status [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Single	43.71	22.91	28		43.38	27.74	28		63.73	49.54	28	
Married	41.84	17.88	77		46.07	30.73	77		58.95	37.50	77	
Re-married	40.86	19.91	6	0.52	34.99	45.44	6	0.94	67.35	36.88	6	0.71
Separated	-	-	1		-	-	1		-	-	1	
Divorced	42.73	31.78	7		40.83	55.86	7		69.20	61.62	7	
Widowed	40.79	21.75	220		44.93	34.32	220		62.36	40.23	220	
Lives alone [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	42.37	20.96	188	0.68	46.01	30.49	188	0.76	63.77	42.45	188	0.89
No	41.89	17.43	116		44.90	30.79	116		60.26	38.81	116	
Housing type [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Standard housing	42.10	19.66	262		44.93	30.39	262		**60.98**	**41.33**	**262**	
Sheltered housing with warden	41.90	17.56	41	0.10	50.13	34.95	41	0.11	**67.37**	**36.37**	**41**	**0.01**
Institution	33.81	24.11	36		35.96	37.90	36		**45.68**	**34.19**	**36**	
Alcohol status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current drinker	41.43	19.65	222		44.39	32.07	222		60.72	41.69	222	
Occasional drinker	43.54	18.27	62	0.91	44.93	27.18	62	0.96	61.77	28.37	62	0.88
Previous drinker	44.37	26.53	14		51.11	48.22	14		67.40	69.17	14	
Never drinker	38.89	27.27	43		45.70	43.64	43		56.67	64.88	43	

Smoking status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current smoker	41.32	29.98	15		53.82	45.51	15		58.67	66.45	15	
Past smoker	41.88	20.20	213	0.57	44.89	32.41	213	0.75	62.78	38.28	213	0.53
Never smoker	40.83	18.56	113		42.85	30.48	113		60.15	39.41	113	
Takes non-prescribed medicines, supplements and/or herbal remedies [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.45	19.33	155	0.32	44.80	31.17	155	0.50	62.51	45.28	155	0.38
No	41.77	21.93	186		44.57	33.02	186		60.50	38.09	186	
Takes prescribed medication [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.69	19.48	325	0.58	44.80	32.68	325	0.51	61.03	39.50	325	0.98
No	39.89	28.06	14		40.48	21.75	14		56.26	60.58	14	
Self-reported physical activity score [P3]	r		n	p	r		n	p	r		n	p
	0.03		341	0.57	0.05		341	0.35	0.04		341	0.47
Uses aid(s)/appliance(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.45	19.43	281	0.20	44.30	33.27	281	0.62	60.71	39.89	281	0.30
No	42.10	23.01	60		46.77	24.14	60		62.70	38.84	60	
Social isolation score [P3]	r		n	p	r		n	p	r		n	p
	-0.06		339	0.25	0.00		339	1.00	-0.02		339	0.69
Key event impact [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Positive key event(s)	42.36	20.11	63		47.65	41.61	63		62.62	50.57	63	
Negative key event(s)	41.69	18.52	95		43.41	28.81	95		62.20	36.40	95	
Positive and negative key event(s)	41.90	24.20	63	1.00	39.79	28.72	63	0.80	58.95	29.73	63	0.78
Key event(s) but impact unknown	42.30	18.99	36		45.81	46.77	36		67.91	50.54	36	
No key event	41.00	20.67	84		44.58	33.15	84		60.47	44.11	84	
Has problems sleeping [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.13	18.73	89	0.98	44.55	33.48	89	0.85	62.62	41.42	89	0.47
No	41.77	20.19	252		44.64	31.58	252		60.67	39.32	252	
Body mass index [P3]	r		n	p	r		n	p	r		n	p
	0.01		304	0.80	0.00		304	0.97	-0.01		304	0.85
Fasted blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	41.90	19.85	331	0.09	44.60	32.33	331	0.52	61.24	40.73	331	0.17
No	34.89	16.07	10		43.92	36.50	10		53.77	32.62	10	
Season of blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Spring	40.73	16.95	88		**49.29**	**37.59**	**88**		64.40	52.19	88	
Summer	42.69	19.71	105	0.33	**44.59**	**33.68**	**105**	**0.03**	59.82	33.27	105	0.08
Autumn	38.77	26.47	76		**39.00**	**28.03**	**76**		54.88	42.71	76	
Winter	42.64	23.70	72		**47.85**	**29.72**	**72**		65.85	39.80	72	

C.

Potential confounders	Mitochondrial membrane potential (AU)											
	PBMCs				Lymphocytes				Monocytes			
Gender [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Male	2.80	0.97	131	0.10	3.87	2.07	131	0.56	2.46	1.06	131	0.14
Female	2.93	1.05	216		3.82	1.99	216		2.57	1.20	216	
Mitochondrial haplogroup [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
H	2.82	1.00	132		3.91	1.98	132		2.41	1.00	132	
T	2.96	1.29	34		3.74	1.84	34		2.59	1.09	34	
J	3.01	0.93	35		3.99	2.53	35		2.44	1.25	35	
U	2.77	0.85	51		3.71	1.89	51		2.63	1.26	51	
K	3.25	1.54	24	0.33	3.59	3.02	24	0.48	2.86	1.43	24	0.70
X	3.07	3.01	4		4.16	3.12	4		2.74	2.81	4	
W	2.59	0.87	10		2.82	2.09	10		2.48	0.74	10	
I	3.30	2.58	4		3.78	1.68	4		2.97	2.39	4	
V	2.61	1.31	8		4.44	2.48	8		2.72	2.45	8	
Age of natural parents death (years) [P1]	r		n	p	r		n	p	r		n	p
Mother	0.00		336	0.95	0.01		336	0.90	-0.03		336	0.54
Father	-0.03		322	0.59	-0.08		322	0.18	0.02		322	0.77
Has/had a sibling(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.88	0.99	314	0.92	3.85	1.99	314	0.54	2.52	1.13	314	0.66
No	2.76	0.96	32		3.89	2.91	32		2.38	1.64	32	
Had full-time higher education [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.79	1.04	53	0.54	3.46	1.78	53	0.11	2.60	0.92	53	0.56
No	2.89	1.04	294		3.88	2.02	294		2.51	1.22	294	
Number of years in main job/role [P1]	r		n	p	r		n	p	r		n	p
	-0.12		271	0.05	**-0.17**		**271**	**0.01**	**-0.14**		**271**	**0.02**
Has offspring [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.85	0.99	289	0.10	3.88	2.00	289	0.61	2.49	1.21	289	0.17
No	3.05	0.98	57		3.73	2.00	57		2.70	1.00	57	

Marital status [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Single	2.89	0.94	30		3.49	1.03	30		2.41	1.19	30	
Married	2.87	1.13	78		4.00	1.89	78		2.50	1.16	78	
Re-married	3.17	1.72	7	0.18	4.96	1.86	7	0.07	3.63	2.00	7	0.05
Separated	-	-	1		-	-	1		-	-	1	
Divorced	3.26	2.05	8		3.57	1.12	8		2.93	3.00	8	
Widowed	2.84	1.00	221		3.86	2.20	221		2.47	1.03	221	
Lives alone [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.91	0.93	194	0.68	3.77	2.22	194	0.53	2.52	1.12	194	0.38
No	2.83	0.96	117		3.92	1.88	117		2.46	1.18	117	
Housing type [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Standard housing	2.86	0.90	263		3.79	2.04	263		2.51	1.10	263	
Sheltered housing with warden	2.95	1.19	47	0.38	4.00	2.13	47	0.75	2.58	1.29	47	0.78
Institution	2.58	1.27	35		3.87	1.81	35		2.71	1.35	35	
Alcohol status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current drinker	2.84	0.98	228		3.78	2.02	228		2.50	1.11	228	
Occasional drinker	3.05	1.09	62	0.51	4.00	2.01	62	0.76	2.62	1.18	62	0.69
Previous drinker	2.77	1.02	14		3.72	1.71	14		2.47	1.51	14	
Never drinker	2.81	0.97	43		3.95	2.07	43		2.49	1.22	43	
Smoking status [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Current smoker	2.72	0.99	15		3.23	1.30	15		2.16	0.68	15	
Past smoker	2.84	0.97	216	0.38	3.84	2.20	216	0.41	2.47	1.12	216	0.09
Never smoker	2.95	1.21	116		3.93	2.01	116		2.62	1.17	116	
Takes non-prescribed medicines, supplements and/or herbal remedies [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.87	0.94	158	0.56	3.70	2.03	158	0.46	2.52	1.20	158	0.76
No	2.86	1.08	189		3.95	1.98	189		2.51	1.12	189	
Takes prescribed medication [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.87	0.98	330	0.93	3.85	2.02	330	0.85	2.51	1.13	330	0.46
No	2.83	1.73	15		3.68	2.83	15		2.65	1.92	15	
Self-reported physical activity score [P3]	r		n	p	r		n	p	r		n	p
	0.03		347	0.52	0.10		347	0.08	0.06		347	0.26
Uses aid(s)/appliance(s) [P1]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.85	1.00	286	0.78	3.83	1.98	286	0.84	2.48	1.14	286	0.11
No	2.94	0.94	61		3.88	1.88	61		2.70	1.09	61	
Social isolation score [P3]	r		n	p	r		n	p	r		n	p
	0.04		345	0.44	0.09		345	0.11	0.08		345	0.15

Key event impact [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Positive key event(s)	2.90	1.21	63		3.59	1.79	63		2.50	1.23	63	
Negative key event(s)	2.80	0.86	101		3.73	1.96	101		2.46	0.89	101	
Positive and negative key event(s)	2.83	0.88	63	0.87	3.71	1.84	63	0.27	2.60	1.20	63	0.47
Key event(s) but impact unknown	3.02	1.45	35		4.11	2.45	35		2.81	1.84	35	
No key event	2.99	1.22	85		4.18	1.99	85		2.59	1.28	85	
Has problems sleeping [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.86	1.04	91	0.94	4.00	2.05	91	0.63	2.55	1.24	91	0.96
No	2.87	0.99	256		3.83	2.02	256		2.51	1.16	256	
Body mass index [P3]	r		n	p	r		n	p	r		n	p
	0.02		310	0.68	0.05		310	0.39	0.02		310	0.70
Fasted blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Yes	2.88	1.06	336	0.25	3.84	1.99	336	0.48	2.52	1.19	336	0.05
No	2.66	0.42	11		4.38	2.45	11		2.09	0.64	11	
Season of blood sample [P3]	Median	IQR	n	p	Median	IQR	n	p	Median	IQR	n	p
Spring	**3.15**	**1.32**	**86**		**4.48**	**2.21**	**86**		**2.58**	**1.20**	**86**	
Summer	**2.71**	**0.86**	**109**	**0.00**	**3.25**	**1.43**	**109**	**0.00**	**2.32**	**0.95**	**109**	**0.00**
Autumn	**3.01**	**0.89**	**80**		**3.87**	**1.50**	**80**		**2.43**	**0.89**	**80**	
Winter	**2.90**	**1.56**	**72**		**4.54**	**3.41**	**72**		**3.15**	**2.93**	**72**	

D.

Potential confounders	8-Iso Prostaglandin F2α (ng/ml) [P3]			
Gender [P1]	Median	IQR	n	p
Male	**1.38**	**1.68**	**145**	**0.02**
Female	**1.57**	**2.64**	**234**	
Mitochondrial haplogroup [P1]	Median	IQR	n	p
H	1.55	1.91	156	
T	1.52	4.04	34	
J	1.45	1.95	35	
U	1.52	1.63	52	
K	1.97	5.58	27	0.45
X	0.92	1.08	5	
W	1.50	1.85	9	
I	3.04	12.42	4	
V	1.27	1.86	11	
Age of natural parents death (years) [P1]	r		n	p
Mother	0.03		366	0.54
Father	0.05		351	0.33
Has/had a sibling(s) [P1]	Median	IQR	n	p
Yes	1.50	2.11	340	0.38
No	1.44	1.68	38	
Had full-time higher education [P1]	Median	IQR	n	p
Yes	1.33	2.22	58	0.27
No	1.52	1.99	321	
Number of years in main job/role [P1]	r		n	p
	-0.08		297	0.18
Has offspring [P1]	Median	IQR	n	p
Yes	1.48	1.86	318	0.59
No	1.59	2.38	60	
Marital status [P3]	Median	IQR	n	p
Single	1.44	6.23	28	
Married	1.52	2.44	91	
Re-married	2.65	5.94	7	0.58
Separated	-	-	1	
Divorced	1.26	4.61	6	
Widowed	1.50	1.71	244	
Lives alone [P3]	Median	IQR	n	p
Yes	1.64	2.10	215	0.31
No	1.46	1.87	127	
Housing type [P3]	Median	IQR	n	p
Standard housing	1.51	2.09	296	
Sheltered housing with warden	1.79	3.06	45	0.13
Institution	1.12	1.05	36	
Alcohol status [P1]	Median	IQR	n	p
Current drinker	1.47	1.88	258	
Occasional drinker	1.49	1.86	68	0.49
Previous drinker	1.58	2.28	15	
Never drinker	1.94	4.04	38	
Smoking status [P1]	Median	IQR	n	p
Current smoker	1.13	0.99	15	
Past smoker	1.54	2.24	251	0.48
Never smoker	1.45	1.90	113	
Takes non-prescribed medicines, supplements and/or herbal remedies [P1]	Median	IQR	n	p
Yes	1.47	1.96	179	0.70
No	1.53	2.07	200	
Takes prescribed medication [P1]	Median	IQR	n	p
Yes	1.50	1.98	360	0.76
No	1.45	3.93	16	
Self-reported physical activity score [P3]	r		n	p
	-0.04		379	0.45
Uses aid(s)/appliance(s) [P1]	Median	IQR	n	p
Yes	1.49	2.11	306	0.71
No	1.50	1.81	73	

Social isolation score [P3]	r		n	p
	-0.02		376	0.73
Key event impact [P3]	Median	IQR	n	p
Positive key event(s)	1.41	1.60	64	
Negative key event(s)	1.53	2.57	118	
Positive and negative key event(s)	1.54	1.87	72	0.77
Key event(s) but impact unknown	1.23	1.40	38	
No key event	1.58	3.12	87	
Has problems sleeping [P3]	Median	IQR	n	p
Yes	1.58	2.93	97	0.28
No	1.47	1.87	282	
Body mass index [P3]	r		n	p
	0.02		343	0.70
Fasted blood sample [P3]	Median	IQR	n	p
Yes	1.48	2.09	369	0.69
No	1.60	2.47	10	
Season of blood sample [P3]	Median	IQR	n	p
Spring	**1.35**	**1.49**	**91**	
Summer	**1.54**	**3.06**	**121**	**0.01**
Autumn	**1.82**	**3.26**	**93**	
Winter	**1.27**	**1.66**	**74**	

Table 5.9. Potential confounders of potential oxidative stress-related BoA.
A. superoxide levels, B. mitochondrial mass, C. mitochondrial membrane potential and D. 8-Iso Prostaglandin $F_{2\alpha}$. All had a non-normal distribution in the study population, assessed using the Kolomogorov-Smirnov test for normality ($p<0.05$). Spearman's correlation was used to the relationship between two continuous variables, Mann-Whitney U was used to test the difference between two groups and Kruskal-Wallis was used to test for the difference between three or more groups. ([P1]: Phase 1 data, [P3]: Phase 3 data).

does not support the findings of the next results of this study which shows an association with increased superoxide production with mitochondrial dysfunction. It could be that the younger population have much more defences against the high levels of ROS produced in leukocytes that are needed to kill microorganisms during infection, such as higher levels of antioxidants and oxidative repair mechanisms, and this could be why the younger population have a better mitochondrial function than the very old population. Another explanation for the decreased superoxide levels between the young and the old of this study could be that fact that the very old represent successful survivors and thus have better resistance to oxidative stress (Ungvari *et al.*, 2011). However this does not explain why they have an increased mitochondrial dysfunction, therefore the former explanation is more likely. A limitation to this study is that the DHE stain is only specific to superoxide where as many other ROS could be involved in the decline of mitochondrial function with age. There are other studies that have shown an increased ROS generation with chronological age in various tissues, including leukocytes (Martins Chaves *et al.*, 2000; Chen *et al.*, 2001; Sasaki *et al.*, 2008). A difference between the latter study and this study is that they used a chemiluminescence assay, and thus method and type of ROS detection could be a reason for their disagreement with this study. ROS are short lived and thus chemiluminescence could be a better detection method than flow cytometry due to the handling and preparation needed. Also, a weakness to this study is that it is of low sample size and of limited age range. Also, chronological age is not the best validation assessment of a BoA due to variations in biological age between individuals.

There was a tendency for 8-Iso Prostaglandin $F_{2\alpha}$ to decrease with chronological age when comparing the younger to the very old population, however this did not reach statistical significance. Some studies agree with these findings in humans and show no significant changes in plasma F_2-isoprostanes with chronological age (Chang *et al.*, 2012), however other studies in mice have reported an increase with chronological age (Ward *et al.*, 2005). There are however differences between the methods used in these two studies and this study where the former study used LCMS and the latter study used GCMS, which could be a reason for the disagreements between studies, including differences in the specificity of the methods to specific F_2-isoprostanes isomers as discussed in Chapter 4. Section 4.4. Interestingly however, in another study in mice, an association between increased mitochondrial dysfunction and decreased plasma F_2-isoprostanes was shown (Lapointe and Hekimi, 2008). They explain that this could be

because the majority of the oxidative metabolites of lipids that find their way into the plasma do not originate in the mitochondria but in cellular membranes, and thus the reduction in F_2-isoprostanes are likely because of a decrease in cytoplasmic ROS generating processes (Lapointe and Hekimi, 2008). Another surprising results was that the longitudinal changes in 8-Iso Prostaglandin $F_{2\alpha}$ in the very old was not linear and declined between phase 1 and 2 (85 to 86.5 years) however increased between phase 2 and phase 3 (86.5 to 88 years). An explanation for the decreased in 8-iso Prostaglandin $F_{2\alpha}$ between phase 1 and phase 2 could reflect less anxiety about participating in the study for the second time.

The next step of the study was to find evidence for association between ROS production, mitochondrial function and oxidative stress. Mitochondria are not only sources of ROS, a by-product of aerobic respiration, but, if antioxidant defences are compromised or unable to cope with excess ROS, they are also a target for their damage. It is accepted that ROS production is associated with mitochondrial function, observed by changes in mitochondrial mass and membrane potential (Forman and Kim, 1989; Pieri *et al.*, 1993). This study supports this by showing a positive association between superoxide levels vs. mitochondrial mass (PBMCs and Lymphocytes) and an association between superoxide levels vs. mitochondrial membrane potential (negative in PBMCs, positive in Lymphocytes and monocytes). The opposite associations between superoxide levels vs. mitochondrial membrane potential in PBMCs and PBMC subpopulations is likely to be because of the differences in superoxide levels and mitochondrial membrane potential between PBMC subpopulations, as shown in Chapter 4. Section 4.3.2. and therefore the positive association between superoxide levels vs. mitochondrial membrane potential in separate PBMC subpopulations rather than the combined PBMC population is more reliable. Mitochondrial produced ROS is generated at the electron transport chain due to electron leakage, and thus a higher membrane potential is expected to generate more ROS, as shown in this study. However, it has been shown that in senescent and therefore dysfunctional cells, superoxide levels are associated with a decrease in membrane potential (Ksiazek *et al.*, 2008). In normal functioning cells, ROS can be converted to non-radical derivatives such as hydrogen peroxide which is subsequently removed by antioxidant systems, maintaining cellular homeostasis. However, when this system is compromised, as suggested in cellular senescent cells, ROS are inefficiently removed, causing oxidative damage to the mitochondrial membrane and thus its depolarisation. Therefore this study suggests that

in the majority of PBMC subpopulations in the very old population samples, there are more cells where mitochondrial function is not compromised than cells with mitochondrial dysfunction, i.e. more non-senescent cells than senescent cells, which have efficient systems in place to prevent damage to the mitochondrial membrane and thus the relationship between ROS production and mitochondrial parameters reflect the non-senescent cells. It would be ideal that non-senescent cells could be separated from senescent cells to determine these relationships between ROS production and mitochondrial function. This was carried out at an individual sample level later in this study to compare ROS production and mitochondrial function in separated non-senescent (CD27+) and senescent (CD27-) lymphocytes, and found that senescent lymphocytes do have increased superoxide production compared to non-senescent lymphocytes. There was also a tendency for mitochondrial mass to be increased and mitochondrial membrane potential to be decreased in senescent lymphocytes, although this did not reach significance. A low sample size may have reduced the power of these findings. The relationship between ROS production and mitochondrial dysfunction would be difficult to investigate in a large population study since a large volume of blood sample is needed to gain the required number of cells for measuring ROS production and mitochondrial function by flow cytometry, especially since cell loss may occur during the cell separation step, and also due to time issues during this process. Alternately, future studies could be to compare the relationship between ROS production and mitochondrial parameters in normal and frail participants to determine these relationships. It is therefore hypothesized that in frail subjects or specifically defined senescent cells, superoxide levels would be associated with a decline in mitochondrial membrane potential. The positive association between superoxide levels vs. mitochondrial membrane potential, and thus the explanation the majority of cells within the very old samples do not have dysfunctional mitochondria however does not explain the positive association between superoxide levels vs. mitochondrial mass, which alternately suggests that mitochondria are dysfunctional since increased mitochondrial mass is commonly found in cellular senescence (Hagen *et al.*, 1997; Hagen *et al.*, 1999; Kokoszka *et al.*, 2001; Moiseeva *et al.*, 2009). However it seems that changes in mitochondrial mass and mitochondrial membrane potential are independent of each other, since no significant relationship was shown between these two parameters within the very old population. Alternately this could potentially be due to a non-linear relationship that is worth further investigation. There was no association found between 8-Iso Prostaglandin $F_{2\alpha}$ and ROS production or mitochondrial

parameters, which as discussed above, could be the fact that F_2-isoprostanes are likely to reflect cytoplasmic ROS generating processes rather than mitochondrial processes (Lapointe and Hekimi, 2008). 8-iso prostaglandin $F_{2\alpha}$ in plasma represents whole body exposures to oxidative stress whereas ROS production and mitochondrial function is specific to PBCMs, thus a lack of association could reflect different levels of oxidative stress and thus senescence in other tissues, suggesting different ageing rates in different tissues. Therefore although the measurement of ROS production and mitochondrial function may be valid BoA in PBMCs, it may not be an appropriate surrogate for predicting age-related outcomes concerning other tissues.

The next step of the study was to find evidence for the validity of potential oxidative stress-related BoA in terms of their implication in the oxidative stress-driven pathway to cellular senescence in the very old population. The agreement between potential oxidative stress-related BoA vs. other markers implicated in the oxidative stress-driven pathway to cellular senescence in the very old was then investigated. It was hypothesized that the potential oxidative stress-related BoA would correlate with a decreased telomere length, an increased DNA damage, a decreased DNA repair, and an increase in inflammatory mediators since these parameters are cellular senescence phenotypes as discussed in Chapter 1. Section 1.3.8. There were some significant agreements between potential oxidative stress-related BoA vs. other markers implicated in the oxidative stress-driven pathway to cellular senescence. However, although some support the hypothesized associations above including increased superoxide levels vs. decreased DNA repair, and increased inflammatory cytokines (il-6 and TNF-α) vs. increased 8-iso Prostaglandin $F_{2\alpha}$, some were in the opposite association as hypothesized. These included an increase in superoxide levels vs. decreased inflammatory cytokines, an increase in mitochondrial mass vs. an increase in telomere length, and an increase in mitochondrial membrane potential vs. decreased CRP. Reasons for this and also disagreements with other senescent markers could be heterogeneity in within and between cells, independent initiators of cellular senescence, method reliability, instability of these parameters in the very old and also the possibility that the correlations arose by chance since they were of very low magnitude.

The next step of the study was to investigate the relationship between potential oxidative stress-related BoA and immunosenescence in the very old population. It was hypothesised that those participants with an increased percentage of immunosenescent

cells, and thus a decreased immune function, should have higher oxidative stress levels. Therefore it was expected that there would be an increase in oxidative stress-related BoA with increasing percentages of various immunosenescent phenotypes, which are described in Figure 5.1., and thus provide further evidence of construct validity in terms of their association with age-related functional decline. An increased superoxide levels and decreased mitochondrial membrane potential were associated with a decline in the lymphocyte/monocyte ratio and mitochondrial mass was associated with an increase in the memory/naïve CD4 T lymphocyte ratio providing some evidence that mitochondria ROS production and dysfunction play a role in immunosenescence and thus immune dysfunction. There is however the possibility that these correlations arose by chance since they were of very low magnitude.

This study also attempted to validate potential oxidative stress-related BoA by determining their agreement with informative BoA in the oldest old. These are markers that were validated in previous study of the Newcastle 85+ cohort, where a large panel of candidate BoA were investigated and defined as valid if they were associated with two or more age-related outcomes: cognitive impairment, disability score, disease count and survival (Martin-Ruiz *et al.*, 2011). Increased superoxide levels and mitochondrial membrane potential in Phase 3 significantly correlated with decreased serum Vitamin D levels in Phase 1. Although a moderate intra-individual variation in vitamin D over approximately five years has been shown, a high correlation was observed, supporting the use of a one-time measurement of vitamin D within 5 years (Meng *et al.*, 2012). It is therefore expected that vitamin D levels at phase 3 should show the same association to superoxide levels and mitochondrial membrane potential as phase 1 levels. However vitamin D supplementation and season of blood draw would affect the reliability of repeat measures of vitamin D. The association between superoxide levels and mitochondrial membrane potential and vitamin D levels could therefore be stronger if there were measurements at the same time point, however this was not available within the study. This could be worth further investigation. Mitochondrial membrane potential and 8-iso Prostaglandin $F_{2\alpha}$ also significantly correlated with free T3. Since vitamin D levels and thyroid hormones vary with the season in the older population (Houston *et al.*, 2013), this suggests that potential oxidative-stress related BoA could also be affected by seasonality. 8-iso Prostaglandin $F_{2\alpha}$ at phase 3 was also associated with FEV_1, for which measurement was only available at phase 1, however since 8-iso

Prostaglandin $F_{2\alpha}$ was also available at phase 1, this was also investigated but no association was revealed (data not shown). There were however no other associations found between the potential oxidative-stress related BoA and the other seven remaining informative BoA. Therefore the very few associations, which were of low magnitude of correlation, suggests there is the possibility that they arose by chance. Since the validation of the ten informative BoA in the very old population, in terms of association with cognitive function, disability and multi-morbidity was cross-sectional, longitudinal studies should follow to determine their ability to predict age-related outcomes and thus further validate then as informative BoA.

Lastly this chapter investigated potential confounding factors that may affect the intra-individual variability of potential oxidative stress-related BoA *in vivo* (e.g. fasting status), and also grouped variability (i.e. fixed characteristics such as gender), by looking at their association between various participant characteristics. Since the effect of various participant characteristics on potential oxidative stress-related BoA was unknown and therefore were not controlled for before the study commenced (i.e. participants refrain from various activities before blood withdrawal) it was thought that an indirect investigation, by comparing the whole population, would provide some indication of factors that may need to be controlled in future predictive validation analyses. An interesting association with seasonality was found for all the potential oxidative stress-related BoA and thus this factor should be controlled for in future analyses. There were no other consistencies between potential oxidative stress-related BoA and other characteristics. A further interesting association was that superoxide levels at phase 3 were reduced in those participants who reported taking non-prescribed medicines, supplements and/or herbal remedies at phase 1. This not only adds to the construct validity of superoxide levels as a candidate BoA, but since there is an association seen approximately three years between these parameters then it is likely that taking non-prescribed medicines, supplements and/or herbal remedies is a regular occurrence of the individuals. Controlling for this factor is therefore not advised since this could reduce the power of future predictive validation analyses.

5.5. Conclusion

These results provide some evidence for the construct validity of potential oxidative stress-related BoA in the very old population in terms of associations with

chronological age, associations with some markers of oxidative stress-induced cellular senescence and a role in immunosenescence. The reliability and validity of the age-related construct variables investigated against the oxidative stress-related BoA within this study could be a limitation. This could be further explored in future work. Seasonal variation is a potential confounder and should be controlled for in future predictive validation analyses.

Chapter 6. The role of ROS production and mitochondrial dysfunction in a mouse model of ageing

6.1. Abstract

Background Dietary restriction has been shown reduce the rate of mitochondrial superoxide production and the susceptibility of many types of tissue to oxidative stress. It is therefore a suitable model to investigate the construct validity of potential oxidative stress-related BoA. **Aims** This study aimed to provide evidence of construct validity of ROS production and mitochondrial function as candidate BoA in leukocytes by investigating the effects of dietary restriction in peripheral blood and bone marrow of mice. **Methods** Superoxide levels, mitochondrial mass and mitochondrial membrane potential were measured in PBMCs of 25 month old C57Bl/6 mice that were AL (n=5) or DR (n=6) and also in bone marrow of 2 month old AL (n=6), 25 month old AL (n=12) and DR (n=11), and 32 month old AL (n=10) and DR (n=10) C57Bl/6 mice. **Results** There was a tendency for superoxide levels and mitochondrial mass to be decreased and mitochondrial membrane potential to be increased in 25 month old DR mice compared to AL controls in all subpopulations of peripheral blood; however, none reached statistical significance ($p \geq 0.07$). There was a significant increase in superoxide levels with age in all bone marrow ($p=0.04$) and lymphoid cells ($p=0.03$) but not in myeloid cells ($p=0.07$), which was significantly reduced in both 25 and 32 month old DR mice in all cell subpopulations ($p \leq 0.04$), with the exception of lymphoid cells in 32 month old mice ($p=0.13$). Mitochondrial mass had a non-linear association with age in bone marrow where in 2 month and 32 month old mice it was higher than in 25 month old mice in all subpopulations ($p \leq 0.02$). The high mitochondrial mass at 32 months of age was significantly reduced by DR in all bone marrow subpopulations ($p \leq 0.01$). There was no significant association between mitochondrial membrane potential and age in bone marrow; however, mitochondrial membrane potential was increased by DR in 32 month old mice compared to AL controls in all subpopulations ($p<0.01$). Superoxide levels, mitochondrial mass and mitochondrial membrane potential were associated with lymphoid/myeloid cell ratio. Lymphoid/myeloid cell ratio was reduced with age and increased by DR. **Conclusion** This study provides evidence for the construct validity of ROS production and mitochondrial function in leukocytes as potential oxidative stress-related BoA.

6.2. Introduction

The use of interventions that are designed to delay age-related outcomes in animal models has been an important step in the validation of candidate BoA in humans (Turturro *et al.*, 1999). Not only does this provide construct validation of the candidate BoA investigated, in terms of its reflection in the biological mechanisms of ageing, but also the assumption that it is valid across different species and that the intervention could also be extrapolated to humans. There are many advantages of validating candidate BoA using an animal model of ageing for use in large human population studies including the reduction of time, costs and ease of access to materials (Rifai *et al.*, 2006). Also, extrinsic factors in human studies are difficult to control, or may even be uncontrollable, since some information is far too intense to capture including the effects of diet, medications and physiological parameters such as metabolism and genetic heterogeneity which may lead to measurement rather than genuine inter-individual variability (Lee *et al.*, 2005). The use of animal models reduces the difficulties associated with the effects of these confounding extrinsic variables due to tightly controlled environmental surroundings and genetic homogeneity.

Dietary restriction is one of the most frequently employed interventions in animal models studies that aim to understand the biological mechanisms of ageing. This is because of its robustness in extending life expectancy and delaying a wide range age-related outcomes including physiologic alterations in behaviours, learning, immune responses, gene expression, enzyme activities, hormonal actions, glucose intolerance, DNA repair capacities and protein synthesis in a variety of different species (Weindruch and Walford, 1988). Early suggestions for the potential mechanisms of dietary restriction on biological ageing include: 1) the delay of growth and development (McCay *et al.*, 1989), which is now contested since dietary restriction started in middle age increases life-span and reduces age-related outcomes (Weindruch and Walford, 1982; Wang *et al.*, 2010); 2) the reduction in body fat, where it is suggested that dietary restriction decreases the chronic effects of peptides, cytokines, complementary factors and substrates which play an important role in the regulation of metabolism and are secreted by fat cells which could have a beneficial effect on other tissues (Barzilai and Gupta, 1999; Tchkonia *et al.*, 2010); and 3) the reduction in body temperature, which is thought to relate to metabolic process, although the exact mechanism is not understood (Lane *et al.*, 1996). The reduction of metabolic rate seems to play an important role in

all the proposed mechanism of dietary restriction. In 1977, George A. Sacher suggested that lowering metabolic rate would enhance longevity (Sacher, 1977). It was then discovered that energy metabolism generates ROS (Turrens and Boveris, 1980) and thus the link between the reduction of metabolic rate by dietary restriction and delayed ageing could be made. It is therefore hypothesised that the mechanism behind dietary restriction is through the amelioration of oxidative stress by reducing the rate of mitochondrial superoxide production, which has been supported by studies in rodents (Sohal *et al.*, 1994a). This mechanism however is not fully understood. Increasing the activity of antioxidant enzymes has been ruled out since studies have not shown a consistent pattern in response to dietary restriction (Sohal *et al.*, 1994a); however, studies have shown that there is a decreased susceptibility of tissues to oxidative stress including reduced damage to lipids (Matsuo *et al.*, 1993; Dandona *et al.*, 2001), proteins (Dubey *et al.*, 1996; Forster *et al.*, 2000) and DNA (Sohal *et al.*, 1994b; Wang *et al.*, 2010). This suggests that repair of oxidative damage could be improved by dietary restriction. It has also been suggested that dietary restriction promotes mitochondrial turnover to prevent the accumulation of damaged mitochondria, which could explain the reduced superoxide production by dietary restriction and thus reduced oxidative damage to tissues (Miwa *et al.*, 2008). It is also becoming increasingly recognised that dietary restriction may also reduce cellular senescence (Wang *et al.*, 2010). Dietary restriction is therefore a suitable model to investigate the validity of potential oxidative stress-related BoA.

Since this study is interested in using peripheral blood as a surrogate tissue for measuring mitochondrial ROS production and dysfunction as potential oxidative stress-related BoA in humans, evidence that these are reduced by dietary restriction in leukocytes is required for their validation. However, a structured literature review revealed that this information is sparse (Table 6.1). According to this review only a couple of studies have investigated this in terms of ROS production and mitochondrial membrane potential for which studies were inconsistent (Avula and Fernandes, 2002; Ueno *et al.*, 2005). No study investigating the effects of dietary restriction on leukocyte mitochondrial mass was identified. In the study by Avula and Fernandes in 2002, it was shown that basal ROS production increases and mitochondrial membrane potential stimulated with H_20_2 was decreased with age in splenic lymphocytes from AL mice and 40% dietary restriction prevented these changes (Avula and Fernandes, 2002). The

Oxidative stress and cellular senescence-related BoA	Reference	Diet group	Species	Gender	Age at start of diet	Age at end of diet	Duration	n	Cell/tissue	Marker	Levels	Units	p	Conclusion
ROS production	(Avula and Fernandes, 2002)	AL	C57BL/6J mice	Female	6 w	6 m 2 w	5 m	5	Splenocytes	Dichlorofluorescein	[E] 55	Mean % stained ± SEM	ns	▼
		CR (40%)									[E] 50			
		AL				19 m 2 w	18 m				[E] 75		*	
		CR (40%)									[E] 53			
	(Ueno *et al.*, 2005)	AL	Institute of Cancer Research mice	Male	7-8 w	8-9 w	1 w	24	Circulating myeloid cells (PMNs and monocytes)	Dihydrorhodamine 123	[E] 5	Mean fluorescence intensity	ns	x
		CR (30%)						24			[E] 5.25			
		CR (60%)						25			[E] 5			
Mitochondrial mass	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Mitochondrial membrane potential	(Avula and Fernandes, 2002)	AL	C57BL/6J mice	Female	6 w	6 m 2 w	5 m	5	Splenocytes	Rhodamine 123	[E] 65	Mean fluorescence ± SEM	ns	▲
		CR (40%)									[E] 70			
		AL				19 m 2 w	18 m				[E] 55		*	
		CR (40%)									[E] 62			
Telomere length	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
DNA Damage	(de la Maza *et al.*, 2004)	Weight maintainers (Weight change of 3kg or less)	Humans	Male	u/k	u/k	10 y	5	Lymphocytes	8-hydroxydeoxyguanosine	u/k	u/k	ns	x
		Weight gainers (Weight increment of 6kg or more)						8						

	(Gedik *et al.*, 2005)	AL CR (30%) PR (50%) CPR (30%)	Hooded-Lister rats (Rowett strain)	Male	1 m	1,3,5,7,9,11,13,15,17 m	0,2,4,6,8,10,12,14,16 m	6-8	Lymphocytes	DNA strand breaks	u/k	u/k	ns	x
	(Hofer *et al.*, 2008)	Baseline CR (20%)	Humans	Mixed	50-60 y	51-61 y	1 y	9	White blood cells	Oxidised DNA (8-oxoGua/10^6 dGuo)	4.24 ± 0.39 2.19 ± 0.34	Mean ± SE	*	▼
	(Ribeiro *et al.*, 2004)	AL CR (25%)	Balb/c mice	Male	12 w	12 w 19 d	19 d	5	White blood cells	Tail moment	2.2 ± 0.2 3.6 ± 0.2	Mean ± SE	*	▲
DNA repair	(Gedik *et al.*, 2005)	AL CR (30%) PR (50%) CPR (30%)	Hooded-Lister rats (Rowett strain)	Male	1 m	1,3,5,7,9,11,13,15,17 m	0,2,4,6,8,10,12,14,16 m	6-8	Lymphocytes	Percentage repair of H_2O_2-induced DNA strand breaks	u/k	u/k	ns	x
	(Raji *et al.*, 1998)	NBMI (≥20) LBMI (<18) NBMI (≥20) LBMI (<18) NBMI (≥20)	Humans	Mixed	8 -14 y 20-35 y ≥55 y	u/k	u/k	20	Lymphocytes	Unscheduled DNA synthesis	10.3 ± 5.3 9.2 ± 3.4 15.7 ± 8.8 22.0 ± 15.4 12.1 ± 9.2	Mean femtomoles of [3H] thymidine incorporated per mg DNA ± SD	ns * *	▲

		LBMI (<18)									26.7 ± 12.6		
		NBMI (≥20)			8-14 y					DNA-polymerase β activity	546 ± 227	Mean picomoles [3H] TMP incorporated per milligram of protein per hour ± SD	ns
		LBMI (<18)									659 ± 259		
		NBMI (≥20)			20-35 y						244 ± 91		*
		LBMI (<18)									292 ± 53		
		NBMI (≥20)			≥55 y						202 ± 96		ns
		LBMI (<18)									232 ± 61		
		NBMI (≥20)			8-14 y					Activities of UV DNase	249 ± 53	Mean mg of DNA-P liberated per milligram DNA ± SD	*
		LBMI (<18)									312 ± 61		
		NBMI (≥20)			20-35 y						190 ± 67		*
		LBMI (<18)									292 ± 136		
		NBMI (≥20)			≥55 y						148 ± 67		*
		LBMI (<18)									222 ± 68		

		NBMI (≥20)			8-14 y					Activities AP DNase	390 ± 97	Mean mg of DNA-P liberated per milligram DNA ± SD	ns	
		LBMI (<18)									406 ± 81			
		NBMI (≥20)			20-35 y						268 ± 97		ns	
		LBMI (<18)									304 ± 129			
		NBMI (≥20)			≥55 y						156 ± 70		*	
		LBMI (<18)									269 ± 73			
Interleukin-6	(Apte *et al.*, 2002)	AL	Spague-Dawley rats	Male	u/k	u/k	21 d	4	Plasma	-	≈10	Mean pg/ml ± SE	*	▲
		DR (35%)									≈40			
	(Bosutti *et al.*, 2008)	AL	Humans	Male	24 y	24 y 14 d	14 d	4-5	Plasma	-	11.4 ± 3.4	Mean pg/ml ± SE	*	▼
		DR (^{E} 20%)									1.7 ± 0.3			
	(Komatsu *et al.*, 2011)	AL	Wister rats	Male	1 m 2 w	6 m	4.5 m	4-8	Serum	-	≈u/k	Mean ng/ml ± SE	ns	x
		DR (30%)												
	(Lane *et al.*, 1995)	AL	Rhesus monkeys	Male	1.5-2 y	6.5-7 y	4.5-7 y	5	Plasma	-	^{E} 4.75	Mean pmol/l ± SE	*	▼
		DR (30%)						6			^{E} 3.24			
		AL			3-5 y	8.5-10 y		5			^{E} 4.75		*	
		DR (30%)						6			^{E} 2			
	(Messier *et al.*, 2012)	E	Humans	u/k	65.6 y	65.6 y 18 m	18 m	u/k	Serum	-	^{E} u/k	Mean pg/ml ± SE	*	▼
		D									^{E} u/k			

	(Piccio *et al.*, 2008)	AL	C57BL/6 mice	Female	5 w	10 w	5 w	9	Plasma	-	^{E} 10	Mean pg/ml ± SE	*	▼
		DR (40%)						5			^{E} 5			
	(Redman *et al.*, 2010)	AL	Humans	u/k	25-50 y	24 y 24 w -50 y 24 w	24 w	u/k	Serum	-	u/k	u/k	ns	X
		DR (25%)												
	(Sherman *et al.*, 2011)	AL	Mice	u/k	u/k	u/k	4 m	u/k	Serum	-	u/k	u/k	*	▼
		DR (u/k %)												
	(Spaulding *et al.*, 1997),	AL	Mice (C3B1 0RF1)	Mixed	28 d	28-36 m	27-35 m	30-19	Serum	-	^{E} 400	Mean pg/ml ± SE	*	▼
		DR (50%)									^{E} 100			
	(Tam *et al.*, 2012)	AL	Humans	Mixed	25-50 y	25 y 24 w -50 y 24 w	24 w	11	Serum	-	145.9 ± 59.5	Mean pg/ml ± SE	ns	X
		DR (25%)						12			66.5 ± 20.2			
	(Volk *et al.*, 1994)	AL	B6 Mice	Male	12 m	18 m	6 m	30	Serum	-	^{E} 350	Mean pg/ml	u/k	u/k
		DR (25%)						35			^{E} 250			
		AL				25 m	13 m	15			^{E} 500			
		DR (25%)						13			^{E} 400			
	(Wang *et al.*, 2007)	AL	Mice	Male	5 m	7 m	u/k	u	Plasma	-	168.34 ±17.76	Mean pg/ml ± SE	*	▼
		DR (30%)									139.71 ± 8.12			
Tumour necrosis factor-alpha	(Avula and Fernandes, 2002)	AL	C57BL/6J mice	Female	6 w	6 m 2 w	5 m	5	CD4 Lymphocytes	-	^{E} 18	Mean % stained ± SEM	*	▼
		CR (40%)									^{E} 9			
		AL				19 m 2 w	18 m				^{E} 22		*	
		CR (40%)									^{E} 10			
		AL				6 m 2 w	5 m		CD8 Lymphocytes		^{E} 10		*	
		CR (40%)									^{E} 3			

		AL				19 m 2 w	18m				^{E} 16		*	
		CR (40%)									^{E} 11			
	(Chiba and Ezaki, 2010)	AL	SHRSP /lzm rats	Male	10 w	12 w	2 w	u/k	Plasma	-	^{E} 0.9	Mean pg/ml ± SE	ns	x
		CR (50%)									^{E} 0.3			
	(Pali and Paszthy, 2008)	Control	Humans	Mixed	u/k	u/k	u/k	10	Macrophages	-	u/k	u/k	ns	x
		Anorexia nervosa						11						
		Obese						12						
	(Paszthy *et al.*, 2007)	Control	Humans	Mixed	u/k	u/k	u/k	19	Monocytes	-	U/K	U/K	ns	x
		Anorexia nervosa						21						
	(Phillips and Leeuwenburgh, 2005)	AL	Fischer 344 rats	u/k	3.5 m	26 m	22.5 m	8	Plasma	-	^{E} 20	Mean pg/ml ± SE	*	▼
		CR (40%)									^{E} 7.5			
	(Redman *et al.*, 2010)	AL	Humans	u/k	25-50 y	24 y 24 w -50 y 24 w	24 w	u/k	Serum	-	u/k	u/k	ns	x
		DR (25%)												
	(Sherman *et al.*, 2011)	AL	Mice	u/k	u/k	u/k	4 m	u/k	Serum	-	u/k	u/k	*	▼
		DR (U/K %)												
	(Spaulding *et al.*, 1997)	AL	Mice (C3B1 0RF1)	Mixed	28 d	28-36 m	27-35 m	30-19	Serum	-	^{E} 350	Mean pg/ml ± SE	*	▼
		DR (50%)									^{E} 30			
	(Tam *et al.*, 2012)	AL	Humans	Mixed	25-50 y	25 y 24 w -50 y	24 w	11	Serum	-	8.0 ± 1.4	Mean pg/ml ± SE	ns	x

		DR (25%)				24 w		12			8.5 ± 2.0			
Lymphoid / Myeloid ratio	(Weindruch and Suffin, 1980)	AL	$B10C3F_1$ mice	Female	21-24 d	6 m	7m	8-9	Thymus cortex	-	2.2 ± 0.15	Mean ratio ± SE	*	▲
		DR (^{E} 30%)									5.6 ± 0.73			
		DR (^{E} 40%)									3.9 ± 0.15			
		AL							Thymus medulla		0.8 ± 0.04		*	
		DR (^{E} 30%)									1.0 ± 0.04			
		DR (^{E} 40%)									1.2 ± 0.05			

Table 6.1. Structured literature search results identifying studies that have investigated the effects of dietary restriction on potential oxidative stress and cellular senescence-related BoA in leukocytes. Full details of the search terms are shown in Appendix B. (AL: Ad libitum, CR: Calorie restricted, PR: Protein restricted, CPR: Calorie and protein restricted, NBMI: Normal body mass index, LBMI: Low body mass index, DR: Dietary restricted, E: Exercise only control, D: Intensive dietary restriction, d: days, w: weeks, m: months, y: years, n: number of participants, PMNs: Polymorphonuclear cells, u/k: Unknown, n/a: No study identified, SEM: Standard error of the mean, SD: Standard deviation of the mean, ^{E}: Estimate provided within literature or if data displayed on chart, *: Statistical significant difference ($p<0.05$), ns: No significant difference, ▲: Increases, **x**: No effect, ▼: Decreases)

remaining study by Ueno *et al* in 2005 investigated ROS production only in circulating myeloid cells; however, found no difference in basal ROS production in AL mice compared to mice with 40% and 60% calorie restriction (Ueno *et al.*, 2005). They did however find that ROS production was significantly enhanced in AL mice compared to dietary restricted mice after 60 minutes of gut ischemic reperfusion, with or without phorbal myristate acetate stimulation. The evidence of an effect of dietary restriction on ROS production and mitochondrial dysfunction therefore only comes from splenic lymphocytes. The effects in peripheral blood cells should be shown since this tissue will be used in human studies.

There is also only a handful of studies investigating the effects of dietary restriction on other potential oxidative stress and cellular senescence-related markers in leukocytes that were described in Chapter 1. Section 1.3.8. and Figure 1.10., for which inconsistencies are also shown (Table 6.1). Some studies have shown that dietary restriction decreases leukocyte DNA damage (Hofer *et al.*, 2008), increases leukocyte DNA repair (Raji *et al.*, 1998) and reduces inflammatory cytokines (Lane *et al.*, 1995; Spaulding *et al.*, 1997; Avula and Fernandes, 2002; Phillips and Leeuwenburgh, 2005; Wang *et al.*, 2007; Bosutti *et al.*, 2008; Piccio *et al.*, 2008; Sherman *et al.*, 2011; Messier *et al.*, 2012); however, other studies contest this and even show opposing effects including increased leukocyte DNA damage (Ribeiro *et al.*, 2004) and increased inflammatory cytokines (Apte *et al.*, 2002). Interestingly, the latter studies were of short term dietary restriction. One hypothesis of the anti-ageing action of dietary restriction is its hormesis effect, first described by Masoro in 1998, defined as the beneficial actions resulting from the response of an organism to a low intensity stressor, in this case dietary restriction (Masoro, 1998). It is though that dietary restriction initially puts the organism under a low intensity stress and this some how prepares the organism to cope with further/more intense stressors (Masoro, 2000). This mechanism is not fully understood but could be through the up-regulation of stress response genes.

One other interesting aspect of dietary restriction is whether it plays a role in improving immune function and if this is associated with a decline in ROS production and improved mitochondrial function in leukocytes. It is know that the number of circulating lymphocytes decreases with age and thus blood becomes increasingly biased towards myeloid cells, which is thought to reflect immune dysfunction (Rothstein, 1993). This has been attributed to a shift in the clonal composition of hematopoietic

stem cells (Cho *et al.*, 2008). Haematopoiesis and thus immune function can therefore be determined by calculating the ratio of lymphoid or lymphoid-derived cells to myeloid or myeloid-derived cells. The structured literature review revealed an early and only study to show that the ratio of volume fractions of thymus cortical and medullary lymphoid cells/cortical and medullary non-lymphoid cells increases in dietary restricted mice (Weindruch and Suffin, 1980). This effect of dietary restriction however has not been shown in peripheral or bone marrow cells. It would be interesting to determine if ROS production and mitochondrial dysfunction in leukocytes are associated with an increase in myeloid bias and thus immune dysfunction with age and that this can be ameliorated by dietary restriction.

This study aimed to investigate the effects of dietary restriction on ROS production and mitochondrial function in peripheral blood and bone marrow of mice to provide further evidence for their validity as candidate BoA and also determine if these markers are associated with the age-associated functional decline in haematopoiesis and thus immune function by investigating peripheral blood and bone marrow cell subpopulation compositions.

6.3. Results

6.3.1. *Comparison of superoxide levels, mitochondrial mass, mitochondrial membrane potential and lymphoid/myeloid derived cell ratio in peripheral blood and bone marrow cells of mice*

This study first set out to measure ROS production and mitochondrial function in peripheral blood from AL and DR mice since this was the tissue that would be used in human studies. However, it was very difficult to get enough PBMCs from the peripheral blood of one mouse sample since these were often of low volume where less than 400µl proved difficult to obtain the number of cells required ($1x10^5$) (Table 6.2.). It was then decided that individual blood samples of low volume would be combined in order to continue the study in this tissue. Combined blood sample were always of the same diet group, age, gender and where possible similar visible pathology (Table 6.2). The latter however was not always possible since the mice displayed varying pathology which may confound future analysis. Another issue was that this then reduced the number of

Mouse sample	Mouse group	Gender	Number of mice	Age at analysis (Months)	Mouse no	Individual Blood volumes (µl)	Tumour	Visible pathology noted
1	AL	Male	4	24.42	1	200	Yes	Tumour on left lung
					2	200	No	Polyp on small intestine
					3	200	No	Polyp on small intestine and abnormally formed testes
					4	200	No	Polyp on small intestine
2	AL	Male	1	24.42	1	400	No	Enlarged seminal tubules and necrosis on liver
3	AL	Male	3	24.92	1	250	No	None
					2	250	No	Two polyps on small intestine
					3	500	Yes	Lymphoma including thymus
4	AL	Male	1	24.92	1	500	Yes	Tumours on pancreas, kidney, lung and thymus
5	AL	Male	1	24.92	1	500	No	None
6	DR	Female	3	24.16	1	200	No	Two polyps and white nodules on spleen
					2	100	No	Two polyps on small intestine
					3	100	No	Polyp on small intestine
7	DR	Female	2	24.16	1	100	No	None
					2	300	No	Four polyps on small intestine
8	DR	Female	2	24.16	1	250	Yes	Polyp on small intestine and ovarian tumour
					2	100	No	Two polyps and white nodules on spleen
9*	DR	Male	2	24.88	1	250	Yes	Pancreatic tumour and enlarged spleen
					2	350	No	Thin, prolapse and enlarge spleen
10	DR	Male	1	24.88	1	600	No	None
11	DR	Male	1	24.88	1	500	No	None

Table 6.2. Details of mice samples for which peripheral blood was derived (*:An error occurred for sample 9 in MitoTracker Green staining hence why there is one less mouse for the peripheral blood 25 month DR group for mitochondrial mass analysis)

samples available for analysis and thus would reduced the power of obtaining statistical significance if this was the case. It was therefore decided that the study should continue using bone marrow samples since plenty of cells could be obtained for the analysis from one mouse sample, avoiding the issues of combining samples. Details of the peripheral blood and bone marrow samples obtained are shown in Table 6.2. and Table 6.3. respectively, with a summary of all samples in Table 6.4.

The comparison between the flow cytometry graphs in peripheral blood and bone marrow of mice, and thus quantification superoxide levels, mitochondrial mass and mitochondrial membrane potential is shown in Figure 6.1. Bone marrow cells were slightly larger and more granular than peripheral blood cells; however the myeloid derived cells could clearly be distinguished from the lymphoid derived cells in peripheral blood and bone marrow based on their larger size and granularity, as in the human peripheral blood samples (See Chapter 2. Figure 2.2.). The same standardised quantification of superoxide levels, mitochondrial mass and mitochondrial membrane potential in cell subpopulations could therefore be used for both human and mice samples.

Normality tests and histograms revealed that superoxide levels, mitochondrial mass and mitochondrial membrane potential in both peripheral blood and bone marrow of mice followed a non-normal distribution (Table 6.5.). Non-parametric statistical tests were therefore used in future analysis.

Using 25 month old AL mice samples only, so they were comparable, superoxide levels were significantly lower in peripheral blood compared to bone marrow cells in all cell subpopulations ($p<0.01$) whereas mitochondrial mass was higher (significant in lymphoid and myeloid derived cell subpopulations only ($p\leq0.02$)) (Figure 6.2.). Mitochondrial membrane potential in peripheral blood was not significantly different compared to bone marrow cells in the combined and lymphoid-derived cell subpopulations ($p\geq0.34$), however was higher compared to bone marrow cells in the myeloid-derived subpopulation ($p=0.01$) (Figure 6.2.). There was a tendency for bone marrow to be more lymphoid derived cell biased compared to peripheral blood, however this did not quite reach statistical significance ($p=0.05$) (Figure 6.2.).

Mouse sample	Mouse group	Gender	Number of mice	Age at analysis (Months)	Mouse no	Individual Blood volumes (µl)	Tumour	Visible pathology noted
12*	AL	Female	1	2.30	-	-	No	None
13	AL	Female	1	2.86	-	-	No	None
14	AL	Female	1	2.86	-	-	No	None
15	AL	Male	1	2.14	-	-	No	None
16	AL	Male	1	2.14	-	-	No	None
17*	AL	Male	1	2.30	-	-	No	None
18	AL	Female	1	24.95	-	-	Yes	Lymphoma
19	AL	Female	1	24.95	-	-	Yes	Tumour on liver, spleen, pancreas and enlarged thymus
20	AL	Female	1	24.95	-	-	No	None
21	AL	Female	1	25.12	-	-	No	None
22	AL	Female	1	25.12	-	-	Yes	Tumour on pancreas and small intestine
23	AL	Female	1	25.12	-	-	Yes	Tumour on ovary, adrenal gland and blood clot on brain
24	AL	Male	1	25.35	-	-	No	None
25	AL	Male	1	25.35	-	-	No	Blood in lungs
26	AL	Male	1	25.35	-	-	Yes	Tumour on thymus and spleen
27	AL	Male	1	25.38	-	-	No	Enlarged seminal vesicles
28	AL	Male	1	25.38	-	-	Yes	Tumour on pancreas, cysts on liver, enlarged spleen and large seminal vesicles
29	AL	Male	1	25.38	-	-	No	Large seminal vesicles
30	DR	Female	1	24.95	-	-	No	None
31	DR	Female	1	24.95	-	-	Yes	Lymphoma
32	DR	Female	1	24.95	-	-	No	None

33	DR	Female	1	25.12	-	-	No	Enlarged right ovary
34	DR	Female	1	25.12	-	-	No	Enlarged right ovary
35	DR	Female	1	25.12	-	-	Yes	Tumour on ovary
36	DR	Male	1	25.35	-	-	No	None
37	DR	Male	1	25.35	-	-	No	None
38	DR	Male	1	25.35	-	-	No	None
39	DR	Male	1	25.38	-	-	No	None
40	DR	Male	1	25.38	-	-	No	Prolapse
41	AL	Female	1	31.82	-	-	Yes	Tumour on kidney
42	AL	Female	1	31.82	-	-	No	Cyst on ovary
43	AL	Female	1	31.82	-	-	Yes	Tumour on liver and lung
44	AL	Female	1	31.82	-	-	No	Cyst on ovary and enlarged spleen
45	AL	Female	1	31.82	-	-	Yes	Tumour on liver and lung
46	AL	Male	1	32.05	-	-	Yes	Tumour on lung and enlarged seminal vesicles
47	AL	Male	1	32.05	-	-	No	Enlarged seminal vesicles
48	AL	Male	1	32.05	-	-	Yes	Tumour on spleen and lung
49	AL	Male	1	32.05	-	-	Yes	Tumour on spleen
50	AL	Male	1	32.05	-	-	Yes	Tumour on liver and lung
51	DR	Female	1	32.08	-	-	Yes	Tumour on spleen and cysts on ovary
52	DR	Female	1	32.08	-	-	No	Cysts on ovary
53	DR	Female	1	32.08	-	-	Yes	Tumour on lung
54	DR	Female	1	32.08	-	-	Yes	Tumour on lung and fibrotic liver
55	DR	Female	1	32.08	-	-	No	None
56	DR	Male	1	32.22	-	-	Yes	Tumour on spleen, kidney and lung

57	DR	Male	1	32.22	-	-	Yes	Tumour on pancreas
58	DR	Male	1	32.22	-	-	No	None
59	DR	Male	1	32.22	-	-	No	None
60	DR	Male	1	32.22	-	-	No	None

Table 6.3. Details of mice samples for which bone marrow was derived. (*:An error occurred for samples 12 and 17 in MitoTracker Green staining hence why there are two less mice for the bone marrow 2 month AL group for mitochondrial mass analysis)

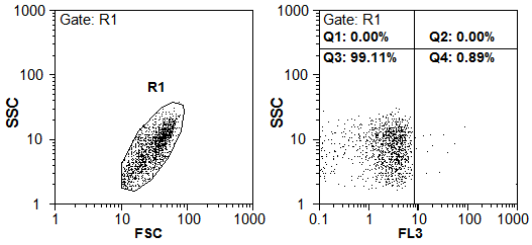
	Peripheral blood		Bone marrow				
Age group	25 Months		2 Months	25 Months		32 Months	
Diet	AL	DR	AL	AL	DR	AL	DR
Total, n	5	6*	6*	12	11	10	10
Male, n (%)	5 (100)	3 (50)	3 (50)	6 (50)	5 (45)	5 (50)	5 (50)
Tumour present, n (%)	3 (60)	2 (33)	0 (0)	6 (50)	2 (18.18)	7 (70)	5 (50)

Table 6.4. Summary of mice samples (*: An error occurred for samples 9, 12 and 17 in MitoTracker Green staining hence why there is one less mouse for the 25 month DR peripheral blood group and two less mice for the 2 month AL bone marrow group for mitochondrial mass analysis)

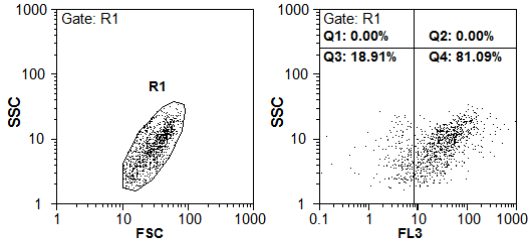
A.

i. Peripheral blood

Unstained

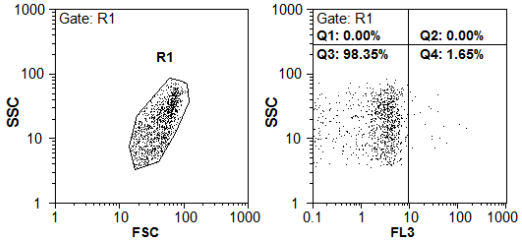


DHE stained

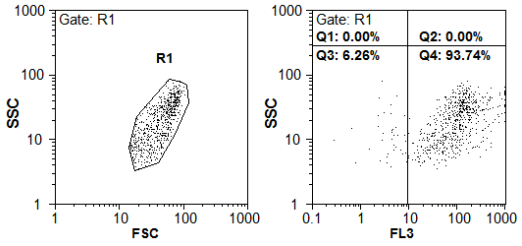


ii. Bone marrow

Unstained



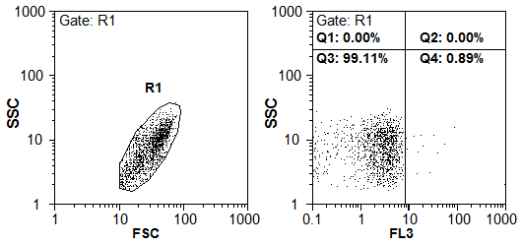
DHE stained



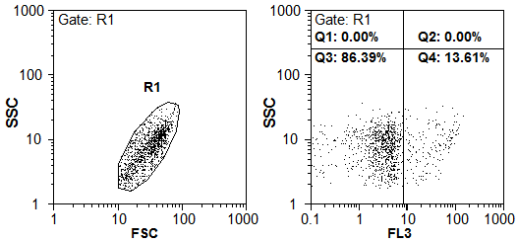
B.

i. Peripheral blood

Unstained

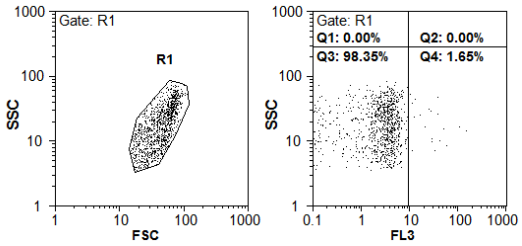


MitoTracker Green FM stained

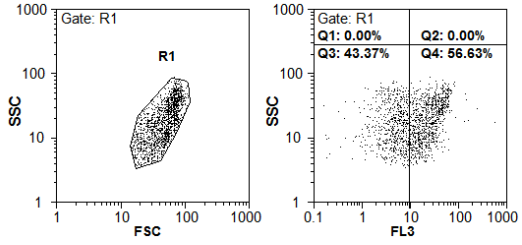


ii. Bone marrow

Unstained



MitoTracker Green FM stained



C.

i. Peripheral blood **ii. Bone marrow**

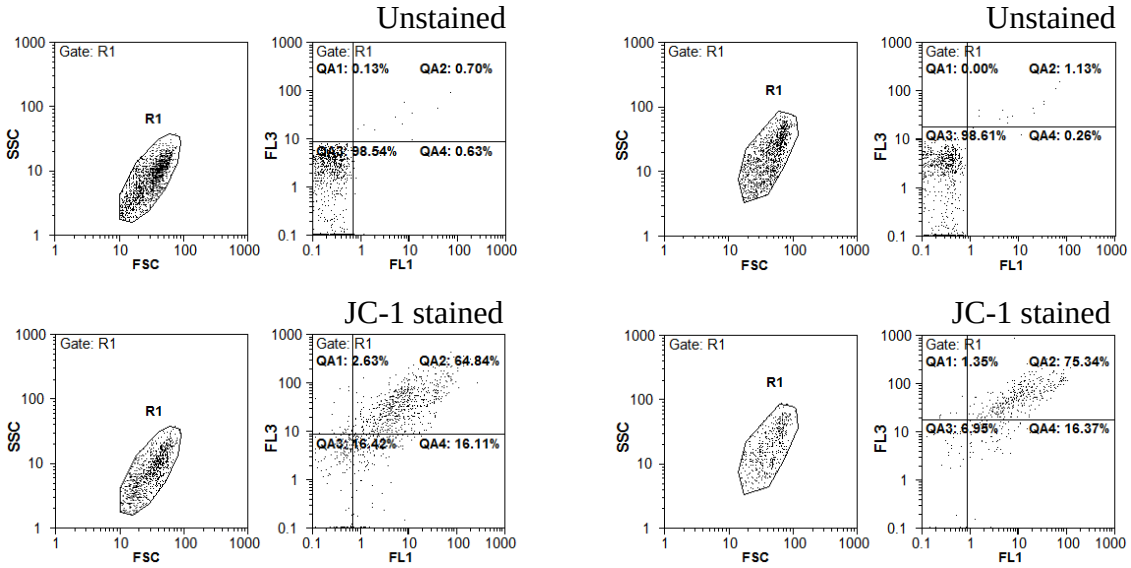


Figure 6.1. Comparison of mouse peripheral blood and bone marrow flow cytometry graphs for quantifying A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential. Representative samples of AL mice aged 25 months are shown.

A. Peripheral blood	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	n	p	Statistic	n	p
Superoxide levels (AU)						
PBMCs	0.19	11	0.20* #	0.91	11	0.27
Lymphocytes	0.27	11	**0.03**	0.80	11	**0.01**
Monocytes	0.20	11	0.20* #	0.91	11	0.22
Mitochondrial mass (AU)						
PBMCs	0.20	10	0.20* #	0.83	10	**0.03**
Lymphocytes	0.20	10	0.20* #	0.94	10	0.58
Monocytes	0.22	10	0.20* #	0.82	10	**0.03**
Mitochondrial membrane potential (AU)						
PBMCs	0.24	11	0.09 #	0.94	11	0.47
Lymphocytes	0.17	11	0.20* #	0.94	11	0.49
Monocytes	0.17	11	0.20* #	0.91	11	0.26
Lymphocyte/monocyte ratio	0.19	11	0.20* #	0.94	11	0.56

B. Bone marrow	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	n	p	Statistic	n	p
Superoxide levels (AU)						
All bone marrow	0.14	49	**0.02**	0.93	49	**0.01**
Lymphoid cells	0.12	49	0.10 #	0.96	49	0.07
Myeloid cells	0.16	49	**0.00**	0.92	49	**0.00**
Mitochondrial mass (AU)						
All bone marrow	0.17	47	**0.00**	0.89	47	**0.00**
Lymphoid cells	0.18	47	**0.00**	0.88	47	**0.00**
Myeloid cells	0.14	47	**0.03**	0.91	47	**0.00**
Mitochondrial membrane potential (AU)						
All bone marrow	0.12	49	0.08 #	0.88	49	**0.00**
Lymphoid cells	0.11	49	0.15 #	0.94	49	**0.01**
Myeloid cells	0.07	49	0.20*	0.99	49	0.90
Lymphoid/myeloid ratio	0.90	49	0.20* #	0.97	49	0.24

Table 6.5. Normality tests of superoxide levels, mitochondrial mass, mitochondrial membrane potential and lymphoid/myeloid derived cell ratio in A. peripheral blood and B. bone marrow of mice. All mice sample were included in this analysis. (**[a]**: Lilliefors Significance Correction, *****: Lower bound of the true significance, **#**: Histograms were positively skewed and therefore analysed as having a non-normal distribution using non-parametric statistical tests)

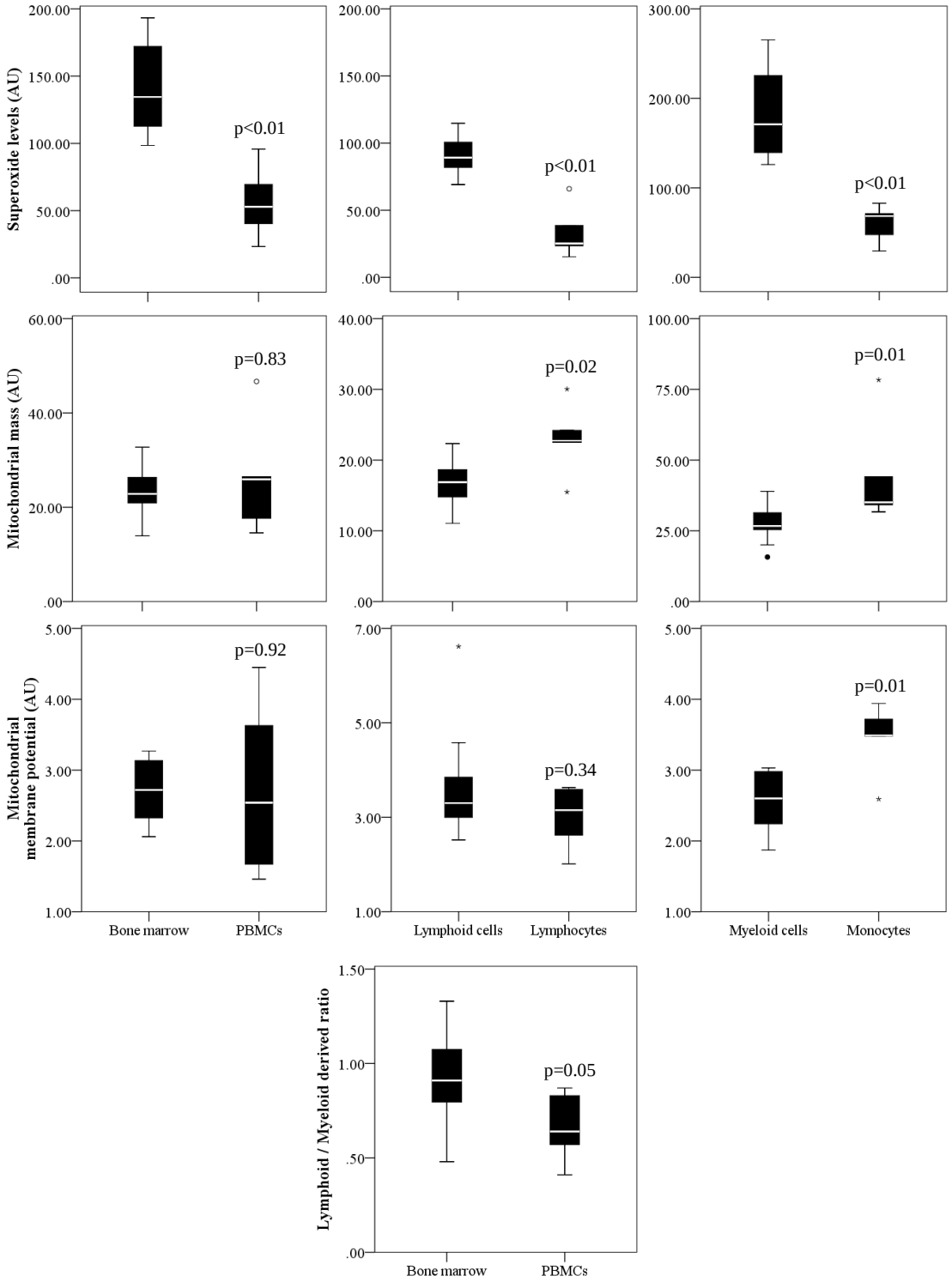


Figure 6.2. Comparison of superoxide levels, mitochondrial mass, mitochondrial membrane potential and lymphoid/myeloid derived cell ratio in peripheral blood and bone marrow of mice. AL mice aged 25 months were included in this analysis: peripheral blood (n=5) and bone marrow (n=12). Mann-Whitney U Test was used to test differences between medians.

6.3.2. *Agreements between superoxide levels, mitochondrial mass, mitochondrial membrane potential in lymphoid and myeloid derived cell subpopulations of mice*

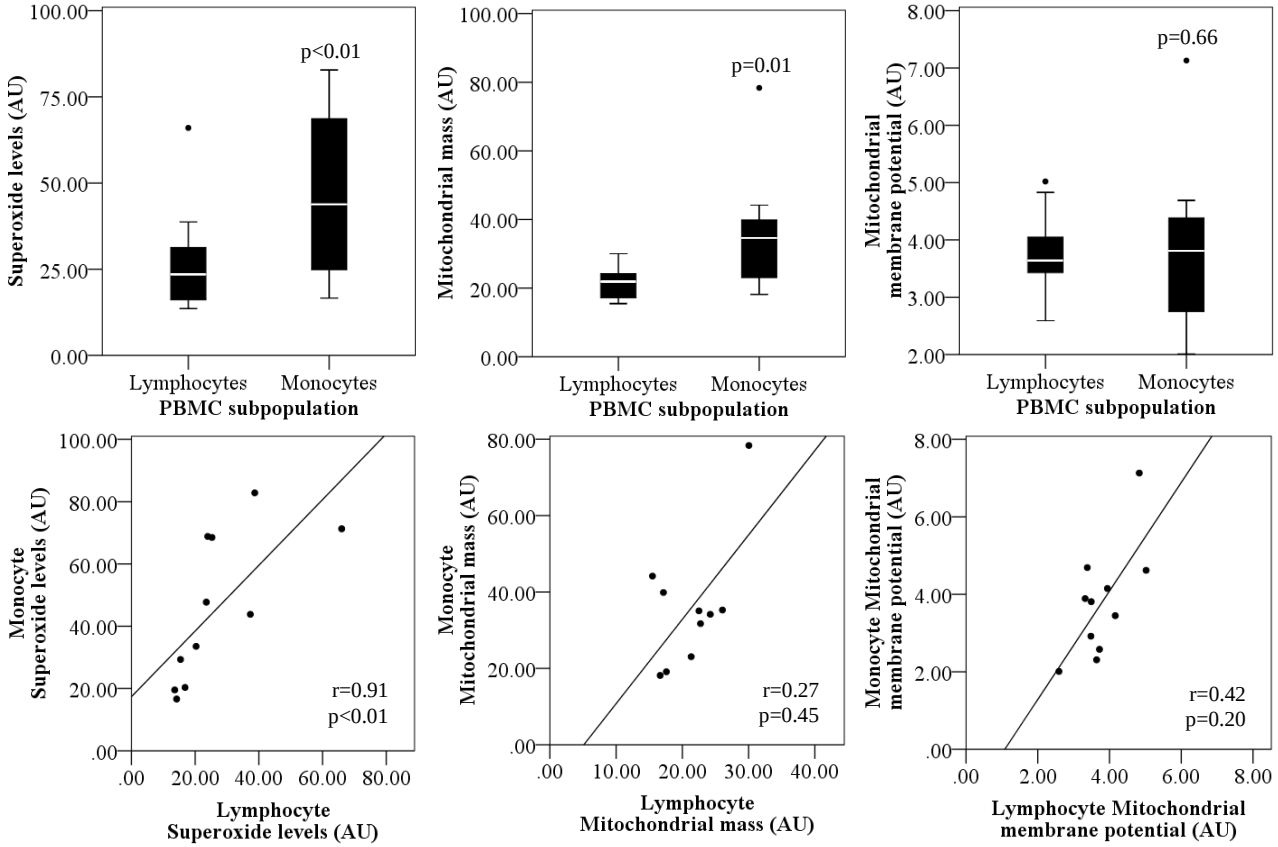
Superoxide levels and mitochondrial mass were significantly higher in myeloid derived cells compared to lymphoid derived cells in both peripheral blood and bone marrow of mice ($p \leq 0.01$) (Figure 6.3. A. and B. respectively). There was no significant difference in mitochondrial membrane potential in myeloid derived cells compared to lymphoid derived cells of peripheral blood ($p=0.66$), however mitochondrial membrane potential was significantly lower in myeloid derived cells compared to lymphoid derived cells of bone marrow ($p<0.01$) (Figure 6.3. A. and B. respectively). There was a significant positive correlation between lymphoid and myeloid derived cells for superoxide levels in both peripheral blood and bone marrow cells, and mitochondrial mass and membrane potential in bone marrow cells only ($r \geq 0.63$, $p \leq 0.01$) (Figure 6.3. A. and B. respectively). There was a trend for a positive correlation between lymphoid and myeloid derived cells for mitochondrial mass and mitochondrial membrane potential in peripheral blood but this did not reach statistical significance ($r \geq 0.27$, $p \geq 0.20$) (Figure 6.3. A.)

6.3.3. *Associations between ROS production, mitochondrial function and lymphoid/myeloid derived cell ratio in peripheral blood and bone marrow cells of mice*

There was no significant association between superoxide levels and mitochondrial mass in lymphocytes and monocytes of peripheral blood in 25 month old mice ($r \geq 0.19$, $p \geq 0.28$), however there was a negative association between superoxide levels and mitochondrial membrane potential which did not quite reach significance in lymphocytes ($r=-0.57$, $p=0.07$) however was significant in monocytes ($r=-0.62$, $p=0.04$) (Figure 6.4. A.). There was no significant association between mitochondrial mass and mitochondrial membrane potential in lymphocytes or monocytes of peripheral blood in 25 month old mice ($r=-0.21$, $p=0.56$) (Figure 6.4. A.).

There was no significant association between superoxide levels and mitochondrial mass or mitochondrial membrane potential in lymphoid or myeloid cells of bone marrow in 25 month old mice ($p \geq 0.06$) (Figure 6.4. B.).

A.


Superoxide levels (AU)
p<0.01
Lymphocytes
Monocytes
PBMC subpopulation
Mitochondrial mass (AU)
p=0.01
Lymphocytes
Monocytes
PBMC subpopulation
Mitochondrial membrane potential (AU)
p=0.66
Lymphocytes
Monocytes
PBMC subpopulation
Monocyte Superoxide levels (AU)
r=0.91
p<0.01
Lymphocyte Superoxide levels (AU)
Monocyte Mitochondrial mass (AU)
r=0.27
p=0.45
Lymphocyte Mitochondrial mass (AU)
Monocyte Mitochondrial membrane potential (AU)
r=0.42
p=0.20
Lymphocyte Mitochondrial membrane potential (AU)

B.

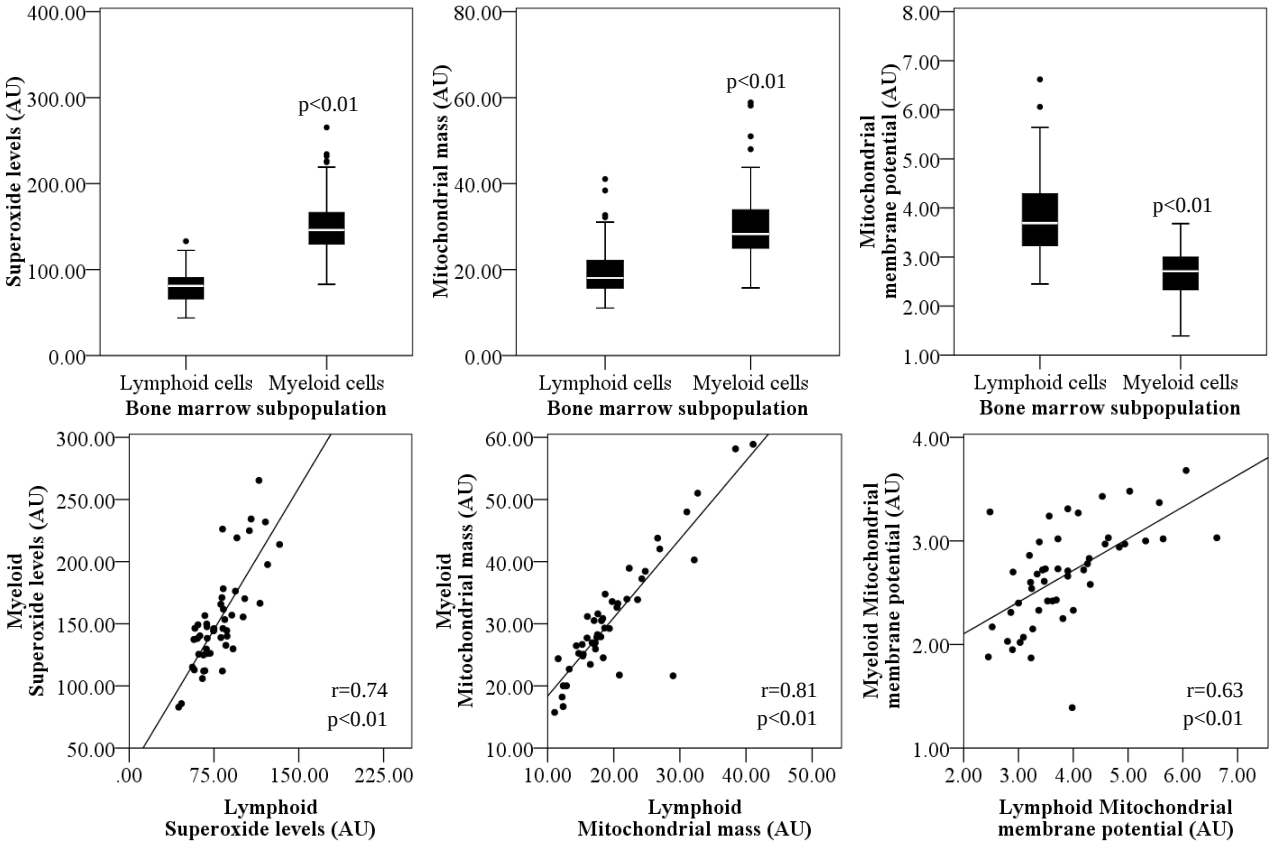
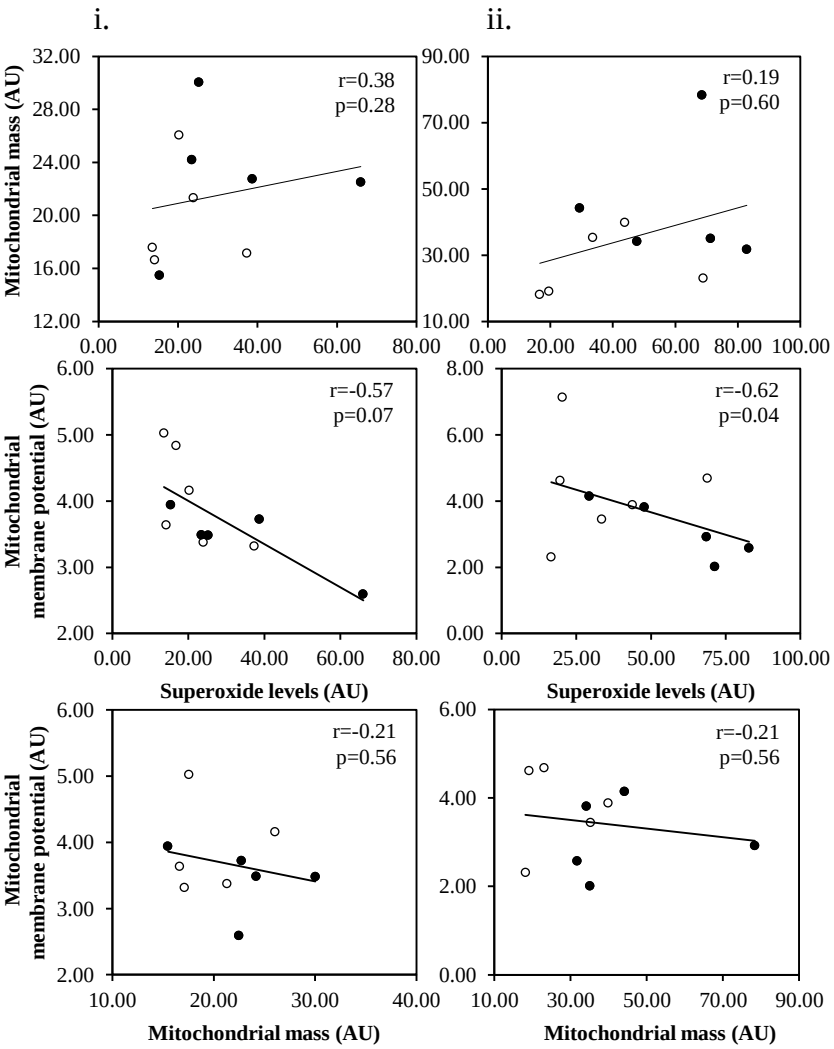


Figure 6.3. Comparing superoxide levels, mitochondrial mass and mitochondrial membrane potential in A. peripheral blood and B. bone marrow lymphoid and myeloid derived cell subpopulations of mice. All mice sample were included in this analysis (see table 6.3.).Wilcoxon Signed-Rank Test was used to test differences between medians and Spearman's correlation coefficient was used to test the strength of associations

A.


i.
ii.
Mitochondrial mass (AU)
r=0.38
p=0.28
r=0.19
p=0.60
Mitochondrial membrane potential (AU)
r=-0.57
p=0.07
r=-0.62
p=0.04
Superoxide levels (AU)
Superoxide levels (AU)
Mitochondrial membrane potential (AU)
r=-0.21
p=0.56
r=-0.21
p=0.56
Mitochondrial mass (AU)
Mitochondrial mass (AU)

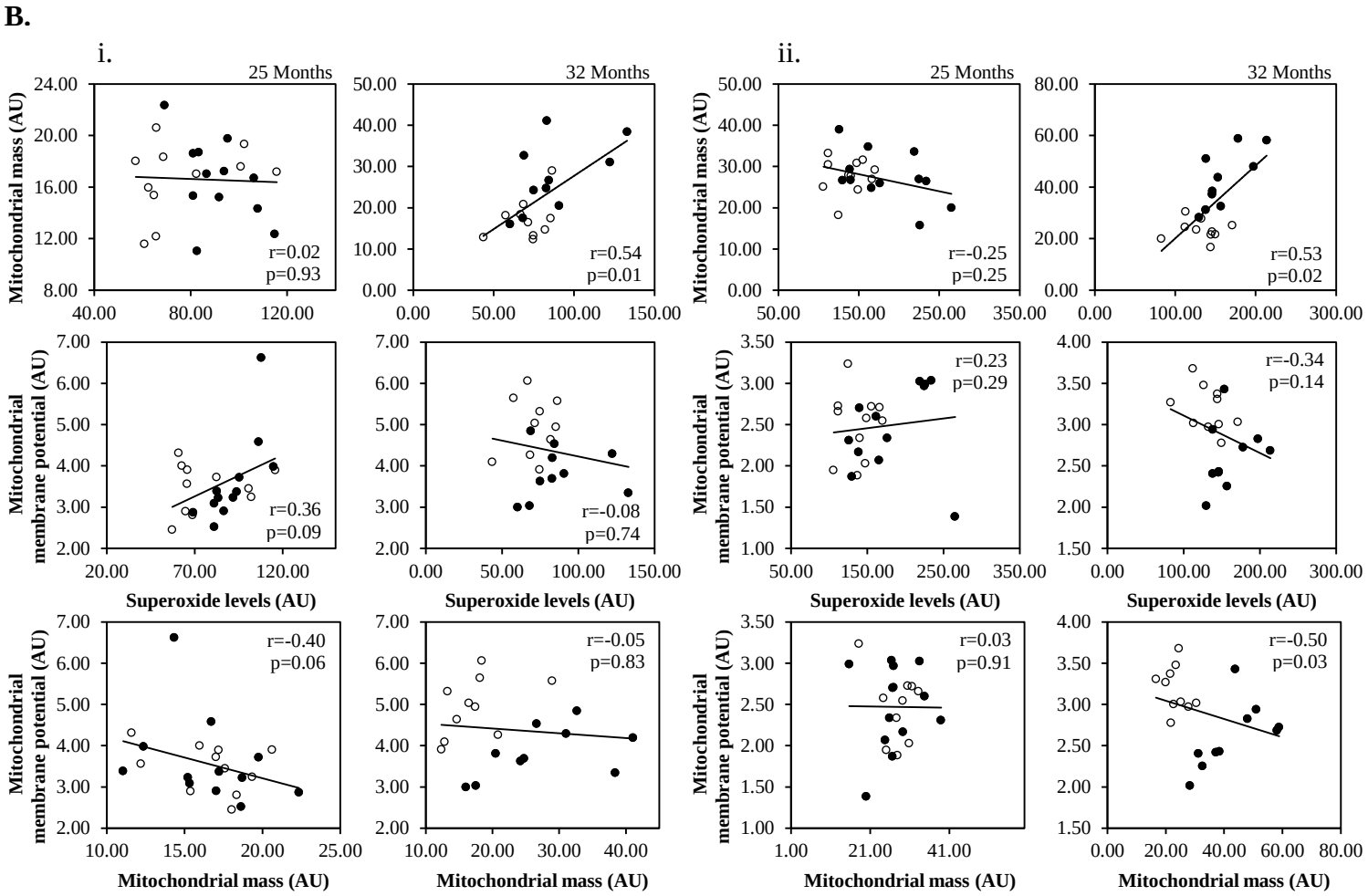


Figure 6.4. The association between superoxide levels, mitochondrial mass and mitochondrial membrane potential in A. peripheral blood and B. bone marrow of mice. i. Lymphoid-derived cells and ii. myeloid derived cells. All mice sample were included in this analysis (see table 6.3.). Spearman's correlation coefficient was used to test the strength of associations**.** (Black dots: AL, White dots: DR)

However, in bone marrow of 32 month old mice, there was a significant positive association between superoxide levels and mitochondrial mass in both lymphoid and myeloid cells ($r \geq 0.54$, $p \leq 0.02$), no significant association between superoxide levels and mitochondrial membrane potential in both lymphoid or myeloid cells ($r \leq -0.34$, $p \geq 0.14$), no significant association between mitochondrial mass and membrane potential in lymphoid cells ($r=-0.05$, $p=0.83$) however this was negatively significant in myeloid cells at 32 years of age ($r=-0.50$, $p=0.03$) (Figure 6.4. B.).

There was a significant negative association between superoxide levels and lymphoid/myeloid derived cell ratio in both peripheral blood and bone marrow at 25 and 32 months of age ($r \geq -0.53$, $p \leq 0.02$) (Figure 6.5. A. and B. respectively). There was also a trend for a negative association between mitochondrial mass and lymphoid/myeloid derived cell ratio which was significant in peripheral blood ($r=-0.72$, $p=0.01$) however was not significant in bone marrow at 25 or 32 months of age ($r=\leq -0.34$, $p \geq 0.14$) (Figure 6.5. A. and B. respectively). There was no association between mitochondrial membrane potential and lymphoid/myeloid derived cell ratio in peripheral blood and bone marrow at 25 months of age ($r \leq 0.49$, $p \geq 0.12$) however there was a significant positive association at 32 months of age in bone marrow cells ($r=0.50$, $p=0.03$) (Figure 6.5. A. and B. respectively).

6.3.4. *Association between superoxide levels, mitochondrial mass, mitochondrial membrane potential and lymphoid/myeloid derived cell ratio and potential confounders of mice*

There was no consistent associations between superoxide levels and gender by diet and age group however mitochondrial mass and mitochondrial membrane potential in bone marrow were significantly higher in female AL mice at 32 months of age compared to males in all subpopulations ($p \leq 0.02$), except mitochondrial membrane potential in lymphoid cells ($p=0.05$) (Table 6.6 A. B. and C. respectively). There was no significant association between lymphoid/myeloid ratio and gender (Table 6.6. D.). There was no significant association between superoxide levels, mitochondrial mass, mitochondrial membrane potential or lymphoid/myeloid ratio and tumour incidence (Table 6.7.).

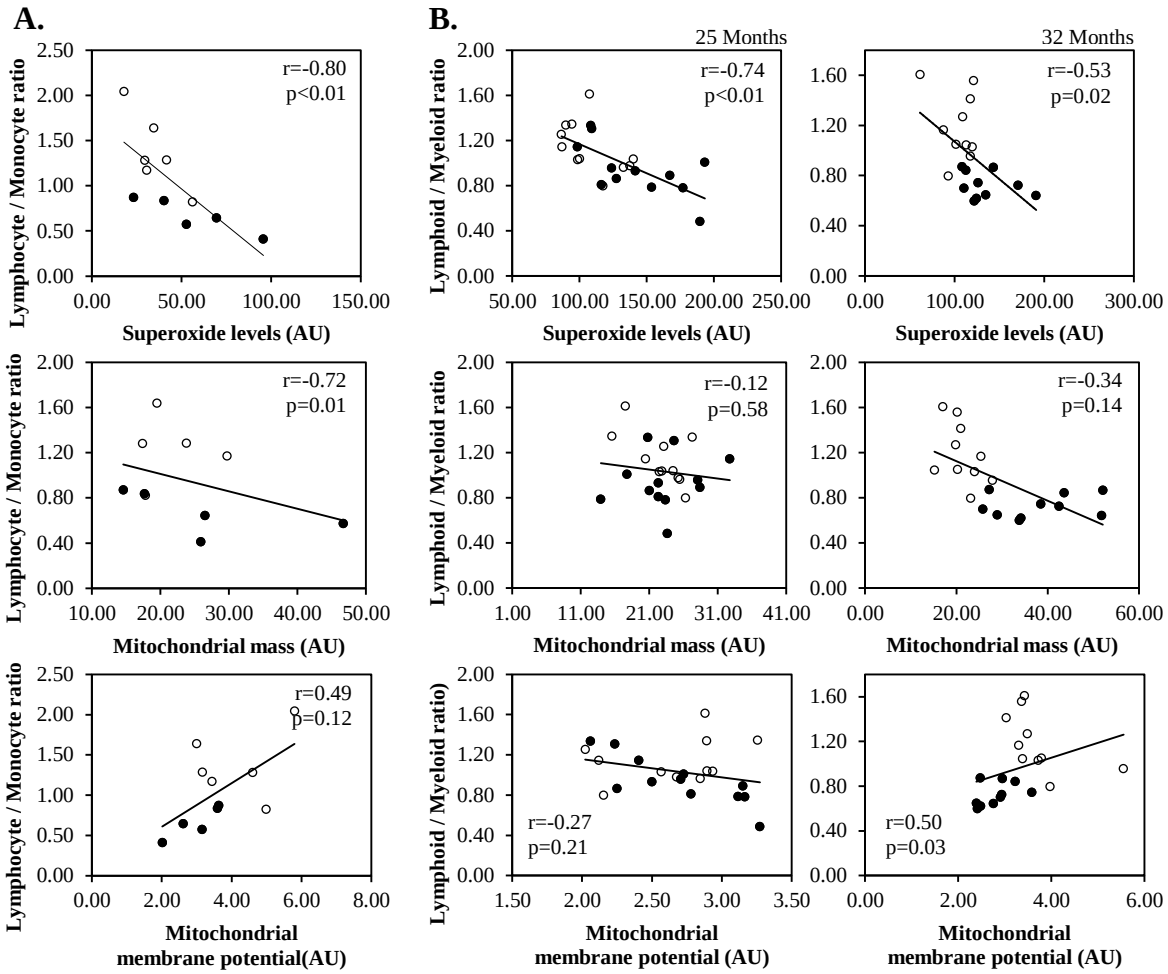


Figure 6.5. The association between superoxide levels, mitochondrial mass, and mitochondrial membrane potential and lymphoid/myeloid ratio in A. peripheral blood and B. bone marrow of mice. Data for combined supopulations is shown. All mice sample were included in this analysis (see table 6.3.). Spearman's correlation coefficient was used to test the strength of associations**.** (Black dots: AL, White dots: DR)

A. Superoxide levels (AU)													
Age	**Diet**	**Gender**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	Female	3	90.61	-	0.51	3	55.85	-	0.51	3	115.09	-	0.51
		Male	3	103.94	-		3	61.42	-		3	146.37	-	
25 Months	AL	Female	6	147.70	81.75	0.52	6	92.89	27.33	0.34	6	201.24	105.44	0.34
		Male	6	125.69	57.90		6	84.95	19.98		6	163.72	84.04	
	DR	Female	6	96.57	23.26	0.10	**6**	**63.66**	**6.48**	**0.01**	6	138.84	27.86	0.36
		Male	5	132.77	43.75		**5**	**100.74**	**34.81**		5	155.47	56.40	
32 Months	AL	Female	5	143.30	61.48	0.08	5	84.52	51.64	0.12	5	178.22	59.85	0.08
		Male	5	121.96	20.19		5	74.96	22.60		5	145.96	17.67	
	DR	Female	5	178.22	59.85	0.25	5	71.60	27.83	0.35	5	126.22	60.64	0.75
		Male	5	145.96	17.67		5	74.64	18.20		5	144.31	24.73	

B. Mitochondrial mass (AU)														
Age	**Diet**	**Gender**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	Female	2	32.84	-	1.00	2	25.28	-	1.00	2	37.96	-	1.00
		Male	2	32.83	-		2	27.08	-		2	37.12	-	
25 Months	AL	Female	6	21.56	7.11	0.11	6	14.78	5.55	0.05	6	26.20	8.38	0.05
		Male	6	25.71	7.49		6	17.87	4.04		6	30.27	9.56	
	DR	Female	6	21.45	6.88	0.07	6	15.67	6.08	0.10	6	26.41	5.80	0.07
		Male	5	25.21	2.70		5	17.59	2.87		5	30.51	4.34	
32 Months	AL	Female	5	**43.60**	**11.43**	**0.01**	5	**32.69**	**10.90**	**0.01**	5	**51.01**	**12.63**	**0.01**
		Male	5	**28.96**	**7.49**		5	**20.48**	**7.74**		5	**32.62**	**8.13**	
	DR	Female	5	20.22	4.37	0.35	5	14.69	4.24	0.17	5	23.46	6.51	0.46
		Male	5	23.13	7.84		5	18.40	10.02		5	21.75	7.02	

C. Mitochondrial membrane potential (AU)													
Age	**Diet**	**Gender**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	Female	3	2.63	–	0.05	3	3.47	–	0.28	3	2.42	–	0.05
		Male	3	2.94	–		3	3.20	–		3	2.86	–	
25 Months	AL	Female	6	2.62	0.97	0.63	6	3.38	1.59	0.42	6	2.25	1.25	0.52
		Male	6	2.75	0.79		6	3.16	1.05		6	2.65	0.73	
	DR	Female	6	2.36	0.88	0.14	6	3.23	1.37	0.58	6	2.18	0.81	0.10
		Male	5	2.89	0.15		5	3.72	0.56		5	2.71	0.13	
32 Months	AL	Female	**5**	**2.95**	**0.56**	**0.02**	5	4.29	0.92	0.05	**5**	**2.83**	**0.49**	**0.01**
		Male	**5**	**2.47**	**0.30**		5	3.62	0.74		**5**	**2.40**	**0.29**	
	DR	Female	5	2.83	0.49	0.47	5	5.03	1.12	0.92	5	3.03	0.37	0.92
		Male	5	2.40	0.29		5	4.94	1.74		5	3.31	0.65	

D. Lymphoid/myeloid cell ratio						
Age	**Diet**	**Gender**	**All bone marrow**			
			n	Median	IQR	p
2 Months	AL	Female	3	0.80	–	0.28
		Male	3	1.23	–	
25 Months	AL	Female	6	0.97	0.60	0.57
		Male	6	0.88	0.20	
	DR	Female	6	1.20	0.44	0.36
		Male	5	1.03	0.23	
32 Months	AL	Female	5	0.74	0.17	0.21
		Male	5	0.64	0.18	
	DR	Female	5	1.27	0.48	0.05
		Male	5	1.03	0.36	

Table 6.6. Association between A. superoxide levels , B. mitochondrial mass, C. mitochondrial membrane potential, D. lymphoid/myeloid cell ratio and gender by age and diet in all bone marrow and cell subpopulations of mice. Mann-Whitney U Test was used to test differences between medians.

A. Superoxide levels (AU)														
Age	**Diet**	**Tumour**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	No	6	103.4	-	-	6	59.76	-	-	6	135.94	-	-
		Yes	0	-	-		0	-	-		0	-	-	
25 Months	AL	No	6	125.69	42.87	0.42	6	84.95	10.50	0.34	6	163.72	83.42	0.52
		Yes	6	159.27	84.24		6	100.10	31.46		6	200.59	106.39	
	DR	No	9	100.07	36.72	0.64	9	65.84	29.90	0.48	9	140.32	40.41	0.48
		Yes	2	115.98	-		2	83.90	-		2	147.56	-	
32 Months	AL	No	3	134.84	-	0.91	3	83.14	-	0.91	3	156.94	.	0.91
		Yes	7	124.48	58.29		7	82.85	53.40		7	146.23	59.29	
	DR	No	5	117.61	20.50	0.46	5	74.90	22.87	0.35	5	144.49	25.17	0.35
		Yes	5	101.61	39.95		5	71.60	23.03		5	126.22	60.10	

B. Mitochondrial mass (AU)														
Age	**Diet**	**Tumour**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	No	4	32.84	8.52	-	c	25.28	8.48	-	4	37.12	7.69	-
		Yes	0	-	-		0	-	-		0	-	-	
25 Months	AL	No	6	21.66	9.06	0.42	6	16.18	4.79	0.87	6	26.70	11.33	0.87
		Yes	6	23.51	5.49		6	16.98	5.71		6	26.71	7.27	
	DR	No	9	23.10	4.41	0.48	9	17.19	2.52	1.00	9	27.90	5.21	0.35
		Yes	2	20.39	-		2	15.77	-		2	23.73	-	
32 Months	AL	No	3	28.96	-	0.57	3	20.48	-	0.73	c	20.48	-	0.73
		Yes	7	38.49	9.80		7	26.63	8.45		7	26.63	8.45	
	DR	No	5	23.98	6.21	0.08	5	18.14	9.56	0.12	5	22.70	7.47	0.46
		Yes	5	20.22	5.57		5	14.69	4.83		5	23.46	6.54	

C. Mitochondrial membrane potential (AU)													
Age	**Diet**	**Tumour**	**All bone marrow**				**Lymphoid cells**				**Myeloid cells**			
			n	Median	IQR	p	n	Median	IQR	p	n	Median	IQR	p
2 Months	AL	No	6	2.89	0.42	_	6	3.37	0.48	_	6	2.67	0.61	_
		Yes	0	-	-		0	-	-		0	-	-	
25 Months	AL	No	6	2.75	0.93	0.75	6	3.23	0.42	0.52	6	2.65	0.98	0.75
		Yes	6	2.62	0.84		6	3.68	2.32		6	2.32	1.01	
	DR	No	9	2.85	0.76	0.34	9	3.72	1.11	0.64	9	2.58	0.73	0.35
		Yes	2	2.97	-		2	3.40	-		2	2.89	-	
32 Months	AL	No	3	2.91	-	0.73	3	3.81	-	0.73	3	2.25	-	0.14
		Yes	7	2.76	0.75		7	3.69	1.19		7	2.68	0.52	
	DR	No	5	3.49	1.47	0.75	5	5.32	1.01	0.35	**5**	**3.00**	**0.32**	**0.05**
		Yes	5	3.42	0.51		5	4.64	1.55		**5**	**3.31**	**0.43**	

D. Lymphoid / Myeloid cell ratio						
Age	**Diet**	**Tumour**	**All bone marrow**			
			n	Median	IQR	p
2 Months	AL	No	6	0.94	0.54	-
		Yes	0	-	-	
25 Months	AL	No	6	0.88	0.24	0.81
		Yes	6	0.97	0.48	
	DR	No	9	1.04	0.30	0.91
		Yes	2	1.16	-	
32 Months	AL	No	3	0.70	-	0.82
		Yes	7	0.72	0.22	
	DR	No	5	1.16	0.35	0.75
		Yes	5	1.05	0.67	

Table 6.7. Association between A. superoxide levels, B. mitochondrial mass, C. mitochondrial membrane potential, D. lymphoid/myeloid cells ratio and tumour presence by age and diet in bone marrow of mice. Mann-Whitney U Test was used to test differences between medians.

6.3.5. *Effect of dietary restriction on ROS production and mitochondrial function in peripheral blood and bone marrow cells*

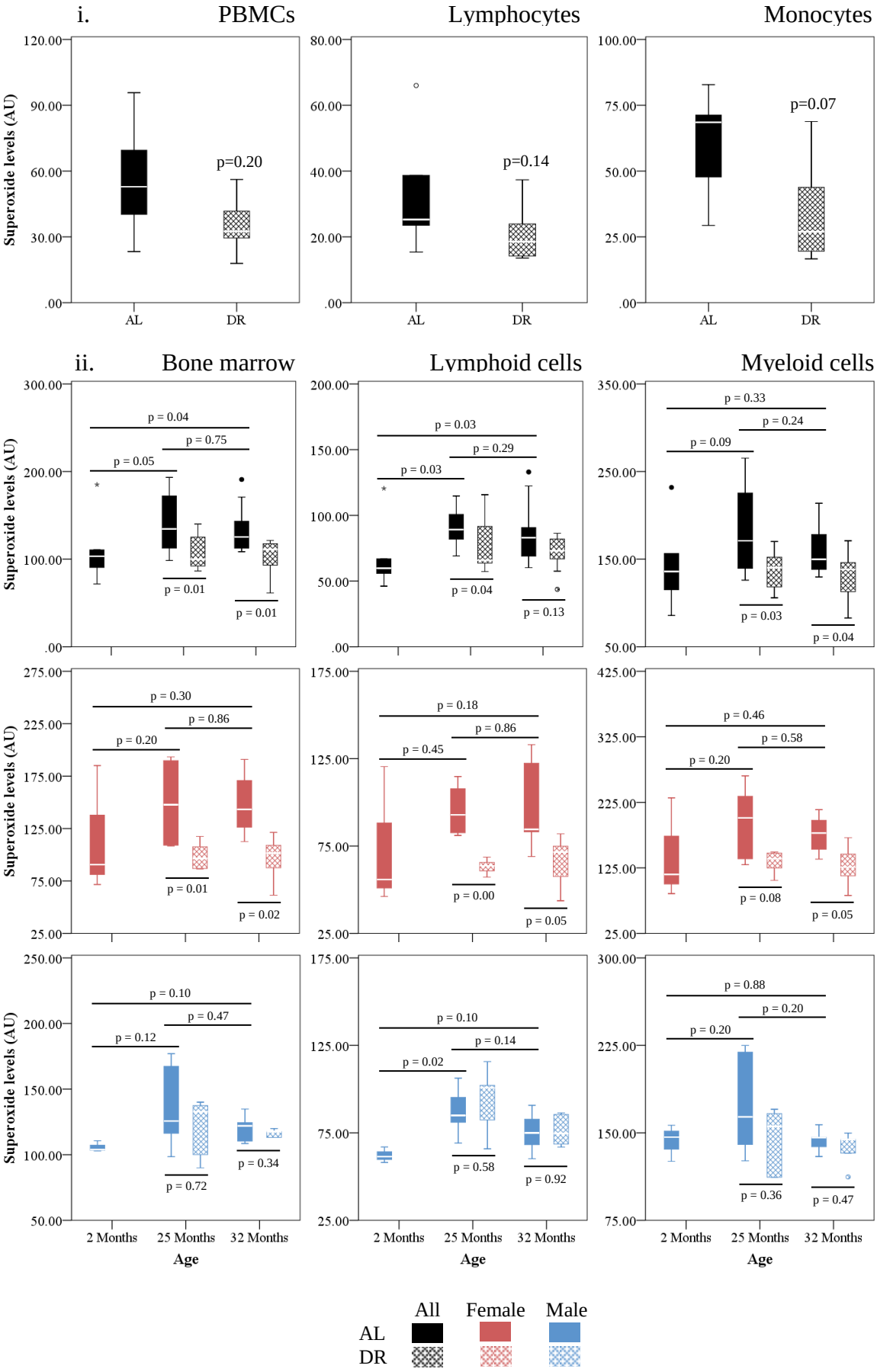
There was a tendency for superoxide levels and mitochondrial mass to be decreased and mitochondrial membrane potential to be increased in 25 month old DR mice compared to AL controls in all subpopulations of peripheral blood; however, none reached statistical significance ($p \geq 0.07$) (Figure 6.6 A. i. B. i. and C. i. respectively).

There was an increase in superoxide levels with age in bone marrow cells (significant in combined bone marrow and lymphoid cells ($p \leq 0.04$) however not in myeloid cells ($p \geq 0.09$)), which was reduced at both 25 and 32 months of dietary restriction (significant in all cell subpopulations of bone marrow compared to AL controls ($p \leq 0.04$) with the exception of lymphoid cells at 32 month of dietary restriction ($p=0.13$) (Figure 6.6. A. ii.). Although there was a trend for an increase in superoxide levels with age in bone marrow cells in both male and females, the significance was removed which could be due to a reduced sample size (Figure 6.6. A. ii.). The reduction of superoxide levels by dietary restriction was only significant in females and not in males (Figure 6.6. A. ii.).

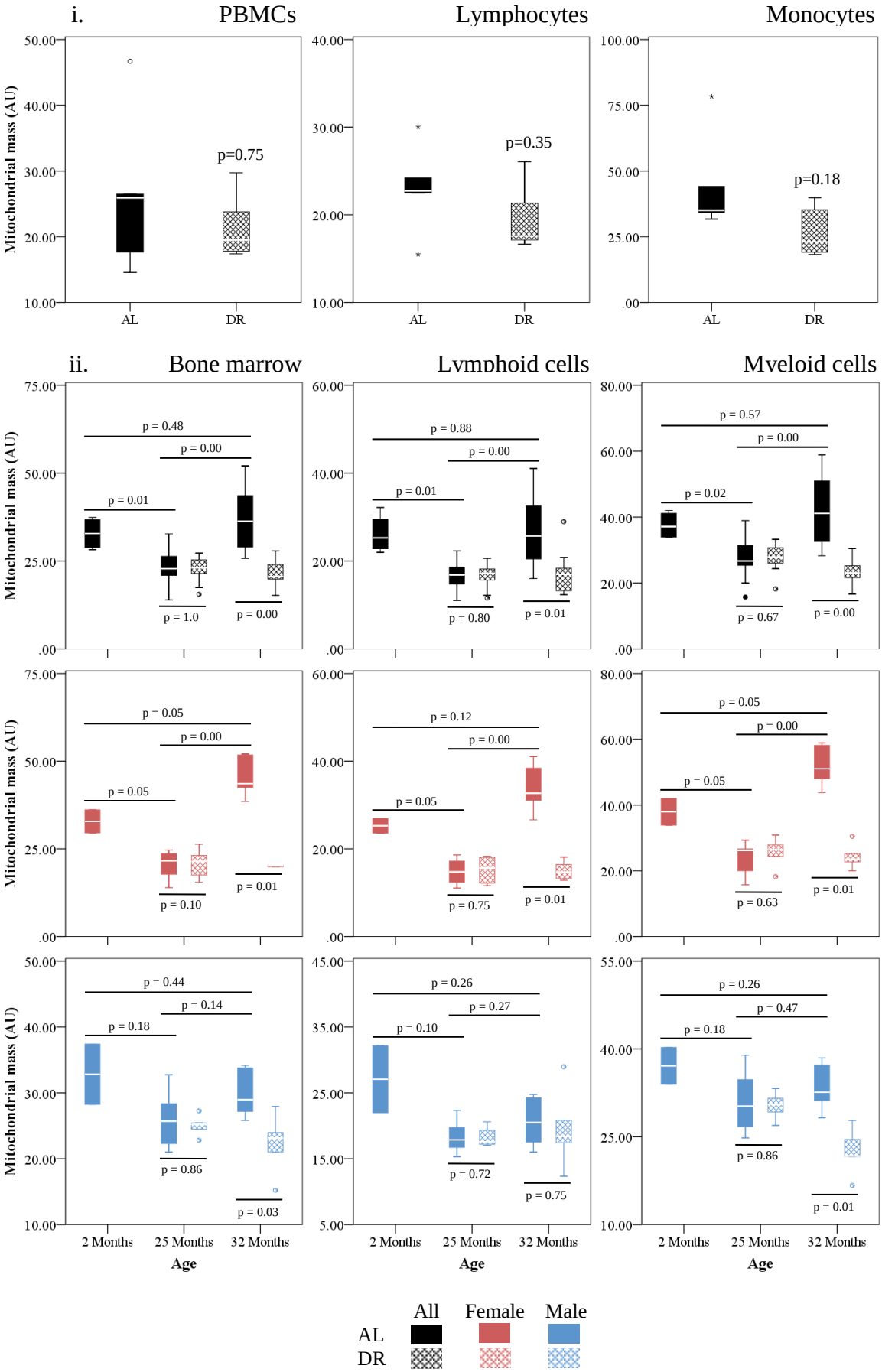
Mitochondrial mass had a non-linear association with age where at 2 month and 32 months of age it was higher than the 25 month old mice in all bone marrow subpopulations ($p \leq 0.02$) (Figure 6.6. B. ii.). There was no effect of dietary restriction on mitochondrial mass at 25 months of age in all bone marrow subpopulations ($p \geq 0.67$); however, the high mitochondrial mass at 32 months of age was significantly reduced by dietary restriction in all bone marrow subpopulations ($p \leq 0.01$) (Figure 6.6. B. ii.). This again was more pronounced in females than in males (Figure 6.6. B. ii.).

There was no significant association between mitochondrial membrane potential and age in all bone marrow subpopulations ($p \geq 0.07$) (Figure 6.6. C. ii.). There was no effect of dietary restriction on mitochondrial membrane potential at 25 months of age in all bone marrow subpopulation ($p \geq 0.74$); however, at 32 months of age, mitochondrial membrane potential was significantly increased by dietary restriction in all bone marrow subpopulations ($p < 0.01$) (Figure 6.6. C. ii.). Interestingly, mitochondrial membrane potential significantly decreased with age and was increased by dietary restriction at 32 months of age in male mice only (Figure 6.6. C. ii.).

A.


i.
PBMCs
Lymphocytes
Monocytes
Superoxide levels (AU)
p=0.20
p=0.14
p=0.07
AL
DR
ii.
Bone marrow
Lymphoid cells
Myeloid cells
p = 0.04
p = 0.75
p = 0.05
p = 0.01
p = 0.01
p = 0.03
p = 0.29
p = 0.03
p = 0.04
p = 0.13
p = 0.33
p = 0.24
p = 0.09
p = 0.03
p = 0.04
p = 0.30
p = 0.86
p = 0.20
p = 0.01
p = 0.02
p = 0.18
p = 0.86
p = 0.45
p = 0.00
p = 0.05
p = 0.46
p = 0.58
p = 0.20
p = 0.08
p = 0.05
p = 0.10
p = 0.47
p = 0.12
p = 0.72
p = 0.34
p = 0.10
p = 0.14
p = 0.02
p = 0.58
p = 0.92
p = 0.88
p = 0.20
p = 0.20
p = 0.36
p = 0.47
2 Months
25 Months
32 Months
Age
All
Female
Male
AL
DR

B.


i.
PBMCs
Lymphocytes
Monocytes
Mitochondrial mass (AU)
p=0.75
p=0.35
p=0.18
AL
DR
ii.
Bone marrow
Lymphoid cells
Myeloid cells
p = 0.48
p = 0.00
p = 0.01
p = 1.0
p = 0.00
p = 0.88
p = 0.80
p = 0.01
p = 0.57
p = 0.02
p = 0.67
p = 0.05
p = 0.10
p = 0.12
p = 0.75
p = 0.63
p = 0.44
p = 0.14
p = 0.18
p = 0.86
p = 0.03
p = 0.26
p = 0.27
p = 0.10
p = 0.72
p = 0.47
2 Months
25 Months
32 Months
Age
All
Female
Male
AL
DR

C.

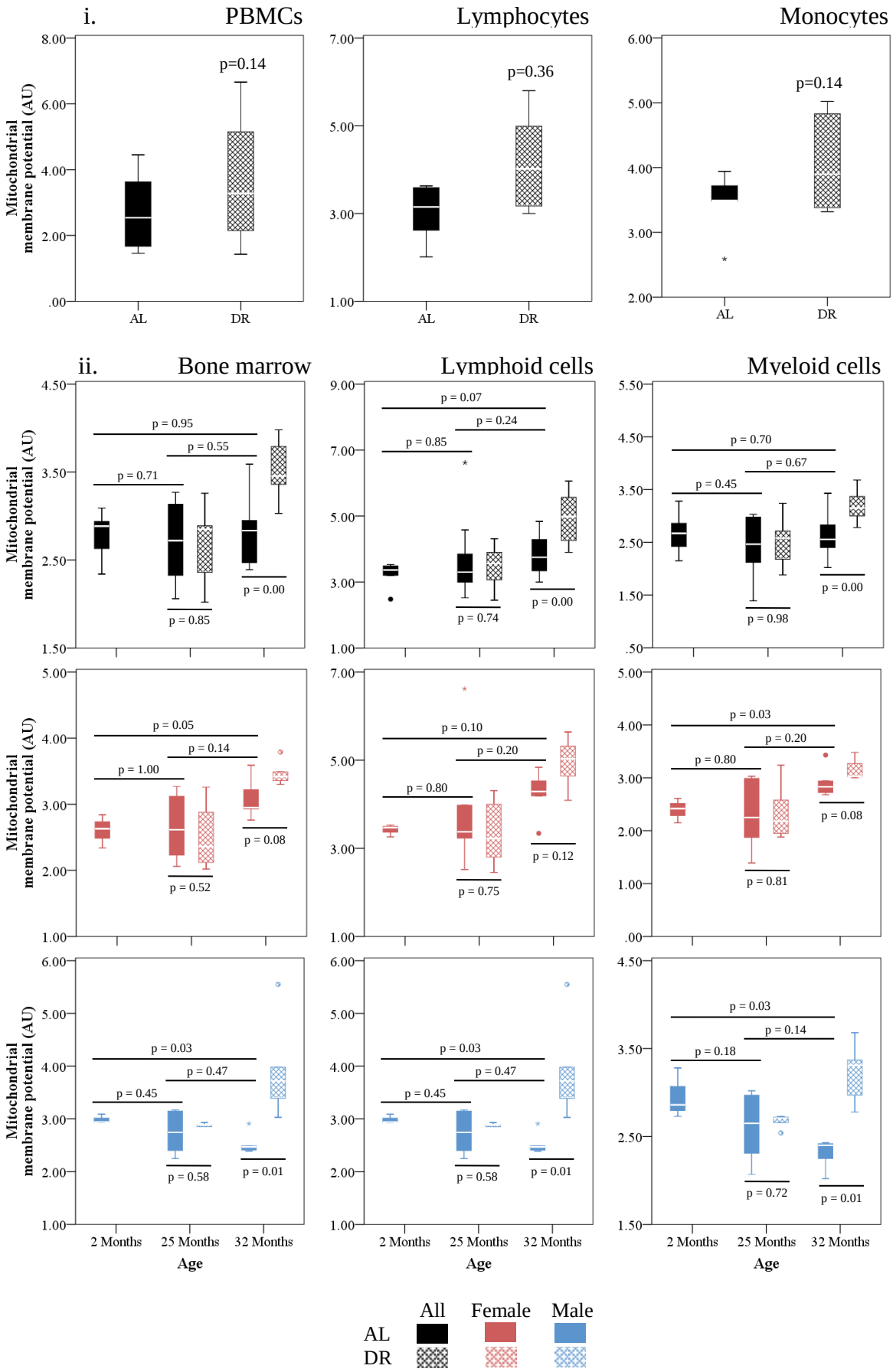

i.
PBMCs
Lymphocytes
Monocytes
p=0.14
p=0.36
p=0.14
Mitochondrial membrane potential (AU)
AL
DR
ii.
Bone marrow
Lymphoid cells
Myeloid cells
p = 0.95
p = 0.55
p = 0.71
p = 0.85
p = 0.00
p = 0.07
p = 0.24
p = 0.85
p = 0.74
p = 0.00
p = 0.70
p = 0.67
p = 0.45
p = 0.98
p = 0.00
p = 0.05
p = 0.14
p = 1.00
p = 0.52
p = 0.08
p = 0.10
p = 0.20
p = 0.80
p = 0.75
p = 0.12
p = 0.03
p = 0.20
p = 0.80
p = 0.81
p = 0.08
p = 0.03
p = 0.47
p = 0.45
p = 0.58
p = 0.01
p = 0.03
p = 0.14
p = 0.18
p = 0.72
p = 0.01
2 Months
25 Months
32 Months
Age
All
Female
Male
AL
DR

Figure 6.6. The effects of chronological age and dietary restriction on A. superoxide levels, B. mitochondrial mass and C. mitochondrial membrane potential in i. peripheral blood and ii. bone marrow of mice. All mice sample were included in this analysis (see table 6.3.). Mann-Whitney U Test was used to test differences between the medians of two groups.

6.3.6. *Effect of dietary restriction on lymphoid/myeloid derived cell ratio*

Lymphocyte/monocyte ratio in peripheral blood was significantly increased by dietary restriction in 25 month old mice (p=0.02) (Figure 6.7. A.).

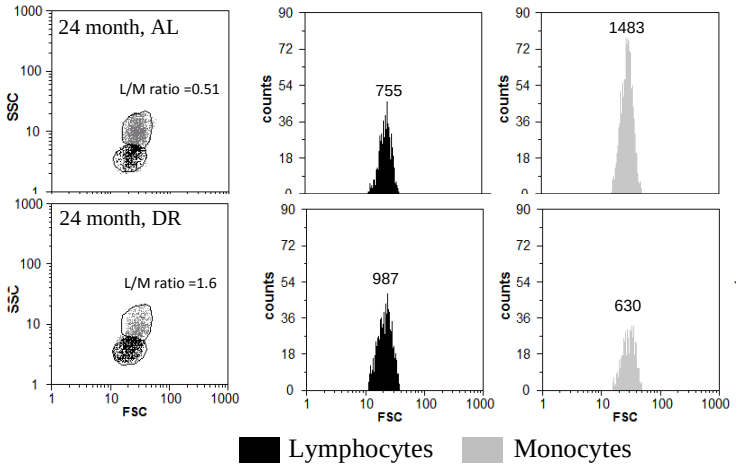
There was a decline in lymphoid/myeloid ratio in bone marrow of mice between 25 and 32 months of age (p=0.01) which was significantly increased by dietary restriction at both 25 and 32 month of age (p=0.03) (Figure 6.7. B.). The decline in lymphoid/myeloid ratio with age was only significant in males; however, there was the same trend for an increase in lymphoid/myeloid ratio by dietary restriction for both males and females, however this only reach significance at 32 month of age (Figure 6.7. B.).

6.4. Discussion

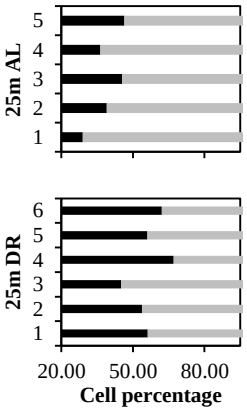
The first analysis of this study was to compare basal levels of superoxide levels, mitochondrial mass and mitochondrial membrane potential between peripheral blood and bone marrow cells of mice. This was because the future analysis of the mice model study, due to difficulties in obtaining enough peripheral blood, would be in bone marrow cells however this study should be comparable to the same tissue used in the human work, which used peripheral blood. Comparisons between these two tissues in terms of ROS production and mitochondrial function, to knowledge, has never been investigated. It was shown that superoxide levels were higher in bone marrow cells than in peripheral blood cells however mitochondrial mass was lower, consistent between lymphoid and myeloid derived cells. There were inconsistencies between mitochondrial membrane potential in cell subpopulations, where there was no difference between peripheral blood and bone marrow in lymphoid derived cells however this was higher in peripheral blood cells compared to bone marrow cells in myeloid derived cells. This therefore goes against the theory that an increase in mitochondrial mass and decreased membrane potential is associated with higher ROS production. However, this

A.

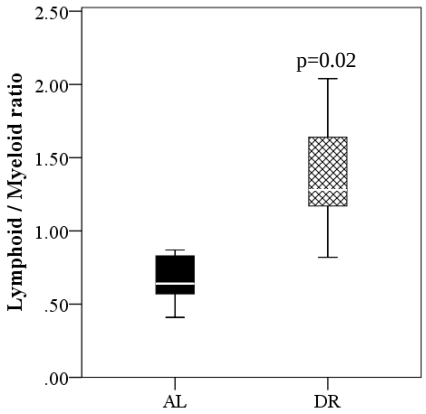
i.


24 month, AL
L/M ratio =0.51
24 month, DR
L/M ratio =1.6
SSC
FSC
counts
755
1483
987
630
Lymphocytes
Monocytes

ii.


25m AL
25m DR
Cell percentage
20.00
50.00
80.00

iii.


Lymphoid / Myeloid ratio
p=0.02
AL
DR

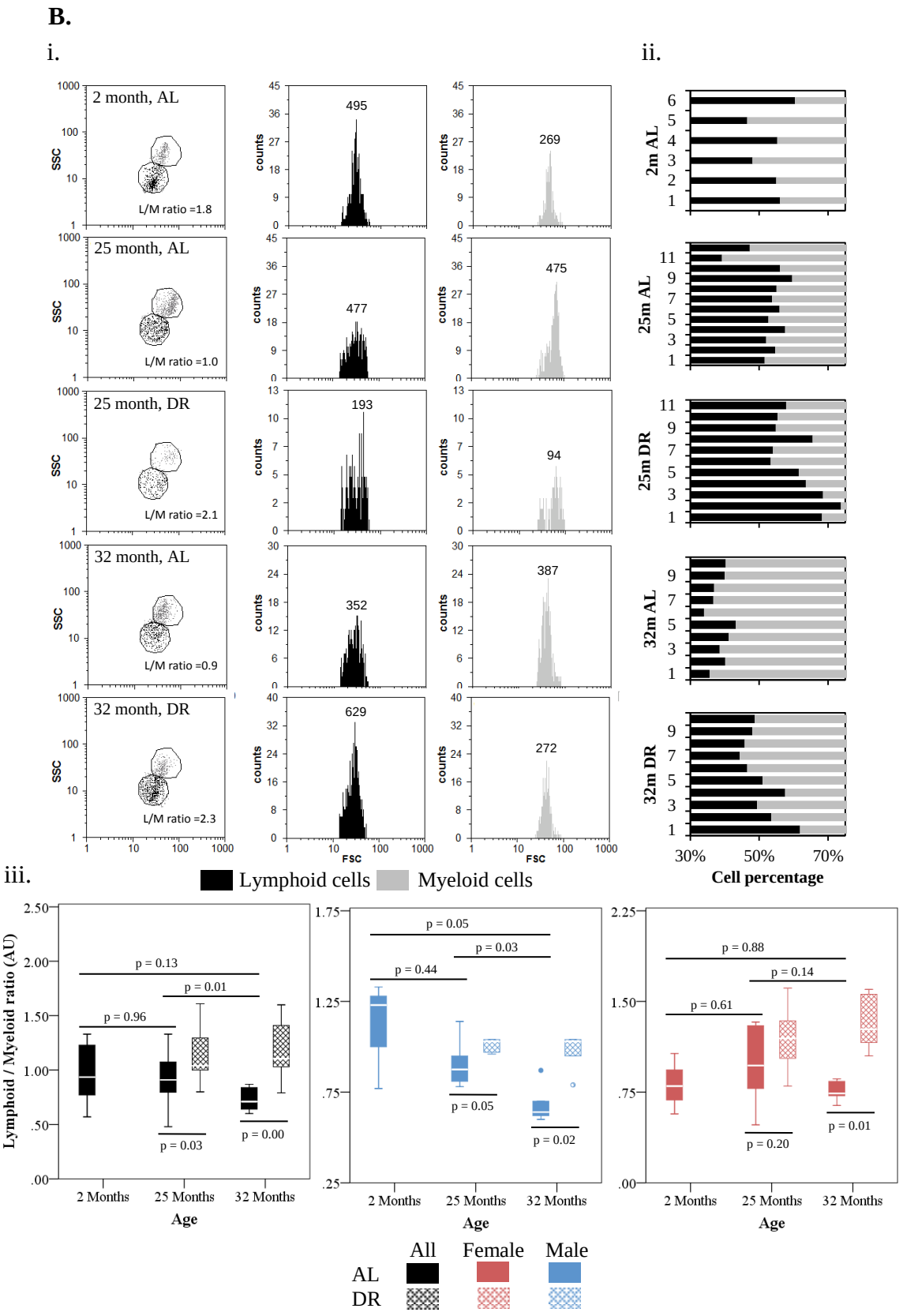


Figure 6.7. The effects of chronological age and dietary restriction on the lymphoid/myeloid derived cell ratio in A. peripheral blood and B. bone marrow of mice. i. Representative flow cytometry graphs for quantification of lymphoid and myeloid derived cell counts, ii. Percentages of lymphoid and myeloid derived cells in individual samples and iii. median comparisons. All mice sample were included in this analysis (see table 6.3.). Mann-Whitney U Test was used to test differences between the medians of two groups.

association may only be seen when cells are dysfunctional (i.e. as seen in senescent cells) and thus a decrease in mitochondrial mass at least could indicate that the mitochondria are less likely to be dysfunctional within these mice cells at 25 months of age. This could be due to effective antioxidant and/or repair mechanisms to remove to prevent mitochondrial damage by ROS. This conclusion will be important in the future findings of this study as discussed below. However, a weakness of this experiment, although mice were matched by age and diet group, is that peripheral blood and bone marrow cells were only compared in separate mice groups of peripheral blood and bone marrow. A better experiment would be to investigate differences within the same mice. This would then tell us that superoxide levels, mitochondrial mass and mitochondrial membrane potential are correlated between different tissues within the same mice, and thus could be tissue independent. This has important relevance of using peripheral blood as a surrogate tissue for other inaccessible tissues. A future experiment could also be to investigate further tissues to determine their agreements in ROS production and mitochondrial function. Despite the differences between peripheral blood and bone marrow cells, it was shown that within the same mice there are agreements between superoxide levels, mitochondrial mass and mitochondrial membrane potential in lymphoid derived and myeloid derived cells, as seen in the human samples (See Chapter 4. Figure. 4.11.) and therefore provides evidence that they are not cell type dependent; whether they are tissue dependent however is in need of further investigation.

The next part of this study was to determine the relationship between ROS production and mitochondrial function. There was no association between superoxide levels and mitochondrial mass in peripheral blood of 25 month old mice. Again in bone marrow of the 25 month old mice there was no association between superoxide and mitochondrial mass or mitochondrial membrane potential; however, at 32 month of age, a positive association between superoxide levels and mitochondrial mass, and a tendency for a negative association between superoxide levels and mitochondrial membrane potential is revealed. It is therefore hypothesised that the cells of the 32 month old mice are more likely to be dysfunctional than at 25 month of age, which could reflect the presence of more senescent cells. It was also revealed that increased ROS production, a trend for increased mitochondrial mass and a decline in mitochondrial membrane potential (at 32 months only in bone marrow) was associated with a decline in lymphoid/myeloid

derived cell ratio suggesting a role for mitochondrial ROS production and dysfunction in the decline of hematopoiesis and thus immune function.

This study then aimed to determine the effect of age and dietary restriction on ROS production and mitochondrial dysfunction, with the expectation that superoxide levels and mitochondrial mass should be reduced and mitochondrial membrane potential should be increased by dietary restriction. It was shown that superoxide levels increase with age however mitochondrial mass displayed a non-linear association with age. The reason mitochondrial mass is not increased at 25 months could support the above hypothesis that the majority of leukocytes at this age do not have dysfunctional mitochondria, and have efficient mechanisms such as antioxidant defenses and/or repair mechanisms to prevent damage to the mitochondria by the increased superoxide levels. At 32 months however, cells become more susceptible to damage by increasing superoxide production with age and thus mitochondria become more dysfunctional, indicated by an increase in mass. Interestingly, this was only the case in females; mitochondrial mass did not significantly change up to 32 month of age in males. It is therefore further hypothesized that the cells of males have less dysfunctional mitochondria/less senescent cells than females at 32 months of age and thus ageing occurs at a faster rate in females than in males. To support this, studies have reported that male mice actually have a longer life expectancy than females in the C57BL/6 strain and this has been attributed to a decrease in oxidative stress (Kunstyr and Leuenberger, 1975; Ali *et al.*, 2006). However, the study by Ali *et al.* in 2006 suggested that non-mitochondrial ROS are responsible for lifespan differences between males and females. This study however, although did not find differences in superoxide levels between males and females, mitochondrial mass and membrane potential were higher in females than males at 32 months of age suggesting a role of the mitochondria in the gender differences in lifespan in mice. To further validate ROS production and mitochondrial function as a BoA, it was confirmed that superoxide levels were decreased by dietary restriction at 25 and 32 months of age, and interestingly, mitochondrial function was only improved at 32 months of age, when it is hypothesised that cells are more dysfunctional and thus senescent. The effect of dietary restriction, in terms of reduction in superoxide levels and mitochondrial mass, was more prominent in females than in males. To support this, studies have shown that life extension by dietary restriction is more prominent in C57BL/6 females than in males (Cameron *et al.*, 2012). However, in terms of increased mitochondrial membrane potential, dietary restriction

was more effective in males than in females at 32 month of age in this study. There are therefore obvious gender differences in the effects of dietary restriction on mitochondrial function.

A further criteria of a BoA that is required to provide evidence of its construct validity is to demonstrate that it can differentiate between groups with established differences in the rate of ageing and/or lifespan (Ingram *et al.*, 2001). However although this is well documented in dietary restriction, this information was not known in this study and therefore it can not be concluded that the dietary restriction regime was affective in terms of life or health expansion. However, it was shown that the decline in the lymphocyte/myeloid ratio with age was improvement by dietary restriction reflecting an improved immune function. It could be interesting that monocytes have a higher ROS production than lymphocytes and become the majority of cells with age, since this could be associated with an increase in oxidative stress within leukocytes and therefore be associated with immune decline with ageing.

6.5. Conclusion

The overall aim of this chapter was to provide further evidence of the construct validity of leukocyte ROS production and mitochondrial function as a candidate BoA. Not only did this study provide evidence of their construct validity, by investigating a well known intervention known to increase life- and health-span, it also permitted their validity in leukocyte cells derived from different tissues, their association with chorological age and their validity in a functional aspect of ageing; the association with a biomarker of haematopoiesis and thus immune function. This is the first study, to knowledge, to report effect of dietary restriction on ROS production and mitochondrial function and also its effect on lymphoid/myeloid ratio in peripheral blood and bone marrow cells.

Chapter 7. The predictive validity of potential oxidative stress-related BoA in the very old population

7.1. Abstract

Background A major requirement of a candidate BoA is to show some evidence of predictive validity in terms of its association with various age-related outcomes and survival.

Aims The final chapter of this study therefore aimed to determine whether PBMC ROS production and mitochondrial function and plasma F_2-isoprostanes are associated with various age-related outcomes in the very old population.

Methods The associations between PBMC superoxide levels, mitochondrial mass and mitochondrial membrane potential and plasma 8-iso Prostaglandin $F_{2\alpha}$ with four age-related outcomes: disability, cognitive impairment, disease only morbidity count and survival was investigated in the very old population.

Results No significant associations between any of the four age-related outcomes were shown, even when adjustments were made for potential confounders.

Conclusion This study does not provide evidence for the predictive validity of various oxidative stress-related BoA in the very old population.

7.2. Introduction

A major requirement of a candidate BoA is that they are able to predict the years of remaining good health and the trajectory towards age-related outcomes and mortality better than chronological age (Sprott, 2010; Martin-Ruiz, 2013). Evidence should therefore at least show that the candidate BoA can distinguish between individuals with different levels of age-related decline.

The final validation assessment of the potential oxidative-stress related BoA therefore aimed to provide evidence to support their predictive validity in terms of their association with various age-related outcomes and survival in the very old population.

7.3. Results

7.3.1. Descriptives

Descriptives of the potential oxidative stress-related BoA, potential confounders and age-related outcomes of the very old from the Newcastle 85+ study are shown in Appendix C. Supplementary Table. 11., 12. and 14. respectively, stratified by all participants and also by participants with superoxide level, mitochondrial mass, mitochondria membrane potential and/or 8-iso Prostaglandin $F_{2\alpha}$ measurements to determine the representativeness of the whole population compared to those participants with oxidative-stress related measures. A description of possible selection bias for potential confounders are discussed in Section 7.3.1. Possible selection bias occurred for: 1) differences in disease only morbidity count which was lower in those with mitochondria mass and 8-iso Prostaglandin measures compared to the whole population; and 2) differences in those who have passed away, where a lower proportion of those who had passed away were present in those with oxidative-stress related measures. An explanation for these differences is that they most likely reflect age-related the changes in these variables. Cut-off values for grouped continuous potential oxidative stress-related BoA and age-related outcomes are shown in Appendix C. Supplementary Table 15 and Table 18 respectively.

7.3.2. Association with age-related outcomes in the very old population

The first assessment was to determine the agreement between potential oxidative stress-related BoA with continuous scores of disability, cognitive impairment and disease only morbidity. Three models of correlation analysis were investigated where data was either 1) unadjusted (Table 7.1), 2) unadjusted with extreme outliers removed as a form of sensitivity analysis (Table 7.2), or 3) adjusted for gender and season with extreme outliers removed (Table 7.3). However, none of theses models provided evidence for the association between any of the four age-related outcomes in the very old population.

A.

Model 1											
Age-related outcome		**Superoxide levels**			**Mitochondrial mass**			**Mitochondrial membrane potential**			**8-iso Prostaglandin $F_{2\alpha}$**
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Disability score	r	0.00	0.01	0.04	-0.03	0.01	0.00	0.02	0.07	0.03	-0.05
	p	0.95	0.90	0.55	0.60	0.83	1.00	0.75	0.21	0.61	0.37
	n	247	247	247	339	339	339	345	345	345	377
SMMSE score	r	0.07	0.08	0.03	0.08	0.04	0.08	0.05	-0.08	-0.02	0.02
	p	0.25	0.19	0.67	0.13	0.42	0.16	0.37	0.12	0.77	0.68
	n	247	247	247	340	340	340	346	346	346	378
Disease count	r	0.08	0.08	0.06	0.04	0.07	-0.01	-0.03	-0.01	-0.03	-0.08
	p	0.20	0.25	0.32	0.46	0.22	0.90	0.55	0.90	0.63	0.15
	n	239	239	239	328	328	328	334	334	334	367

B.

Model 2											
Age-related outcome		**Superoxide levels**			**Mitochondrial mass**			**Mitochondrial membrane potential**			**8-iso Prostaglandin $F_{2\alpha}$**
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Disability score	r	0.01	0.01	0.05	-0.03	0.01	-0.01	0.01	0.06	0.00	-0.05
	p	0.82	0.90	0.43	0.60	0.85	0.80	0.89	0.27	0.98	0.38
	n	244	245	245	339	335	337	341	344	338	357
SMMSE score	r	0.06	0.07	0.01	0.08	0.03	0.06	0.04	-0.09	-0.02	0.00
	p	0.33	0.28	0.90	0.13	0.61	0.30	0.45	0.08	0.70	0.95
	n	241	242	242	335	331	333	337	340	334	352
Disease count	r	0.10	0.08	0.07	0.04	0.06	-0.01	-0.04	-0.02	-0.04	-0.06
	p	0.11	0.19	0.29	0.46	0.24	0.91	0.48	0.77	0.51	0.24
	n	236	237	237	328	324	326	330	333	328	349

C.

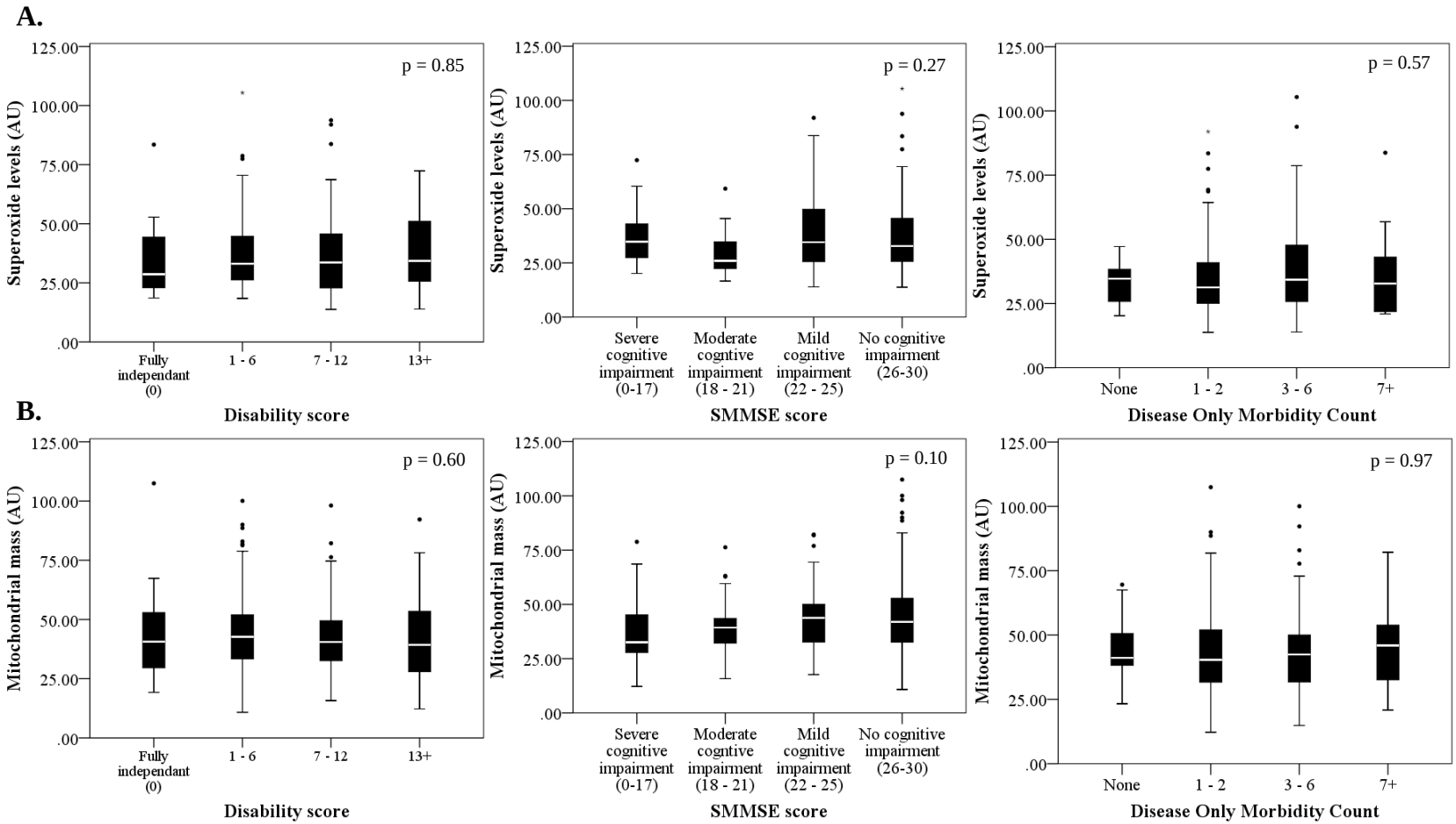
Model 3											
Age-related outcome		**Superoxide levels**			**Mitochondrial mass**			**Mitochondrial membrane potential**			**8-iso Prostaglandin $F_{2\alpha}$**
		PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	PBMCs	Lymphocytes	Monocytes	Plasma
Disability score	r	0.02	0.04	0.04	-0.07	0.00	-0.10	0.01	0.07	0.00	-0.07
	p	0.77	0.56	0.52	0.19	0.98	0.06	0.82	0.17	0.94	0.20
	n	240	241	241	335	331	333	337	340	334	353
SMMSE score	r	0.01	0.00	0.01	0.10	0.04	0.09	0.00	-0.07	-0.05	0.07
	p	0.85	1.00	0.89	0.07	0.43	0.12	0.97	0.23	0.32	0.19
	n	237	238	238	331	327	329	333	336	330	348
Disease count	r	0.11	0.08	0.07	0.03	0.10	0.00	-0.08	-0.02	-0.07	-0.06
	p	0.10	0.20	0.26	0.65	0.07	0.98	0.18	0.69	0.22	0.27
	n	232	233	233	324	320	322	326	329	324	345

Table 7.1: Agreements between continuous potential oxidative-stress related BoA and age-related outcomes. A. Model 1: unadjusted data*, B. Model 2: unadjusted data with extreme outliers removed*, and C. adjusted for gender and season with extreme outliers removed** (r: correlation coefficient (*Spearmans, **Partial), p: probability, n: number of participants).

Next the association between the potential oxidative stress-related BoA and grouped scores of disability, cognitive impairment and disease only morbidity was investigated by comparing median values. However again, there was no significant association shown for any of the age-related outcome (Figure 7.1.). Interestingly however, 8-iso Prostaglandin $F_{2\alpha}$ seemed to show a U shaped relationship with disease only morbidity count however this did not quite reach significance (p=0.09).

7.3.3. Association with survival in the very old population

The final assessment was to determine whether the potential oxidative-stress related BoA were associated with survival. Cox regression analysis was employed using two models: 1) unadjusted data and 2) adjusted for gender and season. No significant association were shown (Figure 7.2.). There was a tendency for those with a high or low mitochondrial membrane potential to have a reduced survival than those with medium mitochondrial membrane potential, however this did not quite reach significance before and after adjusting for confounders (p=0.06 and p=0.05 respectively). There was also a tendency for those with high or medium 8-iso Prostaglandin $F_{2\alpha}$ concentration to have an increased survival than those with low 8-iso Prostaglandin $F_{2\alpha}$ concentration, however this was not significant before and after adjusting for confounders (p=0.13 and p=0.16 respectively).


A.
Superoxide levels (AU)
125.00
100.00
75.00
50.00
25.00
.00
p = 0.85
Fully independant (0)
1 - 6
7 - 12
13+
Disability score
p = 0.27
Severe cognitive impairment (0-17)
Moderate cogntive impairment (18 - 21)
Mild cognitive impairment (22 - 25)
No cognitive impairment (26-30)
SMMSE score
p = 0.57
None
1 - 2
3 - 6
7+
Disease Only Morbidity Count
B.
Mitochondrial mass (AU)
p = 0.60
p = 0.10
p = 0.97

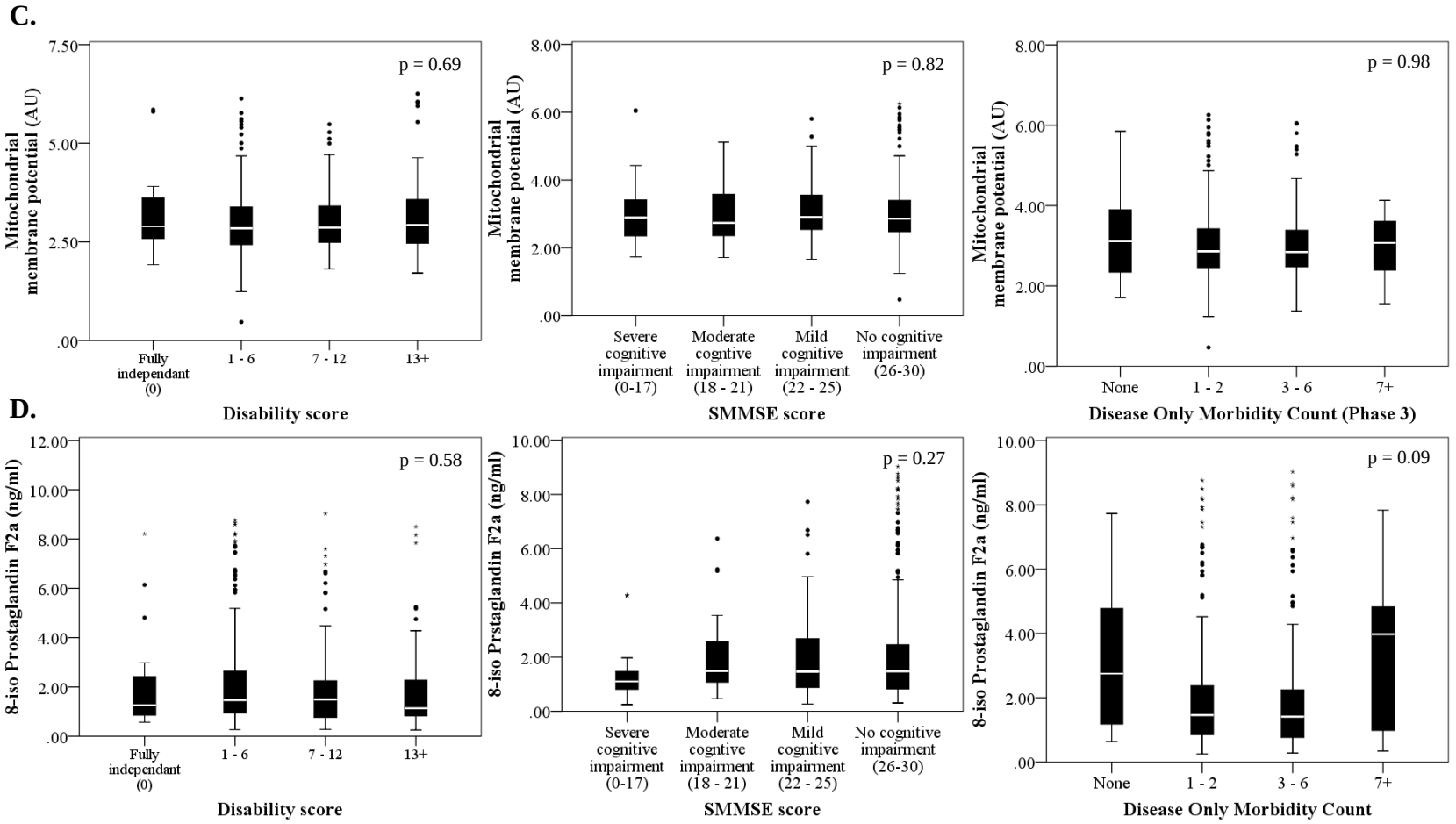
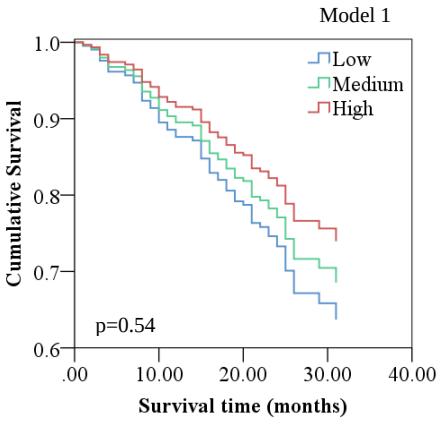
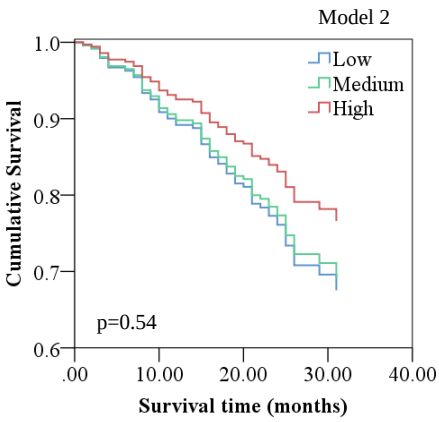


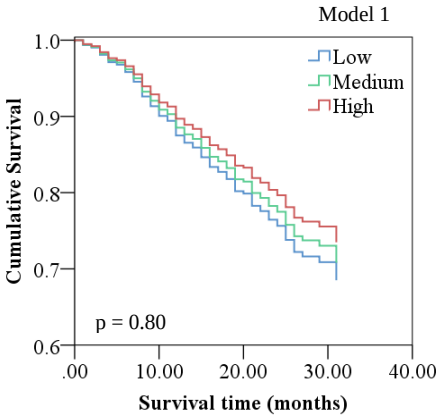
Figure 7.1. Association between continuous potential oxidative-stress related BoA and grouped age-related outcome scores. A. superoxide levels, B. mitochondrial mass, C. mitochondrial membrane potential and D. 8-iso Prostaglandin $F_{2\alpha}$. Kruskal wallis was used to compare medians. Extreme outliers were removed from continuous data. *(Results for PBMCs are shown, there was also no significant association in lymphocyte and monocyte separately, data not shown)*

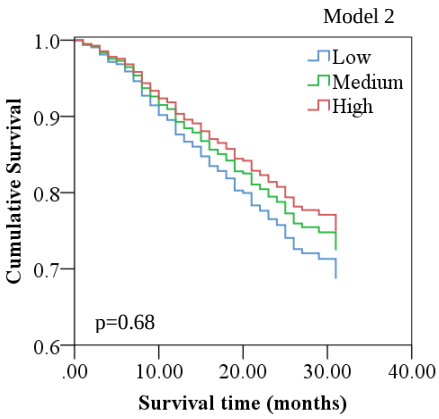
A. Superoxide levels



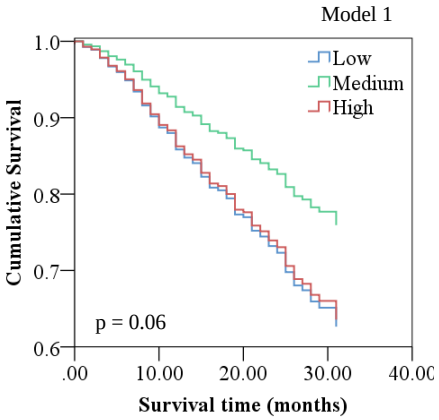


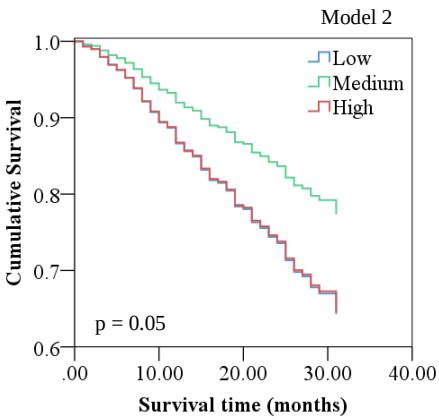
B. Mitochondrial mass





C. Mitochondrial membrane potential





D. 8-iso Prostaglandin $F_{2\alpha}$

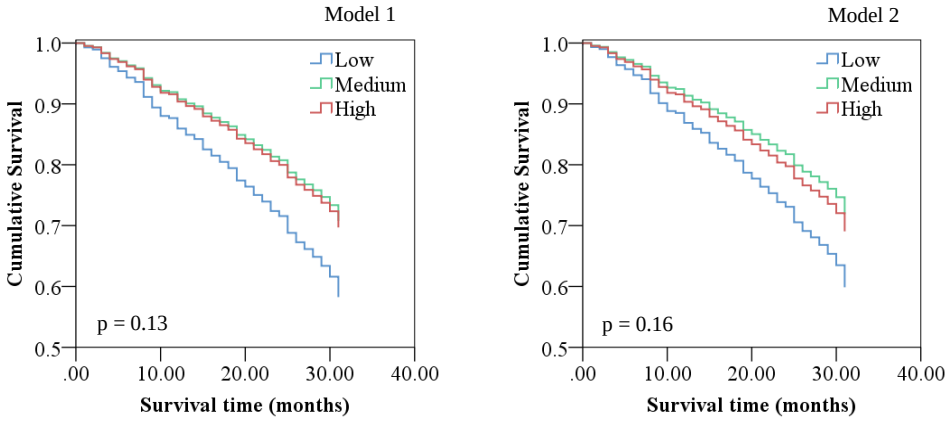


Figure 7.2. Association between potential oxidative stress-related BoA and survival of the very old population. A. superoxide levels, B. mitochondrial mass, C. mitochondrial membrane potential and D 8-iso Prostaglandin $F_{2\alpha}$. Cox regression analysis (Model 1: unadjusted data, Model 2: adjusted for gender and season). *(Results for PBMCs are shown, there was also no significant association in lymphocyte and monocyte separately, data not shown)*

7.4. Discussion

Attempts were made to further validate various oxidative stress-related BoA in terms of their association with various age-related outcomes in the very old population. However, no significant associations were found with cognitive impairment, disability score, disease count or survival, even after adjustment of potential confounders. However, since multi morbidity is common in the very old (Collerton *et al.*, 2009), this study could be confounded by the presence of other age-related outcomes. A more general approach could be to investigate the association between potential BoA and a frailty score. A previous analysis in the Newcastle 85+ study identified the importance of inflammatory markers in frailty in the very old population (Collerton *et al.*, 2012). However, limited evidence was found for the role of immunosenescence and no evidence for the role of telomere length, markers of oxidative damage or DNA damage and repair in frailty was found. Furthermore, a recent analysis in the same cohort found no association between frailty and mitochondrial DNA haplogroups (Collerton *et al.*, 2013). These are maternally inherited variations in mtDNA sequences for which the population can be divided into several groups based on specific nucleotide polymorphisms variants. It is thought that some variants of mitochondrial DNA haplogroups have functional differences including lower rates of ROS production and better capacity to cope with oxidative stress (Chen *et al.*, 2012; Mueller *et al.*, 2012) and several studies have shown associations with various age-related outcomes (van der Walt *et al.*, 2004; Ghezzi *et al.*, 2005; Kofler *et al.*, 2009; Wolf *et al.*, 2010). However there are inconsistencies regarding which variant is more susceptible to age-related outcomes. This study however found no association between mitochondria haplogroup and potential oxidative-stress related BoA in the very old, supporting the study by *Collerton et al., 2013.*

Validating BoA within human studies can be extremely difficult due to variability in unknown intrinsic and extrinsic factors that influence candidate BoA, even in those of the same chronological age, as explained in Chapter 6. Section 6.2. It could be differences in genetic factors that could, for example, influence different rates in the metabolism of various nutrients or medications that influence candidate BoA. Therefore, although information on diet and medication could be captured by questionnaire and then controlled for in future analysis, this variability would mean controlling for these factors would be ineffective. Another genetic factor could be

differences in susceptibilities of tissues to oxidative damage between individuals. Therefore although, for example, superoxide levels could be identical between individuals, this could have different impacts in terms of tissue damage. Environmental exposures could also vary between individuals that are not captured and thus controlled for by the study. An interesting environmental variable discovered to affect all potential oxidative-stress related BoA in this study was seasonal variation. This could be due to changes in sunlight exposure, temperature or other indirect effects of seasonal changes e.g. physical activity. However, even after controlling for this variable, no associations with age-related outcomes were shown. The use of an animal model in chapter 6 was most probably more informative of PBMC ROS production and mitochondrial function as candidate BoA since the mice are in a tightly controlled environment and are genetically similar. Mice used in this study were all of the same genetic background (the C57BL/6 strain), were housed in identical cages, provided with the same bedding materials and housed at the same temperature under the same a 12 hour light/dark photoperiod. Of course genetic mutations and polymorphisms could still occur between these mice, which could be reflected in the different pathologies shown between mice of the same gender and age (See Chapter 6. Table 6.3), however the results of the animal model study provide evidence that intrinsic and extrinsic factors are likely to be eliminated since evidence for the construct validity of ROS production and mitochondrial function is shown. Unknown intrinsic and extrinsic factors are therefore possible reasons for the inconsistencies in the validation of BoA in human studies and where possible efforts should be made to identify theses factors.

Another issue is that it is often thought it is unlikely that a single BoA that can be measured in a surrogate tissue exists because different biological systems age at different rates within the same individual, having varying susceptibility to cellular and molecular damage. This would explain why there is a great variability in the timing, type and number of age-related diseases or disorders amongst individuals. An individual is therefore likely to have a number of different functional ages for specific cells and/or tissues and therefore a number of BoA would be needed. A powerful approach could be to combine scores from a battery of biomarkers, reflecting each biological system, into a single biomarker of ageing (Hochschild, 1990). This however would still require an extensive collection of biomarker measurements from each system, many being impractical to measure, increasing cost and time. The ideal BoA would therefore be one that could be measured in different biological systems at the one time and not be

invasive or difficult to measure. If it was physically possible to track and measure initiators of cellular senescence, such as mitochondrial ROS production, or senescent cells them selves (i.e. p16 positive cells ((Baker *et al.*, 2011)) as a BoA in each system, this would not only have the potential to understand mechanisms, predict timing and test interventions but would also allow the targeting of therapeutic interventions to specific cells/tissues.

A limitation to this study in terms of cognitive impairment, disability score, disease count, is that analysis was cross-sectional and longitudinal analysis would be more informative. Further analysis of this cohort could therefore be to longitudinally investigate the role of ROS production and mitochondrial function in the development of frailty within the very old.

7.5. Conclusion

Further attempts to validate various potential oxidative stress-related BoA in terms of their association with disability, cognitive impairment, disease only morbidity count and survival in the very old population were unsuccessful. Since some evidence for their construct validity has been shown within this study it is suggested that further efforts could be made including; determining whether they are tissue independent, the identification of potential intrinsic and extrinsic confounding variables, the investigation of a more generalised frailty model and also longitudinal analysis.

Appendix A:

Literature search of candidate BoA and their ability to predictor age-related outcomes; specifically mortality, co-morbidity, disability and/or cognitive impairment.

The electronic database MEDLINE via the database provider OvidSP was searched up to 2012 where a typical search for each candidate BoA would consist of:

1. (“Candidate BoA” adj10 predict$ adj10 survival).mp.
2. (“Candidate BoA” adj10 predict$ adj10 death).mp.
3. (“Candidate BoA” adj10 predict$ adj10 mortality).mp.
4. (“Candidate BoA” adj10 predict$ adj10 $morbid$).mp.
5. (“Candidate BoA” adj10 predict$ adj10 disabil$).mp.
6. (“Candidate BoA” adj10 predict$ adj10 cognit$).mp.
7. 1 or 2 or 3 or 4 or 5 or 6
8. limit 26 to (abstracts and humans)

The following “Candidate BoA” with the appropriate words/truncations were searched: (n=number of articles returned)

No	Candidate BoA	Search words/truncations	n
1	Body mass index	((body adj mass) and index)	457
2	Waist to hip ratio	waist adj to adj hip adj ratio	20
3	Fat percentage	fat adj % fat adj percent$	78
4	Fat free mass	fat adj free adj mass	3
5	Total body water	total adj body adj water	1

6	Physical activity	physical adj activity	107
7	Hand grip strength	hand adj grip adj strength	10
8	Timed up and go	timed up and go	5
9	Diastolic blood pressure	diastolic adj blood adj pressure diastolic adj bp	62
10	Systolic blood pressure	systolic adj blood adj pressure systolic adj bp	221
11	Forced expiratory volume	forced adj expiratory adj volume fev	130
12	Forced vital capacity	forced adj vital adj capacity fvc	78
13	Peak expiratory flow	peak adj expiratory adj flow pef	14
14	Oxygen saturation	oxygen adj saturation	31
15	Red blood cell	red adj blood adj cell rbc	42
16	White blood cell	white adj blood adj cell wbc	119
17	Lymphocyte	lymphocyte	148
18	Monocyte	monocyte	23
19	Neutrophil	neutrophil	64
20	Basophil	basophil	0
21	Eosinophil	eosinophil	3
22	Haematocrit	haematocrit	5
23	Haemoglobin	haemoglobin	55
24	Mean cell haemoglobin	mean adj cell adj haemoglobin	0
25	Mean corpuscular volume	mean adj corpuscular adj volume mcv	3
26	Platelet	platelet	160
27	Sodium	sodium	96
28	Potassium	potassium	18

29	Urea	urea	87
30	Urate	urate	7
31	Creatinine	creatinine	431
32	Total protein	total adj protein	5
33	Bilirubin	bilirubin	140
34	Alanin transaminase	alanine adj transaminase alt	23
35	Albumin	albumin	459
36	Calcium	calcium	67
37	Adjusted calcium	adjusted adj calcium	0
38	Phosphate	phosphate	15
39	Alkaline phosphatase	alkaline adj phosphatase	44
40	Glucose	glucose	278
41	Glycosylated harmoglobin	glycosylated adj haemoglobin HbA1c	29
42	Tryglcerides	triglycerid$	5
43	Cholesterol	cholesterol	246
44	High density lipoprotein	high adj density adj lipoprotein hdl	96
45	Low density lipoprotien	low adj density adj lipoprotein ldl	51
46	Apolipoprotein A1	apolipoprotein adj A1	0
47	Apolipoprotein B	apolipoprotein adj B	3
48	Cortisol	cortisol	55
49	Free thyroxine	free adj thyroxine free adj t4	1
50	Free triiodothyronine	free adj triiodothyronine free adj t3	3
51	Thyroid stimulating hormone	thyroid adj stimulating adj hormone tsh	7

52	Thyroid peroxidase	thyroid adj peroxidase	1
53	C reactive protein	c adj reactive adj protein crp	515
54	Rheumatoid factor	rheumatoid adj factor	12
55	Vitamin B2	vitamin adj b2	0
56	Vitamin B6	vitamin adj b6	0
57	Vitamin B12	vitamin adj b12	5
58	Vitamin D	vitamin adj d	34
59	Ferittin	ferritin	15
60	Red cell folate	red adj cell adj folate	0
61	Homocysteine	homocysteine	49
62	Brain natriuretic peptide	brain adj natriuretic adj peptide bnp	335
63	Telomere length	telomere adj length	22
64	DNA damage	dna adj damage	9
65	DNA repair	dna adj repair	18
66	Immunosenescence	immunosenescence	4
67	Interleukin 6	interleukin adj "6" il adj "6"	194
68	Tumour necrosis factor	tumour adj necrosis adj factor tnf	45
69	Mitochondrial Function	mitochondrial adj function	1
70	Reactive oxygen species	reactive adj oxygen adj species	0
71	Oxidative stress	oxidative adj stress	21

Appendix B:

Literature search of the effects of dietary restriction on potential oxidative stress and cellular senescence-related BoA in leukocytes.

The electronic databases MEDLINE via the database provider OvidSP and also PubMeD was searched during July 2012 using the following search terms where a typical search for each potential oxidative stress and cellular senescence-related BoA would consist of:

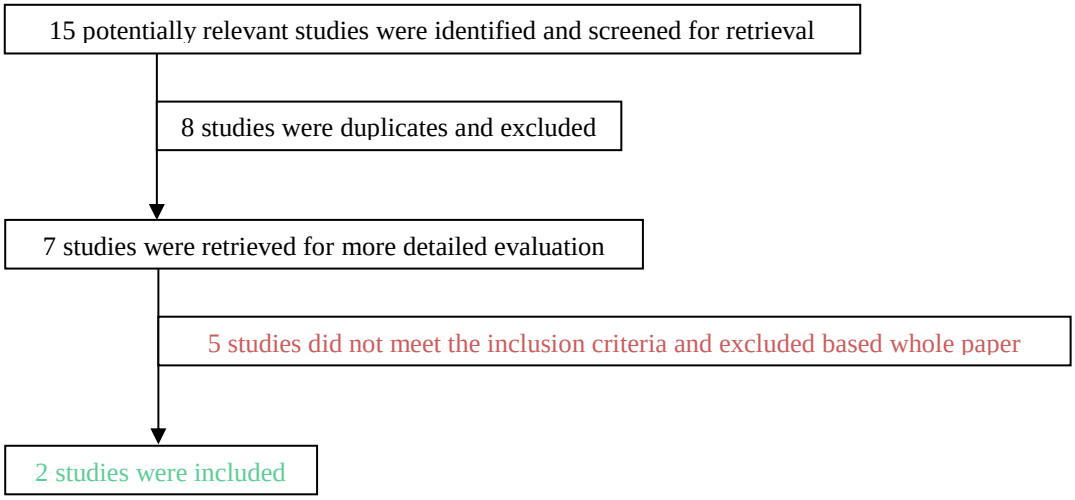
1. Calorie restriction
2. Dietary restriction
3. 1 or 2
4. Peripheral blood
5. White blood cells
6. Lymphocytes
7. Monocytes
8. Bone marrow
9. Lymphoid
10. Myeloid
11. Plasma*
12. Serum*
13. 4 or 5 or 6 or 7 or 8 or 9 or 10
14. "Potential oxidative stress and cellular senescence-related BoA"
15. 3 and 13 and 14
16. limit 13 to abstract

*Interleukin-6 and tumour necrosis factor-alpha only

The following 'Potential oxidative stress and cellular senescence-related BoA" with the appropriate words/truncations were searched, including the stages of identifying the included articles

ROS production and mitochondrial function

Search words/truncations: Reactive oxygen species or ROS or Superoxide or Mitochondria

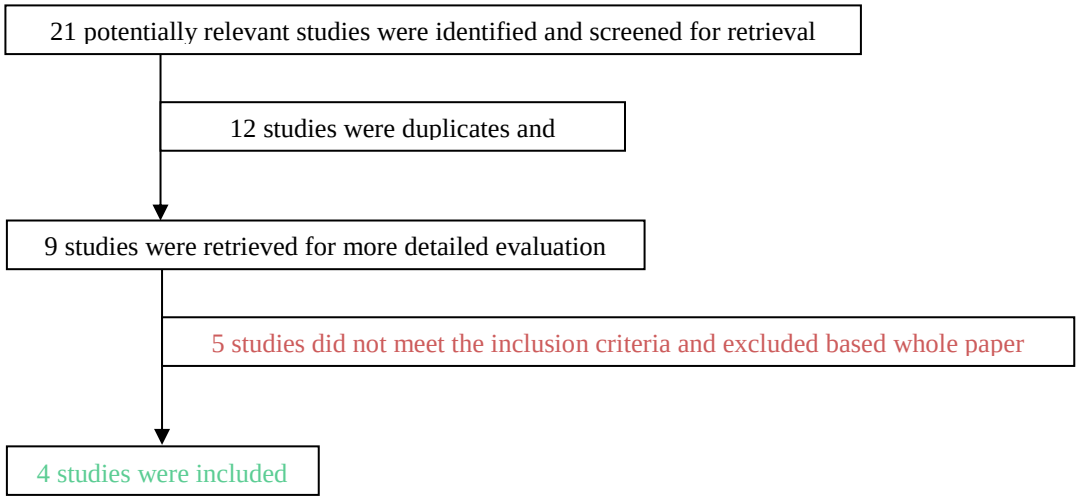


Telomere length

Search words/truncations: Telomere length

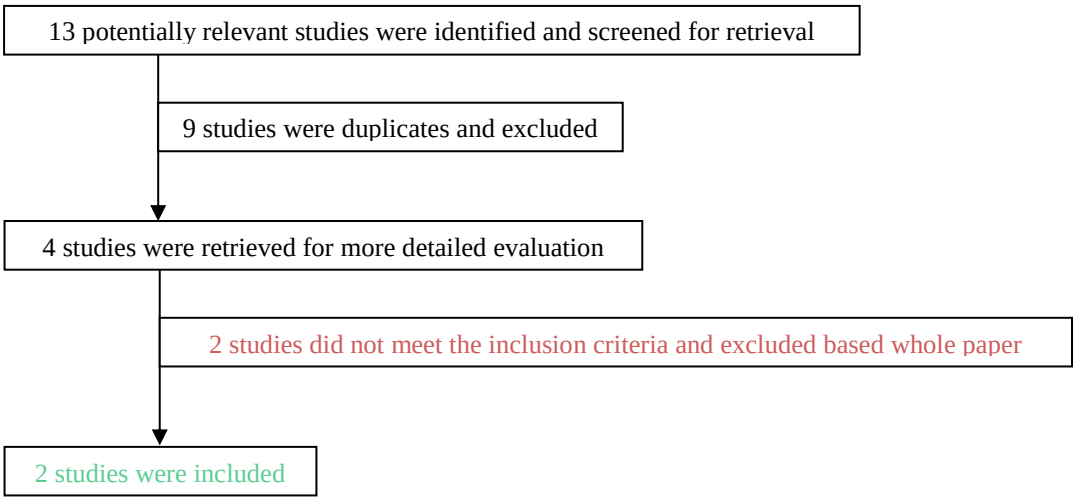
DNA damage

Search words/truncations: DNA damage



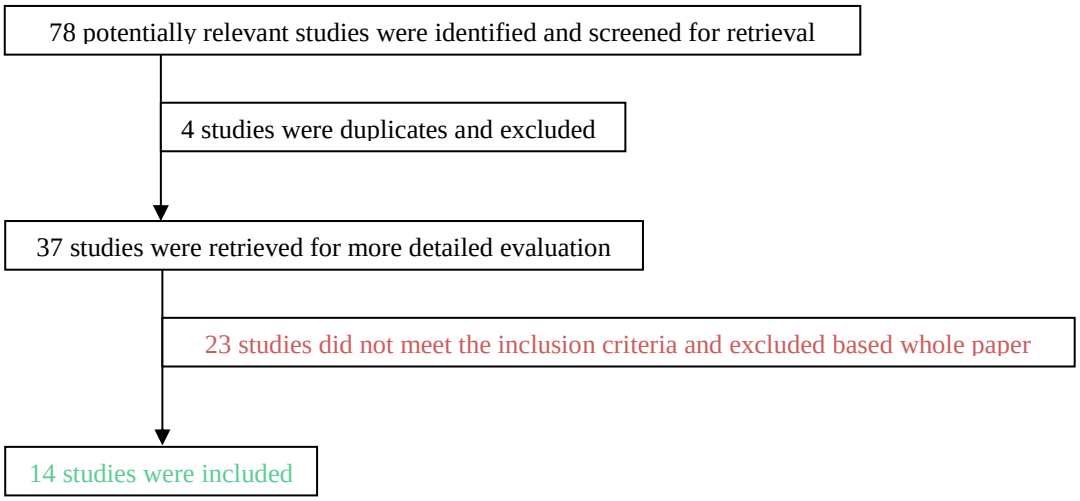
DNA repair

Search words/truncations: DNA repair



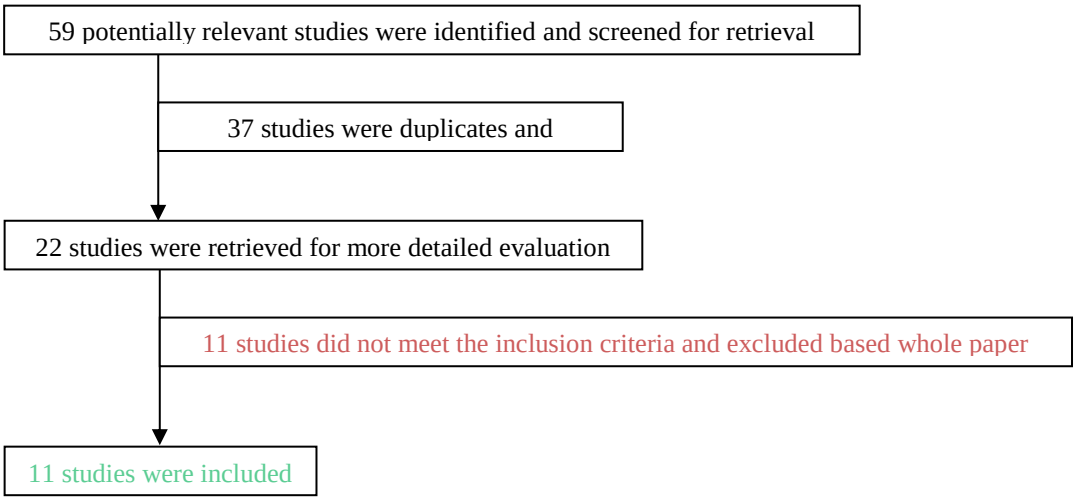
Interleukin-6

Search words/truncations: IL 6 or IL-6 or Interleukin 6 or Interleukin-6



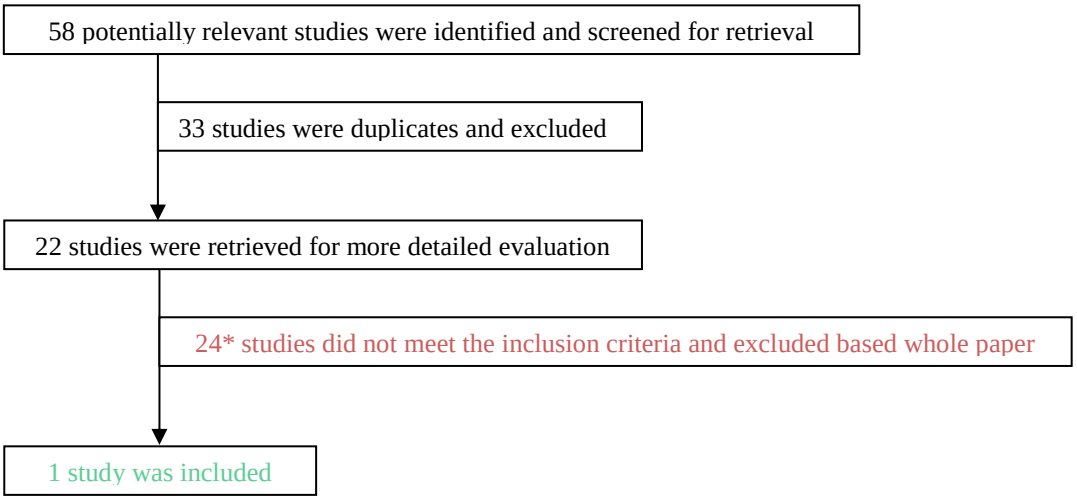
Tumour necrosis factor-alpha

Search words/truncations: TNF or TNF-a or TNF alpha or TNF-alpha or Tumour necrosis factor a or Tumour necrosis factor-a or Tumour necrosis factor alpha or Tumour necrosis factor-alpha



Lymphoid / Myeloid ratio

Search words/truncations: Ratio or Bias or Percentage or Proportion or Fraction



* 7 studies reported a change in lymphoid or lymphocytes but no measurements of myeloid or monocytes changes in DR

Appendix C:

Supplementary data

Cell type	Superoxide levels (AU)			Mitochondrial mass (AU)			Mitochondrial membrane potential (AU)		
PBMCs									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	40.15	48.51	68.32	45.34	51.13	76.66	3.37	3.19	3.76
Repeat 2	43.22	51.08	68.47	50.52	58.37	80.02	3.46	3.28	4.07
Repeat 3	50.32	51.1	61.69	48.46	51.64	82.70	2.73	3.22	3.66
Mean	44.56	50.23	66.16	48.11	53.71	79.79	3.19	3.23	3.83
SD	5.22	1.49	3.87	2.61	4.04	3.03	0.40	0.04	0.21
% CV	11.71	2.97	5.85	5.42	7.52	3.79	12.40	1.38	5.50
Average % CV		6.84			5.58			6.43	
Lymphocytes									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	34.07	36.23	58.9	56.94	89.71	107.29	9.27	8.03	9.61
Repeat 2	36.76	39.6	62.48	56.19	81.48	106.93	7.72	8.78	10.08
Repeat 3	35.76	44.47	57.78	52.95	84.84	105.38	6.61	8.59	9.23
Mean	35.53	40.1	59.72	55.36	85.34	106.53	7.87	8.47	9.64
SD	1.36	4.14	2.45	2.12	4.14	1.01	1.34	0.39	0.42
% CV	3.83	10.33	4.11	3.83	4.85	0.95	16.99	4.57	4.38
Average % CV		6.09			3.21			8.64	
Monocytes									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	109.36	115.77	139.61	94.51	148.89	181.64	1.87	1.67	2.53
Repeat 2	128.43	125.02	142.22	102.56	134.07	228.07	2.02	2.41	2.85
Repeat 3	124.41	126.32	138.75	85.05	159.70	212.50	2.19	2.03	2.30
Mean	120.73	122.37	140.19	94.04	147.55	207.40	2.02	2.04	2.56
SD	10.05	5.75	1.81	8.76	12.87	23.63	0.16	0.37	0.28
% CV	8.33	4.70	1.29	9.32	8.72	11.39	7.77	18.16	10.75
Average % CV		4.77			9.81			12.23	

Supplementary Table 1. (Raw data to complement Figure 4.1.) Intra-assay precision of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis.

Cell type	Superoxide levels (AU)			Mitochondrial mass (AU)			Mitochondrial membrane potential (AU)		
PBMCs									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	14.65	22.83	40.01	44.42	41.40	56.39	73.43	131.07	169.40
Repeat 2	13.51	24.42	41.77	38.13	48.57	51.27	81.74	88.29	186.51
Repeat 3	15.57	20.80	39.41	31.21	53.17	49.59	84.04	126.26	217.42
Mean	14.58	22.68	40.40	37.92	47.71	52.42	79.74	115.21	191.11
SD	1.03	1.81	1.23	6.61	5.93	3.54	5.58	23.43	24.34
% CV	7.08	8.00	3.04	17.42	12.43	6.76	7.00	20.34	12.74
Average % CV		6.04			12.20			22.45	
Lymphocytes									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	11.15	19.62	30.89	52.24	83.36	81.10	6.83	8.57	9.79
Repeat 2	11.49	18.32	27.28	46.65	74.48	82.55	8.01	7.21	9.59
Repeat 3	12.04	17.88	27.69	36.43	75.90	94.40	8.08	9.48	8.15
Mean	11.56	18.61	28.62	45.11	77.91	86.02	7.64	8.42	9.18
SD	0.45	0.90	1.98	8.02	4.77	7.30	0.70	1.14	0.89
% CV	3.89	4.86	6.91	17.77	6.12	8.48	9.21	13.55	9.72
Average % CV		5.22			10.79			10.83	
Monocytes									
Control sample	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Repeat 1	35.45	65.56	94.21	73.43	131.07	169.40	1.58	1.21	2.35
Repeat 2	29.46	70.34	94.18	81.74	88.29	186.51	2.12	2.35	2.34
Repeat 3	33.84	72.07	104.59	84.04	126.26	217.42	1.72	2.40	2.40
Mean	32.92	69.32	97.66	79.74	115.21	191.11	1.81	1.99	2.36
SD	3.10	3.37	6.00	5.58	23.43	24.34	0.28	0.67	0.04
% CV	9.42	4.86	6.15	7.00	20.34	12.74	15.69	33.82	1.57
Average % CV		6.81			13.36			17.02	

Supplementary Table 2. (Raw data to complement Figure 4.2.) Inter-assay precision of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis.

	8-iso-prostaglandin $F_{2\alpha}$ (ng/ml)		
Control sample	**1**	**2**	**3**
Repeat 1	0.69	5.67	8.32
Repeat 2	0.64	5.73	8.72
Repeat 3	0.25	5.71	9.21
Repeat 4	1.68	6.09	9.73
Repeat 5	1.17	5.82	9.70
Repeat 6	0.43	5.74	9.43
Repeat 7	0.47	5.74	8.67
Repeat 8	0.72	5.80	8.22
Repeat 9	0.25	5.86	9.22
Repeat 10	0.36	5.53	8.74
Mean	0.67	5.77	9.00
SD	0.45	0.14	0.54
% CV	67.34	2.51	6.00
Average % CV		25.28	

Supplementary Table 3. (Raw data to complement Figure 4.3.) Intra-assay precision of 8-iso-prostaglandin $F_{2\alpha}$ by AutoDELFIA analysis.

	8-iso-prostaglandin $F_{2\alpha}$ (ng/ml)		
Control sample	**1**	**2**	**3**
Repeat 1	1.27	5.63	9.44
Repeat 2	0.40	6.33	9.41
Repeat 3	0.42	5.51	9.02
Repeat 4	0.36	6.04	9.17
Repeat 5	0.49	5.80	9.31
Repeat 6	0.69	6.52	9.88
Repeat 7	0.20	5.55	9.63
Repeat 8	0.85	6.75	9.46
Repeat 9	0.49	6.17	8.65
Repeat 10	0.31	5.40	8.47
Repeat 11	0.58	5.96	9.45
Repeat 12	0.57	6.30	9.12
Repeat 13	0.79	5.61	8.28
Repeat 14	1.41	5.57	8.71
Repeat 15	0.09	5.81	9.36
Repeat 16	0.51	5.92	9.50
Repeat 17	0.61	4.86	8.18
Repeat 18	0.42	6.24	9.05
Repeat 19	1.30	6.86	9.25
Repeat 20	0.54	6.22	9.31
Mean	0.62	5.95	9.13
SD	0.36	0.49	0.46
% CV	57.73	8.20	4.99
Average % CV		23.64	

Supplementary Table 4. (Raw data to complement Figure 4.4.) Inter-assay precision of 8-iso-prostaglandin $F_{2\alpha}$ by AutoDELFIA analysis.

Cell type	Superoxide levels (AU)						Mitochondrial mass (AU)						Mitochondrial membrane potential (AU)					
PBMCs																		
Contol subject	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Day 1 (Baseline)	33.43	52.82	49.76	63.00	56.70	49.75	37.01	40.45	40.33	32.24	32.09	45.59	5.34	3.53	3.09	2.64	3.20	3.70
Day 7	49.32	58.24	53.98	58.98	59.31	48.21	32.90	41.42	42.69	36.42	32.20	44.22	9.43	6.63	7.98	5.50	4.56	4.57
Mean	41.37	55.53	51.87	60.99	58.01	48.98	34.95	40.93	41.51	34.33	32.15	44.91	7.38	5.08	5.54	4.07	3.88	4.14
SD	11.24	3.83	2.99	2.84	1.85	1.09	2.91	0.68	1.67	2.95	0.08	0.97	2.89	2.19	3.46	2.02	0.96	0.61
% CV	27.17	6.90	5.76	4.66	3.18	2.22	8.32	1.67	4.02	8.60	0.24	2.16	39.20	43.20	62.47	49.68	24.73	14.87
Average % CV	8.31						4.17						39.02					
Lymphocytes																		
Contol subject	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Day 1 (Baseline)	31.01	44.35	40.52	48.41	51.56	43.89	35.31	37.53	35.84	30.60	30.06	44.43	6.57	4.49	5.33	4.62	3.99	4.70
Day 7	39.64	51.42	46.62	45.47	50.42	40.96	28.14	39.57	36.95	33.57	30.10	42.26	10.31	6.88	8.62	6.90	5.09	4.96
Mean	35.32	47.88	43.57	46.94	50.99	42.43	31.72	38.55	36.40	32.08	30.08	43.34	8.44	5.68	6.98	5.76	4.54	4.83
SD	6.10	5.00	4.32	2.08	0.81	2.07	5.07	1.44	0.78	2.10	0.03	1.54	2.64	1.69	2.33	1.61	0.78	0.19
% CV	17.27	10.45	9.91	4.42	1.59	4.88	15.97	3.74	2.16	6.56	0.09	3.55	31.32	29.74	33.35	28.03	17.21	3.88
Average % CV	8.09						5.34						23.92					
Monocytes																		
Contol subject	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Day 1 (Baseline)	85.41	104.61	90.34	96.78	134.59	128.06	65.19	57.44	51.91	36.95	47.16	53.24	3.62	2.10	1.34	1.78	2.42	2.73
Day 7	96.99	130.57	106.29	105.80	120.47	90.42	49.63	57.78	65.92	50.13	43.52	66.51	8.57	5.91	7.18	4.69	3.67	4.05
Mean	91.20	117.59	98.31	101.29	127.53	109.24	57.41	57.61	58.91	43.54	45.34	59.87	6.09	4.01	4.26	3.23	3.04	3.39
SD	8.18	18.36	11.28	6.38	9.98	26.62	11.00	0.24	9.91	9.32	2.57	9.39	3.50	2.70	4.13	2.06	0.88	0.93
% CV	8.97	15.61	11.47	6.30	7.83	24.37	19.17	0.41	16.82	21.41	5.68	15.68	57.46	67.29	97.00	63.70	28.95	27.43
Average % CV	12.42						13.19						56.97					

Supplementary Table 5. (Raw data to complement Figure 4.5.) Short-term day-day precision of superoxide levels, mitochondrial mass and mitochondrial membrane potential in PBMCs and cell subpopulations, lymphocytes and monocytes, by flow cytometry analysis. (Day 1 and day 7 measured independently)

	8-iso-prostaglandin $F_{2\alpha}$ (ng/ml)															
Control subject	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Day 1 (Baseline)	0.58	1.99	0.25	3.74	31.73	1.99	2.18	7.03	2.33	18.89	2.48	0.25	8.00	1.19	2.10	0.25
Day 7	0.25	2.82	8.07	3.99	38.72	0.25	0.25	4.92	0.25	13.43	0.26	0.25	6.72	1.11	1.40	0.25
Mean	0.42	2.40	4.16	3.87	35.22	1.12	1.21	5.98	1.29	16.16	1.37	0.25	7.36	1.15	1.75	0.25
SD	0.24	0.58	5.53	0.18	4.94	1.23	1.36	1.49	1.47	3.87	1.57	0.00	0.91	0.05	0.50	0.00
% CV	56.48	24.35	132.92	4.58	14.03	109.80	112.30	24.96	113.98	23.93	115.02	0.00	12.35	4.59	28.55	0.00
Average % CV	48.62															

Supplementary Table 6. (Raw data to complement Figure 4.7.) Short-term day-day precision of 8-iso-prostaglandin $F_{2\alpha}$ by AutoDELFIA analysis. (Day 1 and day 7 measured on the same occasion)

Potential oxidative stress-related BoA	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	n	p	Statistic	n	p
Superoxide levels (AU) [P3]						
PBMCs	0.12	248	**0.00**	0.85	248	**0.00**
Lymphocytes	0.14	248	**0.00**	0.80	248	**0.00**
Monocytes	0.14	248	**0.00**	0.86	248	**0.00**
Mitochondrial mass (AU) [P3]						
PBMCs	0.07	341	**0.00**	0.96	341	**0.00**
Lymphocytes	0.13	341	**0.00**	0.76	341	**0.00**
Monocytes	0.14	341	**0.00**	0.91	341	**0.00**
Mitochondrial membrane potential (AU) [P3]						
PBMCs	0.17	347	**0.00**	0.68	347	**0.00**
Lymphocytes	0.12	347	**0.00**	0.89	347	**0.00**
Monocytes	0.16	347	**0.00**	0.83	347	**0.00**
8-Iso Prostaglandin $F_{2\alpha}$ (ng/ml) [P3]	0.24	379	**0.00**	0.70	379	**0.00**

Supplementary Table 7. Normality tests of continuous potential oxidative stress-related BoA of the very old population from the Newcastle 85+ Study. ([P3]: Phase 3 data, a: Lilliefors Significance Correction)

Potential confounders	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	n	p	Statistic	n	p
Age of parents death [P1]						
Mother	0.11	810	**0.00**	0.93	810	**0.00**
Father	0.10	776	**0.00**	0.95	776	**0.00**
Number of years in main job/role [P1]	0.07	666	**0.00**	0.97	666	**0.00**
Self-reported physical activity score [P3]	0.16	484	**0.00**	0.90	484	**0.00**
Social isolation score [P3]	0.06	480	**0.00**	0.99	480	**0.01**
Body mass index [P3]	0.04	401	0.10	0.99	401	**0.00**

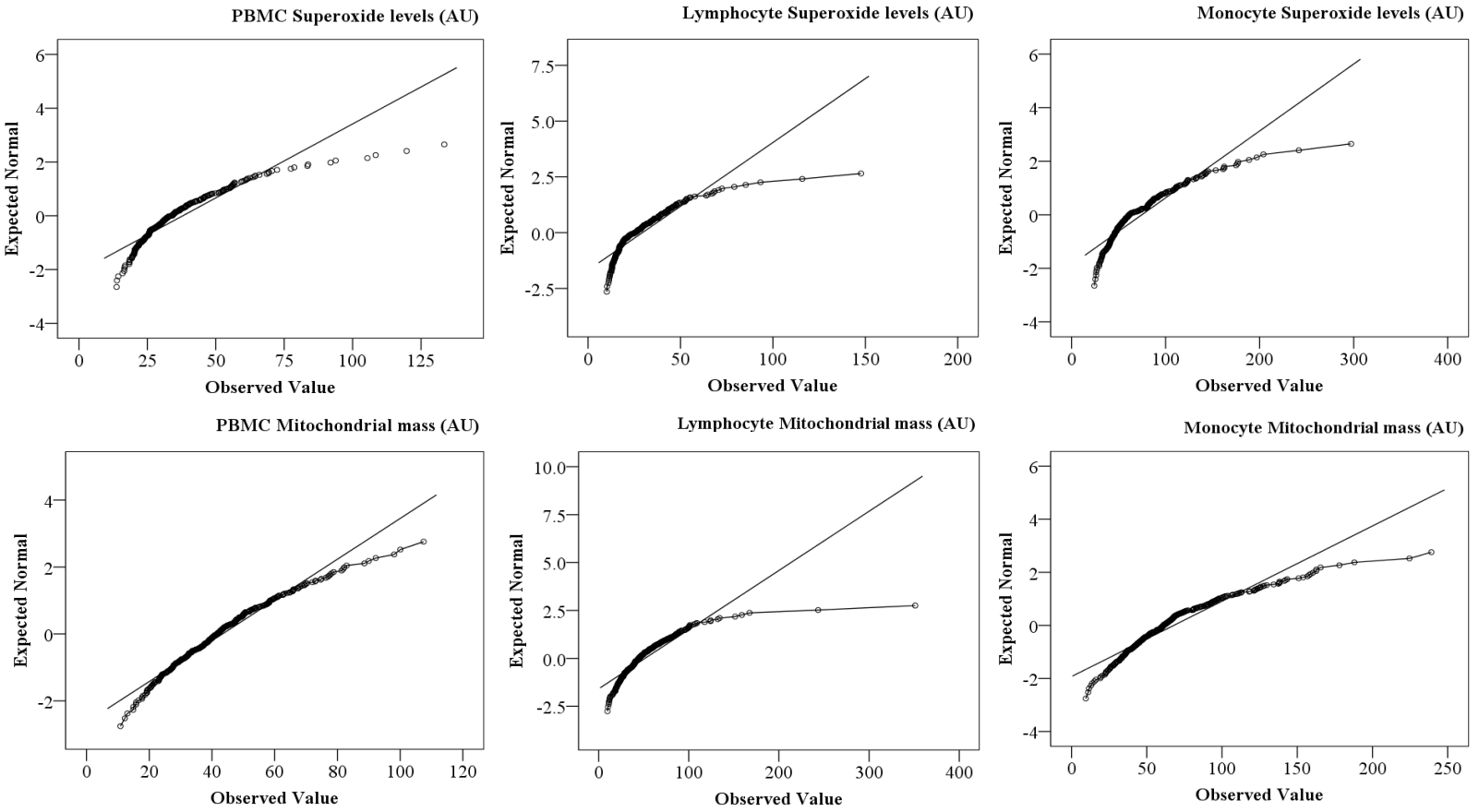
Supplementary Table 8. Normality tests of continuous potential confounders BoA of the very old population from the Newcastle 85+ Study. ([P1]: Phase 1 data, [P3]: Phase 3 data, a: Lilliefors Significance Correction)

Age-related construct variables	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	n	p	Statistic	n	p
PBMC telomere length (bp) [P3]	0.07	430	**0.00**	0.98	430	**0.00**
PBMC DNA damage (%) [P3]	0.04	431	0.06	0.98	431	**0.00**
PBMC DNA repair (%) [P3]	0.07	431	**0.00**	0.96	431	**0.00**
Interleukin-6 (pg/ml) [P3]	0.45	430	**0.00**	0.19	430	**0.00**
Tumour necrosis factor-alpha (pg/ml) [P3]	0.45	430	**0.00**	0.17	430	**0.00**
Glycosylated haemoglobin (%) [P3]	0.13	428	**0.00**	0.89	428	**0.00**
High sensitivity C-reactive protein (mg/l) [P3]	0.34	434	**0.00**	0.36	434	**0.00**
Albumin (g/l) [P3]	0.09	434	**0.00**	0.96	434	**0.00**
Rheumatoid factor (IU/ml) [P1]	0.44	774	**0.00**	0.12	774	**0.00**
Lymphocyte/Monocyte ratio [P3]	0.16	427	**0.00**	0.49	427	**0.00**
CD4 / Upper CD8 T lymphocyte ratio [P3]	0.45	405	**0.00**	0.05	405	**0.00**
Memory / Naive B lymphocyte ratio [P3]	0.39	429	**0.00**	0.19	429	**0.00**
Memory / Naive CD4 T lymphocyte ratio [P3]	0.37	428	**0.00**	0.29	428	**0.00**
Memory / Naive CD8 T lymphocyte ratio [P3]	0.29	402	**0.00**	0.55	402	**0.00**
Senescent (CD27-/RO-) CD4 T lymphocytes (%) [P3]	0.29	429	**0.00**	0.53	429	**0.00**
Senescent (CD27-/RO-) CD8 T lymphocytes (%) [P3]	0.08	403	**0.00**	0.95	403	**0.00**
Hand grip strength (kg) [P3]	0.07	453	**0.00**	0.97	453	**0.00**
Timed up and go test (seconds) [P3]	0.22	402	**0.00**	0.54	402	**0.00**
Forced expiratory volume in 1 second (l) [P1]	0.07	776	**0.00**	0.98	776	**0.00**
Systolic blood pressure (mmHg) [P3]	0.03	462	0.20	1.00	462	0.26
Haematocrit (%) [P3]	0.04	427	0.20	1.00	427	0.24
Haemoglobin (g/dl) [P3]	0.05	427	**0.01**	0.99	427	0.06
Red blood cells (x 10^12/l) [P3]	0.04	427	0.09	1.00	427	0.19
Free triiodothyronine (pmol/l) [P3]	0.08	426	**0.00**	0.99	426	**0.00**
Vitamin D (nmol/l) [P1]	0.11	778	**0.00**	0.93	778	**0.00**
Prohormone brain-type natriuretic peptide (pg/ml) [P3]	0.21	124	**0.00**	0.68	124	**0.00**

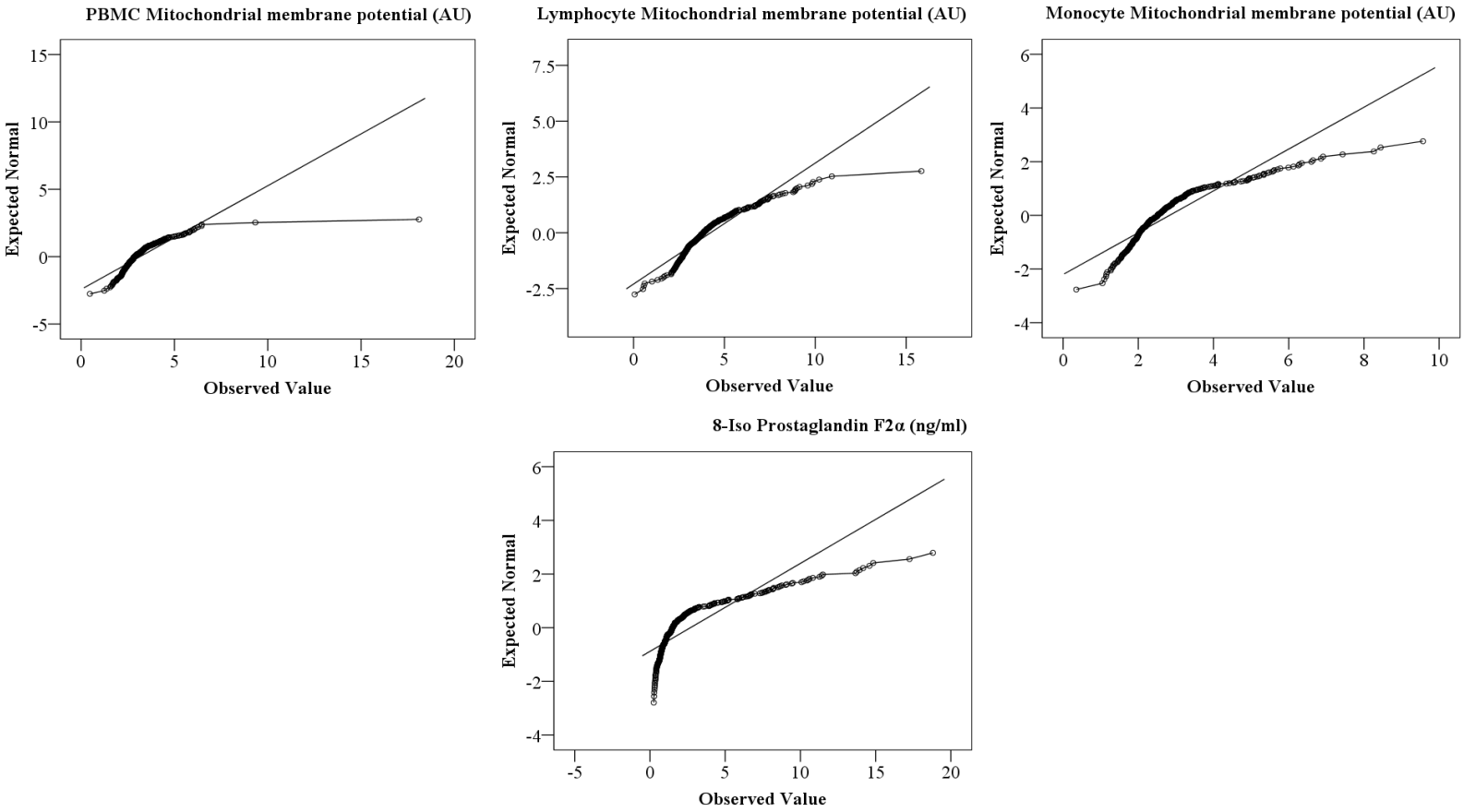
Supplementary Table 9. Normality tests of continuous age-related construct variables of the very old population from the Newcastle 85+ Study. ([P1]: Phase 1 data, [P3]: Phase 3 data, a: Lilliefors Significance Correction)

Age-related outcomes	Normality test					
	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Disability score [P3]	0.11	482	**0.00**	0.94	482	**0.00**
SMMSE score [P3]	0.20	471	**0.00**	0.75	471	**0.00**
Disease Only Morbidity Count [P3]	0.16	751	**0.00**	0.94	751	**0.00**

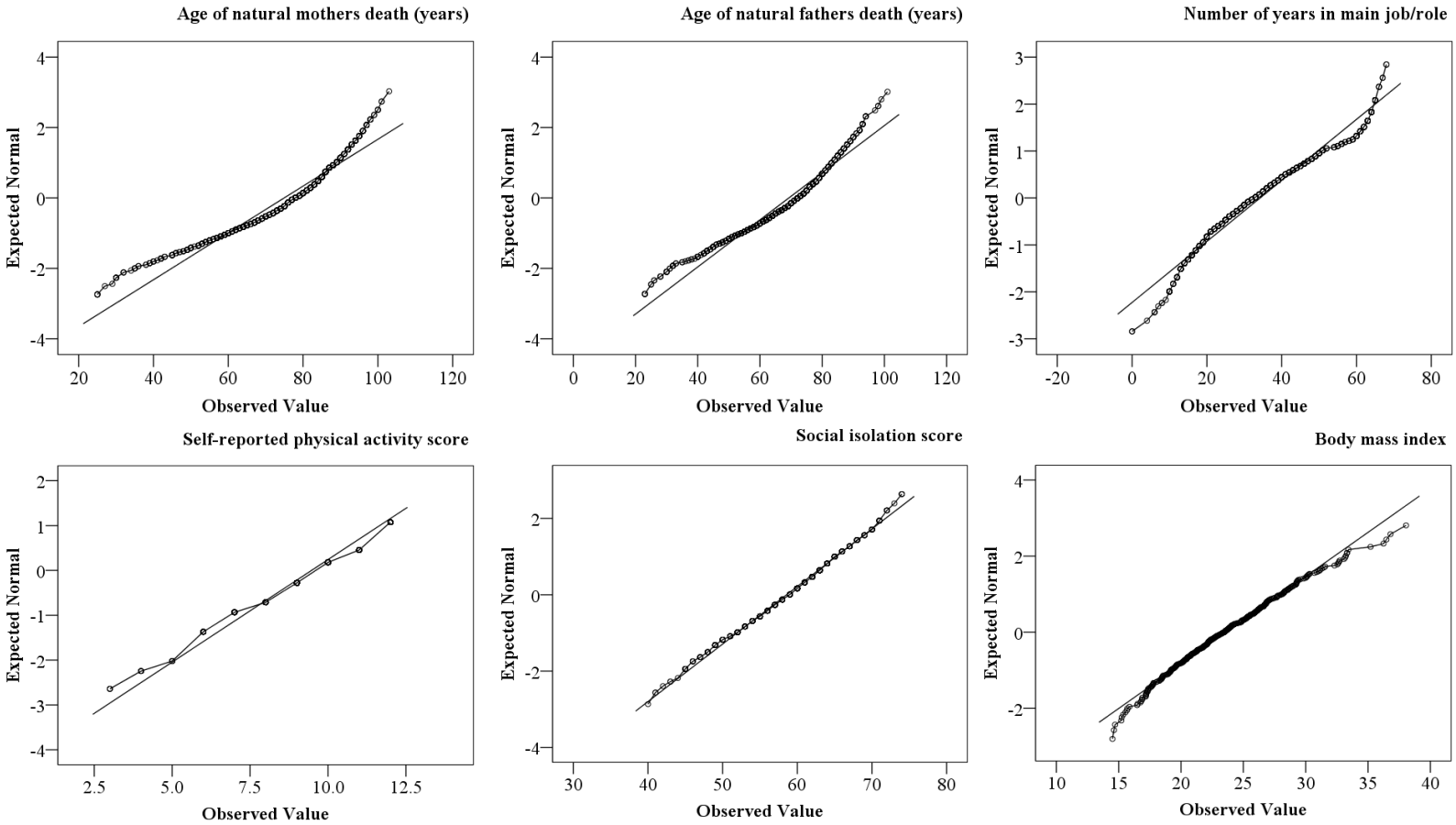
Supplementary Table 10. Normality tests of continuous age-related outcomes of the very old population from the Newcastle 85+ Study. ([P3]: Phase 3 data, a: Lilliefors Significance Correction)


PBMC Superoxide levels (AU)
Expected Normal
Observed Value
Lymphocyte Superoxide levels (AU)
Expected Normal
Observed Value
Monocyte Superoxide levels (AU)
Expected Normal
Observed Value
PBMC Mitochondrial mass (AU)
Expected Normal
Observed Value
Lymphocyte Mitochondrial mass (AU)
Expected Normal
Observed Value
Monocyte Mitochondrial mass (AU)
Expected Normal
Observed Value

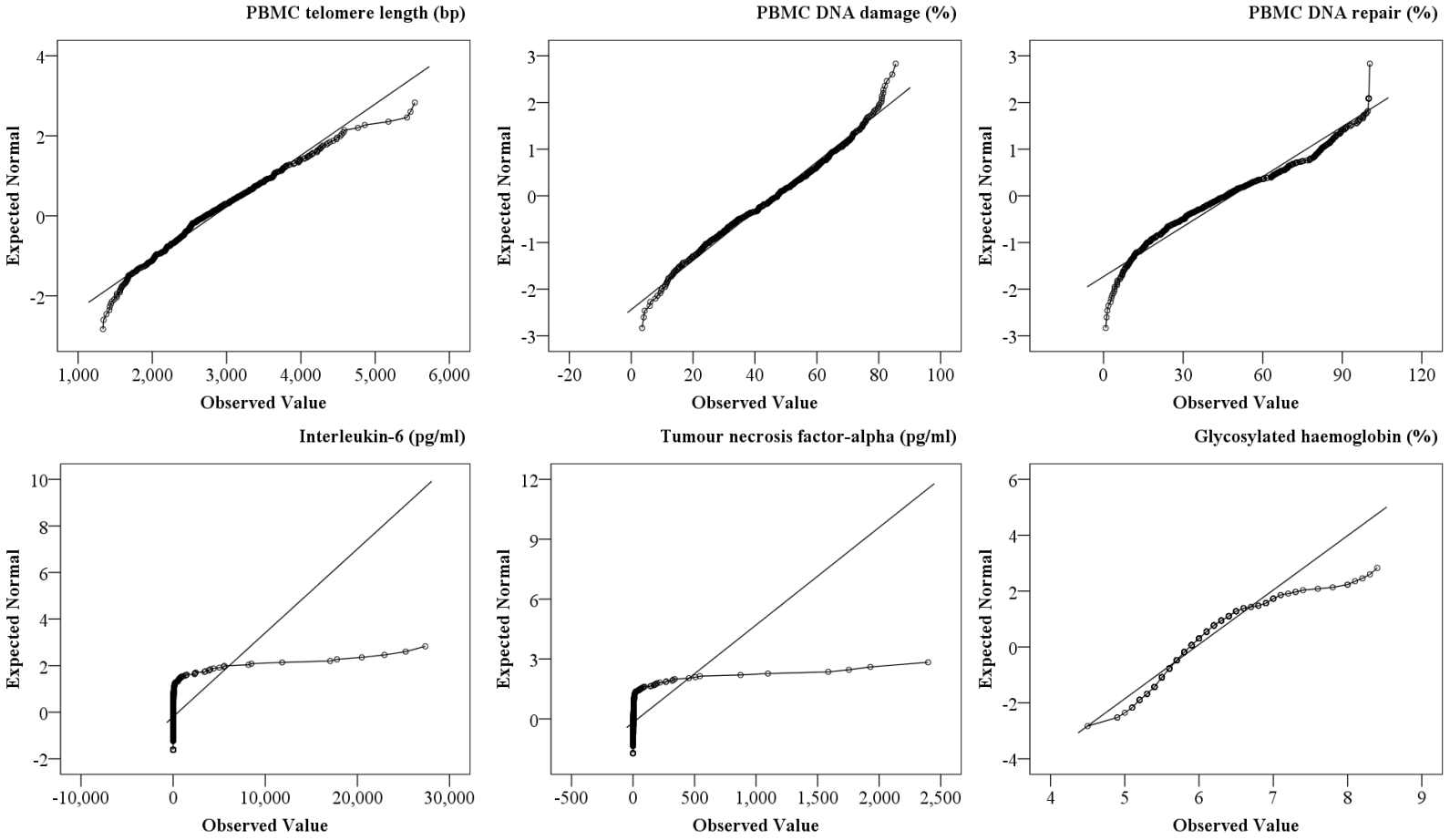
Cont...



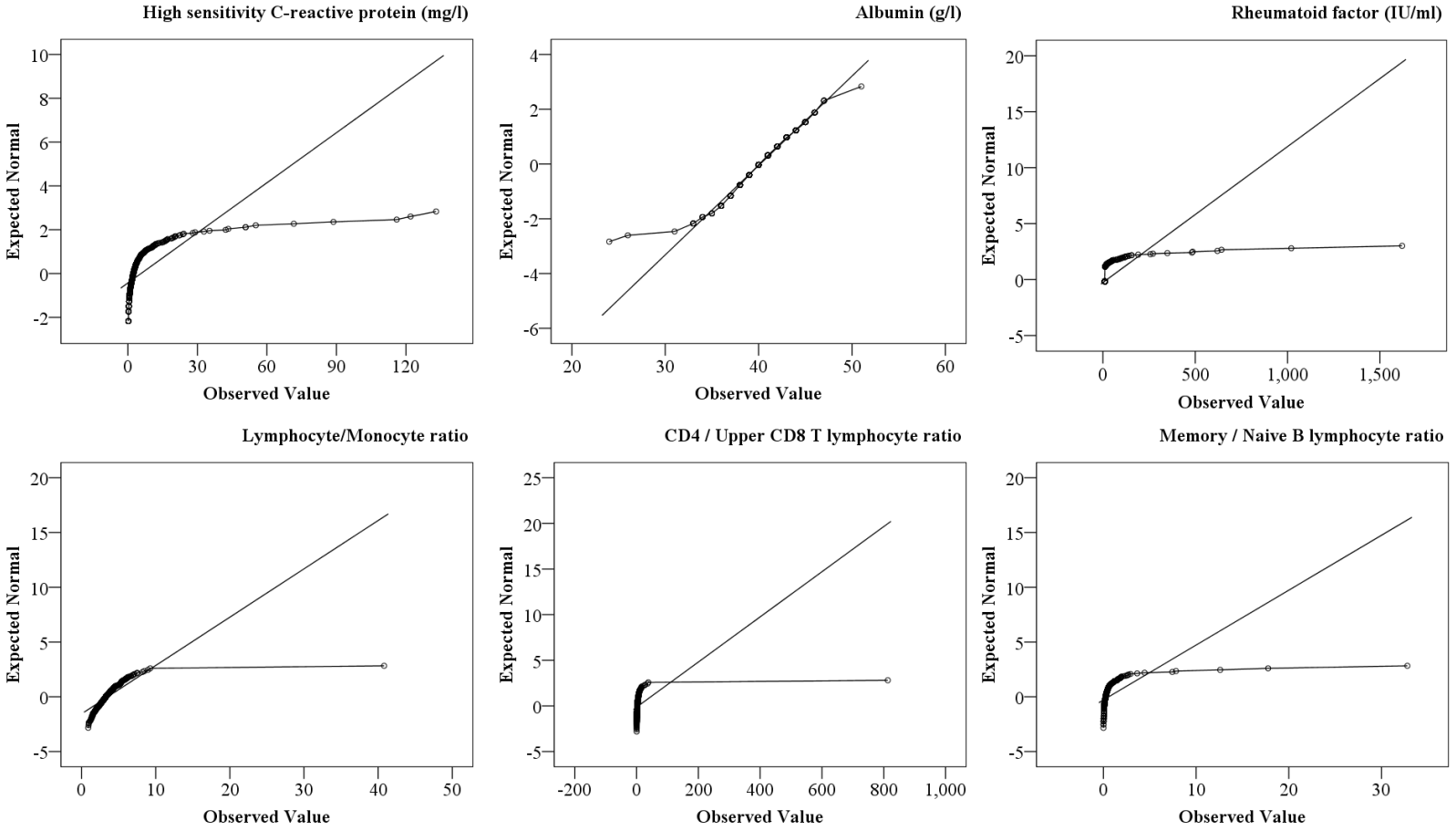
Supplementary Figure 1. Q-Q plots of continuous potential oxidative stress-related BoA of the very old population from the Newcastle 85+ Study.



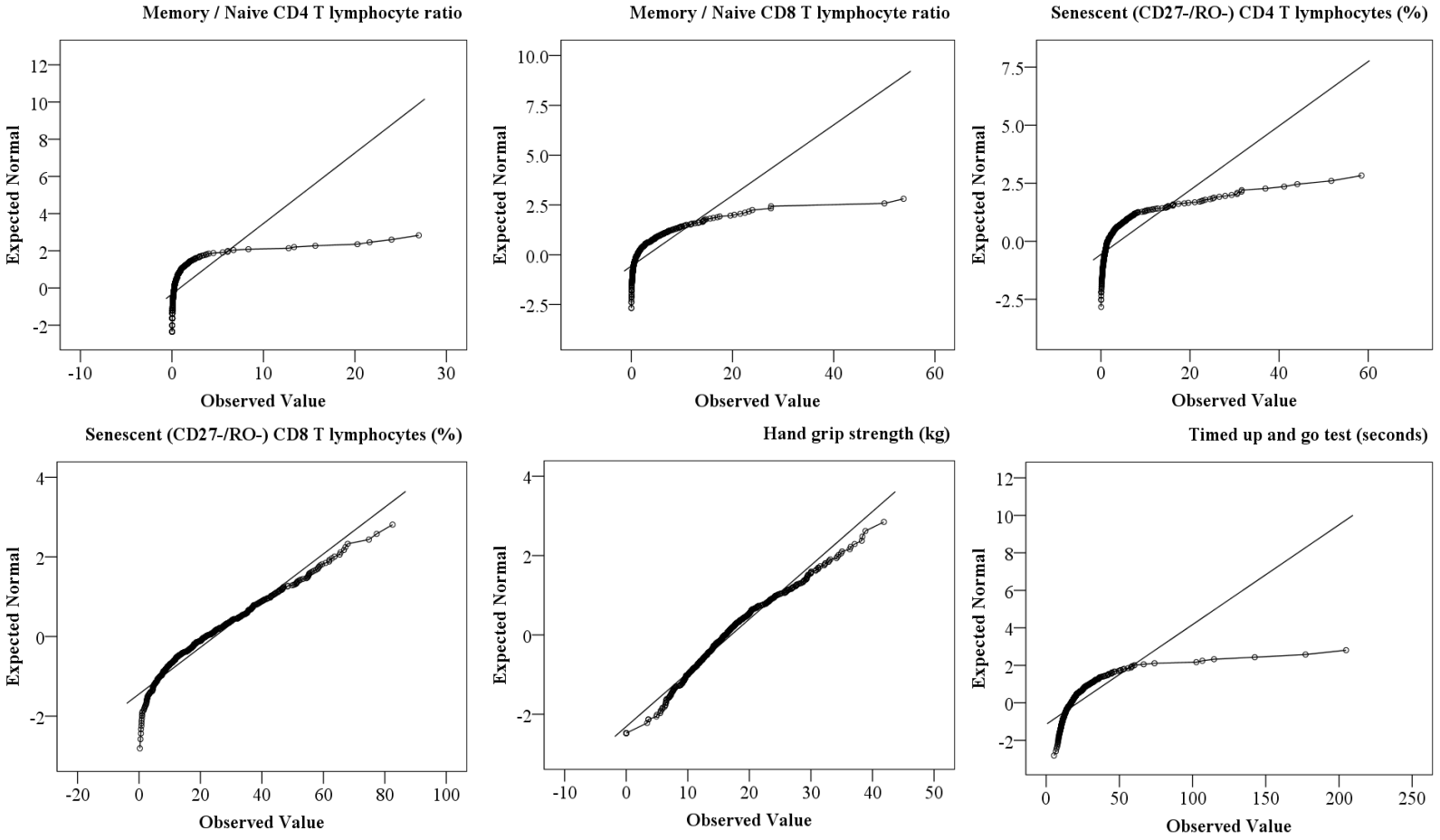
Supplementary Figure 2. Q-Q plots of continuous potential confounders BoA of the very old population from the Newcastle 85+ Study.


PBMC telomere length (bp)
Expected Normal
Observed Value
PBMC DNA damage (%)
Expected Normal
Observed Value
PBMC DNA repair (%)
Expected Normal
Observed Value
Interleukin-6 (pg/ml)
Expected Normal
Observed Value
Tumour necrosis factor-alpha (pg/ml)
Expected Normal
Observed Value
Glycosylated haemoglobin (%)
Expected Normal
Observed Value

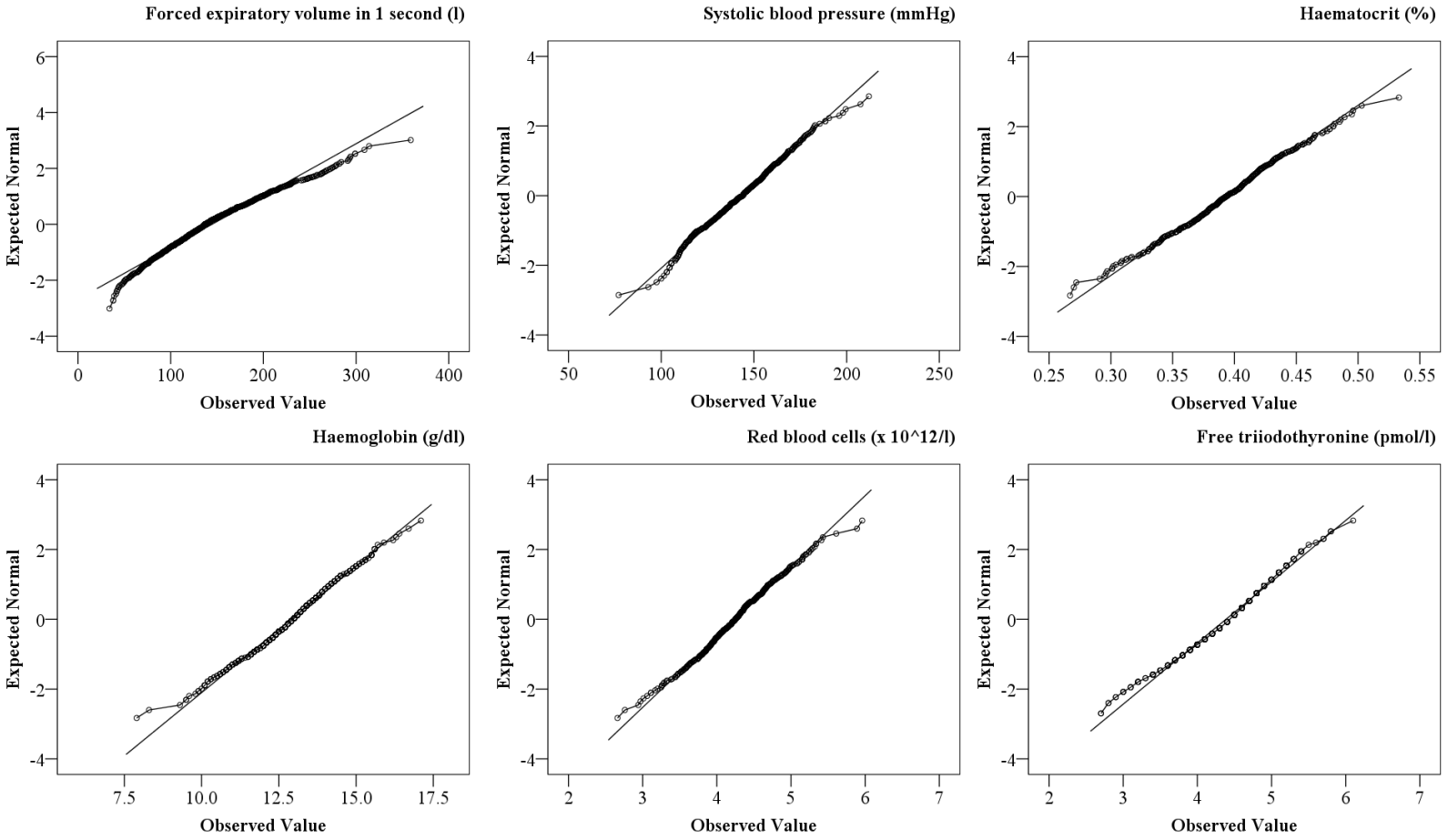
Cont...


High sensitivity C-reactive protein (mg/l)
Expected Normal
Observed Value
Albumin (g/l)
Expected Normal
Observed Value
Rheumatoid factor (IU/ml)
Expected Normal
Observed Value
Lymphocyte/Monocyte ratio
Expected Normal
Observed Value
CD4 / Upper CD8 T lymphocyte ratio
Expected Normal
Observed Value
Memory / Naive B lymphocyte ratio
Expected Normal
Observed Value

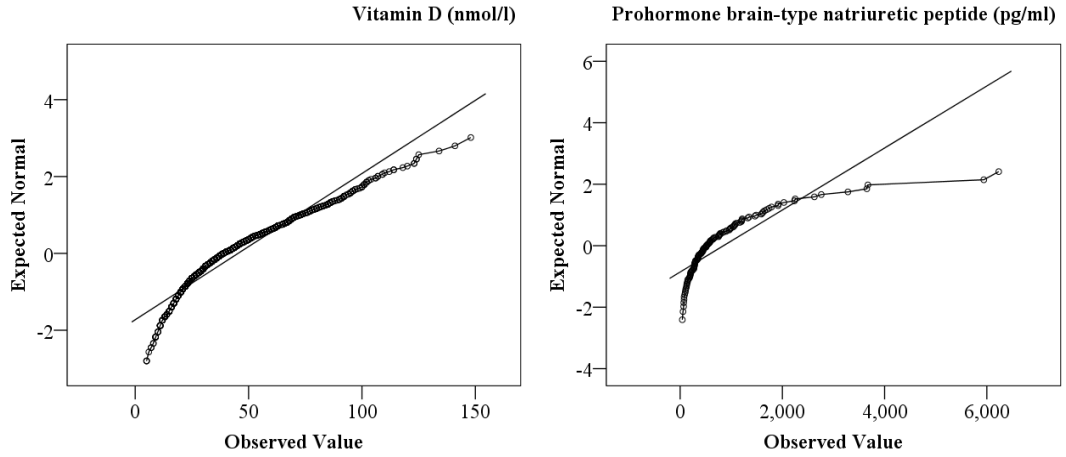
Cont...


Memory / Naive CD4 T lymphocyte ratio
Expected Normal
Observed Value
Memory / Naive CD8 T lymphocyte ratio
Expected Normal
Observed Value
Senescent (CD27-/RO-) CD4 T lymphocytes (%)
Expected Normal
Observed Value
Senescent (CD27-/RO-) CD8 T lymphocytes (%)
Expected Normal
Observed Value
Hand grip strength (kg)
Expected Normal
Observed Value
Timed up and go test (seconds)
Expected Normal
Observed Value

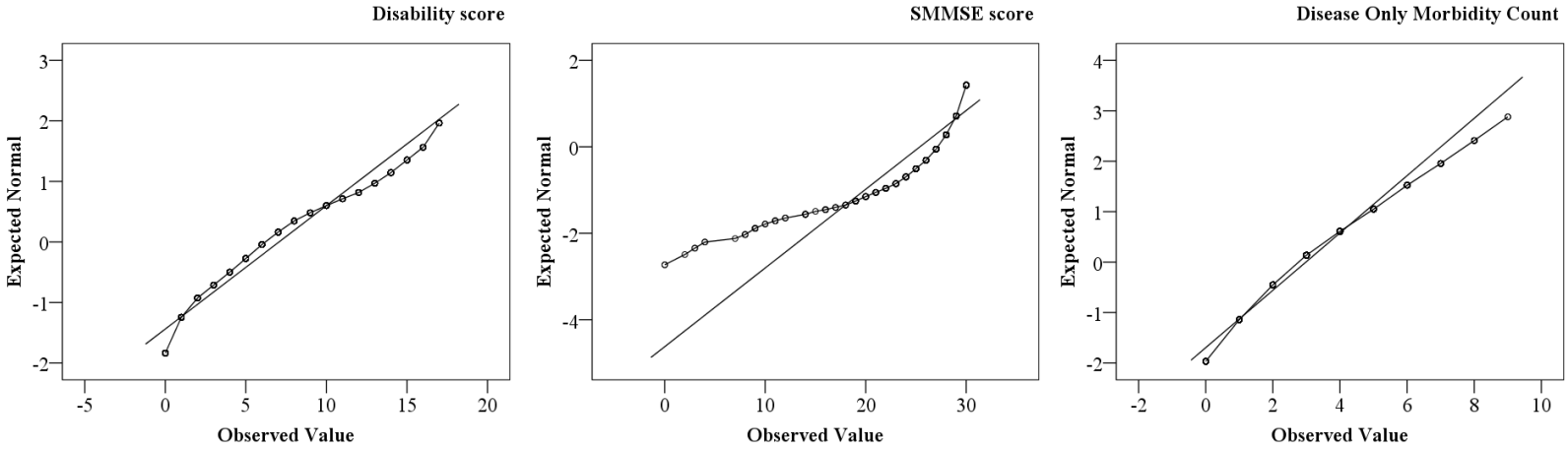
Cont...


Forced expiratory volume in 1 second (l)
Expected Normal
Observed Value
Systolic blood pressure (mmHg)
Expected Normal
Observed Value
Haematocrit (%)
Expected Normal
Observed Value
Haemoglobin (g/dl)
Expected Normal
Observed Value
Red blood cells (x 10^12/l)
Expected Normal
Observed Value
Free triiodothyronine (pmol/l)
Expected Normal
Observed Value

Cont...



Supplementary Figure 3. Q-Q plots of continuous age-related construct variables of the very old population from the Newcastle 85+ Study.



Supplementary Figure 4. Q-Q plots of continuous age-related construct variables of the very old population from the Newcastle 85+ Study.

Potential oxidative stress-related BoA	All			A.				B.				C.				D.			
	Statistic		n	Statistic		n	p	Statistic		n	p	Statistic		n	p	Statistic		n	p
Superoxide levels (AU) [P3]																			
PBMCs	33.94	20.17	(248)	33.94	20.17	(248)	-	34.29	20.01	(243)	0.76	34.29	20.07	(239)	0.91	34.31	19.72	(216)	0.70
Lymphocytes	25.39	20.81	(248)	25.39	20.81	(248)	-	26.05	20.83	(243)	0.79	25.69	20.82	(239)	0.86	26.06	20.75	(216)	0.79
Monocyte	61.49	46.09	(248)	61.49	46.09	(248)	-	62.07	46.24	(243)	0.83	61.66	46.22	(239)	0.87	63.17	46.60	(216)	0.79
Mitochondrial mass (AU) [P3]																			
PBMCs	41.69	19.99	(341)	40.09	18.62	(243)	0.36	41.69	19.99	(341)	-	41.86	19.76	(332)	0.99	41.89	20.64	(302)	0.75
Lymphocytes	44.60	32.06	(341)	44.89	37.43	(243)	0.55	44.60	32.06	(341)	-	44.60	31.49	(332)	0.88	44.57	31.29	(302)	0.96
Monocytes	61.03	40.24	(341)	61.03	45.38	(243)	0.93	61.03	40.24	(341)	-	61.05	38.75	(332)	0.99	61.29	40.47	(302)	0.89
Mitochondrial membrane potential (AU) [P3]																			
PBMCs	2.86	0.98	(347)	2.90	1.07	(239)	0.83	2.88	1.07	(332)	0.79	2.86	0.98	(347)	-	2.87	1.02	(304)	0.83
Lymphocytes	3.85	2.00	(347)	4.02	2.32	(239)	0.07	3.87	2.08	(332)	0.68	3.85	2.00	(347)	-	3.87	2.14	(304)	0.77
Monocytes	2.51	1.16	(347)	2.50	1.18	(239)	0.98	2.52	1.19	(332)	0.97	2.51	1.16	(347)	-	2.52	1.19	(304)	0.79
8-Iso Prostaglandin $F_{2\alpha}$ (ng/ml) [P3]	1.49	2.08	(379)	1.47	2.09	(216)	0.94	1.49	1.99	(302)	0.82	1.47	2.05	(304)	0.96	1.49	2.08	(379)	-

Supplementary Table 11. Descriptive statistics of potential oxidative stress-related BoA of the very old population from the Newcastle 85+ Study. A. Superoxide levels population only, B. Mitochondrial mass population only, C. Mitochondrial membrane potential population only and D. 8-Iso Prostaglandin $F_{2\alpha}$ population only. Continuous data are displayed as median and, inter-quartile ranges since all measures had a non-normal distribution. ([P3]: Phase 3 data)

Potential confounders	All			A.				B.				C.				D.			
	Statistic		n	Statistic		n	p	Statistic		n	p	Statistic		n	p	Statistic		n	p
Gender [P1]																			
Male	36.12	%	(375)	36.69	%	(91)	0.87	36.36	%	(124)	0.94	37.75	%	(131)	0.59	38.26	%	(145)	0.46
Female	63.87	%	(663)	63.31	%	(157)		63.64	%	(217)		62.25	%	(216)		61.74	%	(234)	
Ethnicity [P1]																			
White	99.65	%	(845)	99.19	%	(246)		99.41	%	(339)		99.42	%	(345)		99.21	%	(376)	
Black - Caribbean	0.12	%	(1)	0.40	%	(1)		0.29	%	(1)		0.29	%	(1)		0.26	%	(1)	
Black - African	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Black - Other black groups	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Indian	0.12	%	(1)	0.40	%	(1)	0.57	0.29	%	(1)	0.73	0.29	%	(1)	0.74	0.26	%	(1)	0.80
Pakistani	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Bangladeshi	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Chinese	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
None of these	0.12	%	(1)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.26	%	(1)	
Mitochondrial haplogroup [P1]																			
H	46.18	%	(326)	42.58	%	(89)		42.91	%	(127)		43.71	%	(132)		46.85	%	(156)	
T	10.20	%	(72)	11.96	%	(25)		11.49	%	(34)		11.26	%	(34)		10.21	%	(34)	
J	10.91	%	(77)	10.53	%	(22)		11.49	%	(34)		11.59	%	(35)		10.51	%	(35)	
U	15.44	%	(109)	17.22	%	(36)	0.98	16.89	%	(50)	0.99	16.89	%	(51)	0.97	15.62	%	(52)	1.00
K	7.22	%	(51)	7.18	%	(15)		7.43	%	(22)		7.95	%	(24)		8.11	%	(27)	
X	1.84	%	(13)	1.91	%	(4)		1.69	%	(5)		1.32	%	(4)		1.50	%	(5)	
W	2.83	%	(20)	3.83	%	(8)		3.38	%	(10)		3.31	%	(10)		2.70	%	(9)	

I	1.84	%	(13)	1.44	%	(3)		1.35	%	(4)		1.32	%	(4)		1.20	%	(4)	
V	3.54	%	(25)	3.35	%	(7)		3.38	%	(10)		2.65	%	(8)		3.30	%	(11)	
Age of natural parents death (years) [P1]																			
Mother	78.00	19.00	(810)	80.00	18.00	(239)	0.37	79.00	18.75	(332)	0.68	79.00	18.00	(336)	0.65	78.00	18.00	(366)	0.70
Father	72.00	19.00	(776)	72.00	19.75	(232)	0.99	74.00	19.00	(318)	0.19	74.00	19.00	(322)	0.27	73.00	17.00	(351)	0.42
Place of birth [P1]																			
Country																			
England	91.03	%	(771)	90.32	%	(224)		90.62	%	(309)		90.49	%	(314)		91.03	%	(345)	
Wales	0.71	%	(6)	0.40	%	(1)		0.88	%	(3)		0.86	%	(3)		0.53	%	(2)	
Scotland	4.49	%	(38)	6.05	%	(15)		5.28	%	(18)		5.19	%	(18)		4.75	%	(18)	
Northern Ireland	0.47	%	(4)	0.40	%	(1)	0.70	0.29	%	(1)	0.83	0.29	%	(1)	0.87	0.26	%	(1)	0.86
UK (unknown constituent country)	0.00	%	(0)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Republic of Ireland	0.47	%	(4)	0.00	%	(0)		0.00	%	(0)		0.00	%	(0)		0.00	%	(0)	
Other Europe	1.53	%	(13)	0.81	%	(2)		1.17	%	(4)		1.44	%	(5)		1.85	%	(7)	
Non-Europe	1.30	%	(11)	2.02	%	(5)		1.76	%	(6)		1.73	%	(6)		1.58	%	(6)	
Region of England																			
North East England	84.55	%	(651)	82.14	%	(184)		83.82	%	(259)		83.44	%	(262)		82.90	%	(286)	
Cumbria	0.91	%	(7)	0.45	%	(1)	0.47	0.32	%	(1)	0.53	0.32	%	(1)	0.47	0.58	%	(2)	0.60
Other	14.55	%	(112)	17.41	%	(39)		15.86	%	(49)		16.24	%	(51)		16.52	%	(57)	
Has/had a sibling(s) [P1]																			
Yes	90.40	%	(763)	89.47	%	(221)	0.67	91.18	%	(310)	0.68	90.75	%	(314)	0.85	89.95	%	(340)	0.80
No	9.60	%	(81)	10.53	%	(26)		8.82	%	(30)		9.25	%	(32)		10.05	%	(38)	
Had full-time higher education [P1]																			
Yes	11.86	%	(100)	13.31	%	(33)	0.54	14.96	%	(51)	0.15	15.27	%	(53)	0.11	15.30	%	(58)	0.10
No	88.14	%	(743)	86.69	%	(215)		85.04	%	(290)		84.73	%	(294)		84.70	%	(321)	

Number of years in main job/role [P1]	33.00	23.00	(666)	33.00	21.00	(197)	0.94	34.00	21.75	(264)	0.44	34.00	22.00	(271)	0.39	34.00	22.00	(297)	0.46
Has offspring [P1]																			
Yes	82.78	%	(702)	83.40	%	(206)	0.82	83.53	%	(284)	0.76	83.53	%	(289)	0.76	84.13	%	(318)	0.56
No	17.22	%	(146)	16.60	%	(41)		16.47	%	(56)		16.47	%	(57)		15.87	%	(60)	
Martial status [P3]																			
Single	8.92	%	(43)	7.69	%	(19)		8.26	%	(28)		8.70	%	(30)		7.43	%	(28)	
Married	22.41	%	(108)	21.86	%	(54)		22.71	%	(77)		22.61	%	(78)		24.14	%	(91)	
Re-married	1.66	%	(8)	1.21	%	(3)	0.88	1.77	%	(6)	1.00	2.03	%	(7)	1.00	1.86	%	(7)	0.96
Separated	0.21	%	(1)	0.00	%	(0)		0.29	%	(1)		0.29	%	(1)		0.27	%	(1)	
Divorced	1.87	%	(9)	2.83	%	(7)		2.06	%	(7)		2.32	%	(8)		1.59	%	(6)	
Widowed	64.94	%	(313)	66.40	%	(164)		64.90	%	(220)		64.06	%	(221)		64.72	%	(244)	
Lives alone [P3]																			
Yes	64.47	%	(274)	64.09	%	(141)	0.93	61.84	%	(188)	0.47	62.38	%	(194)	0.56	62.87	%	(215)	0.65
No	35.53	%	(151)	35.91	%	(79)		38.16	%	(116)		37.62	%	(117)		37.13	%	(127)	
Housing type [P3]																			
Standard housing	74.90	%	(361)	75.61	%	(186)		77.29	%	(262)		76.23	%	(263)		78.51	%	(296)	
Sheltered housing with warden	12.66	%	(61)	13.41	%	(33)	0.83	12.09	%	(41)	0.68	13.62	%	(47)	0.57	11.94	%	(45)	0.36
Institution	12.45	%	(60)	10.98	%	(27)		10.62	%	(36)		10.14	%	(35)		9.55	%	(36)	
Alcohol status [P1]																			
Current drinker	59.93	%	(492)	66.13	%	(164)		65.10	%	(222)		65.71	%	(228)		68.07	%	(258)	
Occasional drinker	20.22	%	(166)	16.53	%	(41)	0.05	18.18	%	(62)	**0.03**	17.87	%	(62)	**0.02**	17.94	%	(68)	**0.01**
Previous drinker	8.77	%	(72)	4.44	%	(11)		4.11	%	(14)		4.03	%	(14)		3.96	%	(15)	
Never drinker	11.08	%	(91)	12.90	%	(32)		12.61	%	(43)		12.39	%	(43)		10.03	%	(38)	

Smoking status [P1]																			
Current smoker	5.79	%	(49)	4.03	%	(10)		4.40	%	(15)		4.32	%	(15)		3.96	%	(15)	
Past smoker	63.52	%	(538)	62.10	%	(154)	0.41	62.46	%	(213)	0.50	62.25	%	(216)	0.44	66.23	%	(251)	0.36
Never smoker	30.70	%	(260)	33.87	%	(84)		33.14	%	(113)		33.43	%	(116)		29.82	%	(113)	
Takes non-prescribed medicines, supplements and/or herbal remedies [P1]																			
Yes	41.08	%	(350)	47.58	%	(118)	0.07	45.45	%	(155)	0.17	45.53	%	(158)	0.16	47.23	%	(179)	**0.04**
No	58.92	%	(502)	52.42	%	(130)		54.55	%	(186)		54.47	%	(189)		52.77	%	(200)	
Takes prescribed medication [P1]																			
Yes	95.07	%	(983)	96.75	%	(238)	0.26	95.87	%	(325)	0.55	95.65	%	(330)	0.66	95.74	%	(360)	0.60
No	4.93	%	(51)	3.25	%	(8)		4.13	%	(14)		4.35	%	(15)		4.26	%	(16)	
Self-reported physical activity score [P3]	9.00	4.00	(484)	9.00	3.00	(248)	0.35	9.00	4.00	(341)	0.22	9.00	4.00	(347)	0.18	9.00	4.00	(379)	0.40
Uses aid(s)/appliance(s) [P1]																			
Yes	85.53	%	(727)	85.08	%	(211)	0.86	82.40	%	(281)	0.18	82.42	%	(286)	0.18	80.74	%	(306)	**0.03**
No	14.47	%	(123)	14.92	%	(37)		17.60	%	(60)		17.58	%	(61)		19.26	%	(73)	
Social isolation score [P3]	59.00	9.00	(480)	59.00	10.00	(246)	0.44	58.00	10.00	(339)	0.16	58.00	10.00	(345)	0.17	58.00	10.00	(376)	0.19
Key event impact [P3]																			
Positive key event(s)	16.74	%	(81)	19.76	%	(49)		18.48	%	(63)		18.16	%	(63)		16.89	%	(64)	
Negative key event(s)	30.79	%	(149)	26.21	%	(65)		27.86	%	(95)		29.11	%	(101)		31.13	%	(118)	
Positive and negative key event(s)	17.15	%	(83)	14.11	%	(35)	0.40	18.48	%	(63)	0.89	18.16	%	(63)	0.97	19.00	%	(72)	0.94
Key event(s) but impact unknown	10.33	%	(50)	10.89	%	(27)		10.56	%	(36)		10.09	%	(35)		10.03	%	(38)	
No key event	25.00	%	(121)	29.03	%	(72)		24.63	%	(84)		24.50	%	(85)		22.96	%	(87)	
Has problems sleeping [P3]																			
Yes	26.29	%	(127)	27.02	%	(67)	0.83	26.10	%	(89)	0.95	26.22	%	(91)	1.00	25.59	%	(97)	0.82
No	73.71	%	(356)	72.98	%	(181)		73.90	%	(252)		73.78	%	(256)		74.41	%	(282)	

Body mass index [P3]	23.47	6.10	(401)	23.83	6.31	(222)	0.48	23.56	6.14	(304)	0.77	23.51	6.13	(310)	0.76	23.29	6.17	(343)	0.77
Fasted blood sample [P3]																			
Yes	-	-	-	0.98	%	(242)	-	0.97	%	(331)	-	0.97	%	(336)	-	0.97	%	(369)	-
No	-	-	-	0.02	%	(6)		0.03	%	(10)		0.03	%	(11)		0.03	%	(10)	
Season of blood sample [P3]																			
Spring	-	-	-	35.08	%	(87)		25.81	%	(88)		24.78	%	(86)		24.01	%	(91)	
Summer	-	-	-	30.24	%	(75)	-	30.79	%	(105)	-	31.41	%	(109)	-	31.93	%	(121)	-
Autumn	-	-	-	13.71	%	(34)		22.29	%	(76)		23.05	%	(80)		24.54	%	(93)	
Winter	-	-	-	20.97	%	(52)		21.11	%	(72)		20.75	%	(72)		19.53	%	(74)	

Supplementary Table 12. Descriptive statistics of potential confounders of the very old population from the Newcastle 85+ Study. A. Superoxide levels population only, B. Mitochondrial mass population only, C. Mitochondrial membrane potential population only and D. 8-Iso Prostaglandin $F_{2\alpha}$ population only. Continuous and discrete data are displayed as median and, inter-quartile ranges since all measures had a non-normal distribution and grouped data are displayed as percentage of population. ([P1]: Phase 1 data, [P3]: Phase 3 data)

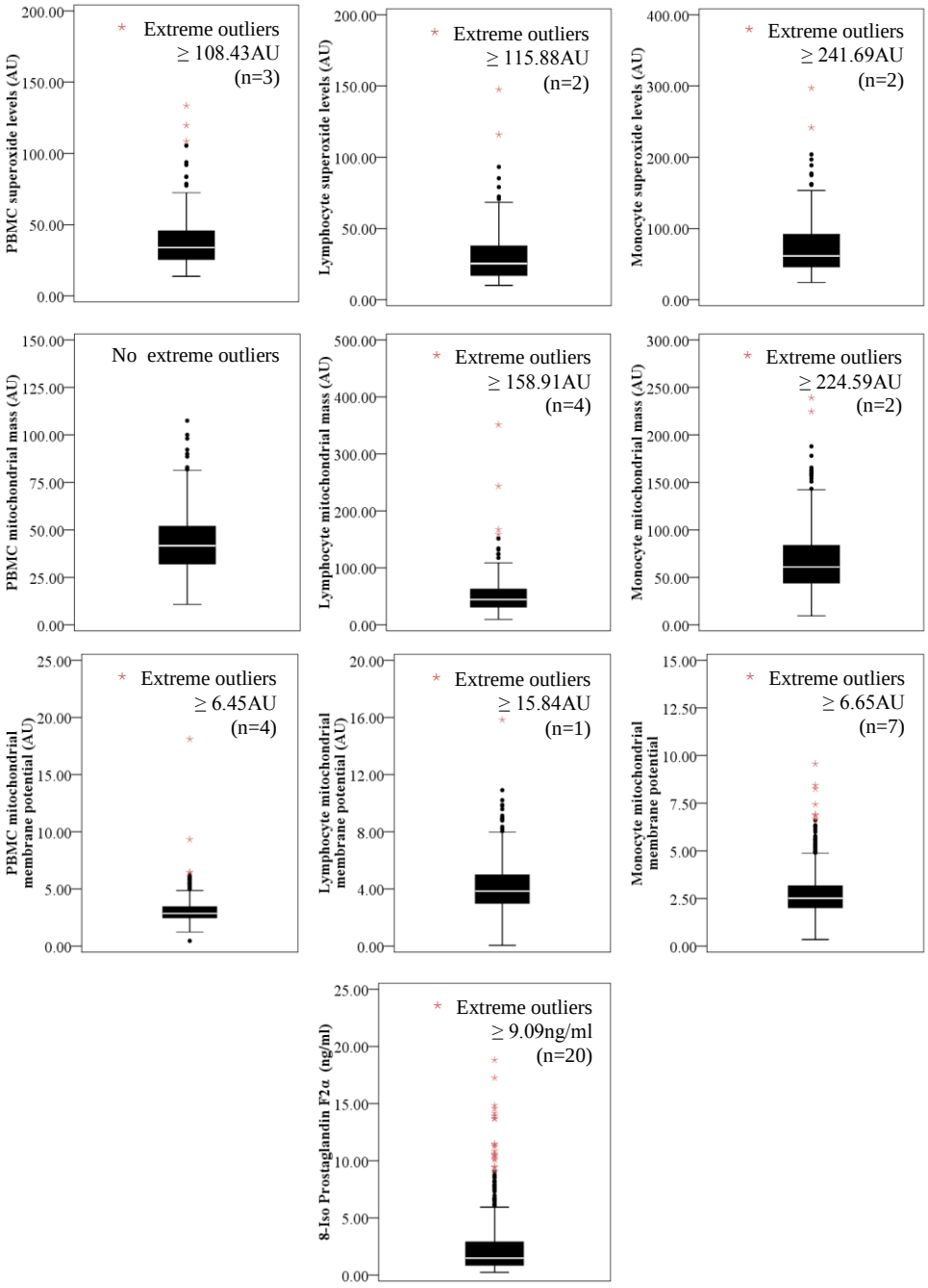
Age-related construct variables	All			A.				B.				C.				D.			
	Statistic		n	Statistic		n	p	Statistic		n	p	Statistic		n	p	Statistic		n	p
Telomere shortening																			
PBMC telomere length (bp) [P3 ~]	2715.77	1057.17	(430)	2786.50	1068.93	(248)	0.62	2722.65	1042.64	(340)	0.88	2709.72	1051.81	(345)	0.98	2738.62	1027.77	(376)	0.73
DNA damage and repair																			
PBMC DNA damage (%) [P3 ~]	47.26	29.65	(431)	44.25	27.56	(248)	0.10	45.37	30.41	(341)	0.56	46.00	29.99	(347)	0.75	47.20	28.84	(379)	0.90
PBMC DNA repair (%) [P3 ~]	46.24	47.27	(431)	43.29	43.94	(248)	0.56	44.79	46.29	(341)	0.76	45.75	46.94	(347)	0.97	46.54	48.37	(379)	0.85
Inflammation																			
Interleukin-6 (pg/ml) [P3 ~]	10.25	31.43	(430)	6.79	33.89	(247)	0.07	9.77	31.19	(340)	0.68	10.21	32.06	(345)	0.84	9.90	31.56	(377)	0.94
Tumour necrosis factor-alpha (pg/ml) [P3 ~]	2.55	4.42	(430)	2.10	4.18	(247)	0.07	2.45	4.26	(340)	0.50	2.48	4.45	(345)	0.61	2.64	4.49	(377)	0.78
Glycosylated haemoglobin (%) [P3 ~]	5.90	0.60	(428)	5.90	0.50	(247)	0.41	5.90	0.60	(339)	0.67	5.90	0.60	(344)	0.74	5.90	0.60	(375)	0.97
High sensitivity C-reactive protein (mg/l) [P3 ~]	2.40	3.70	(434)	2.35	3.28	(248)	0.81	2.40	3.70	(341)	0.73	2.40	3.70	(347)	0.67	2.40	3.60	(379)	0.85
Albumin (g/l) [P3 ~]	40.00	4.00	(434)	40.00	4.00	(248)	0.88	40.00	4.00	(341)	0.70	40.00	4.00	(347)	0.81	40.00	4.00	(379)	0.70
Rheumatoid factor (IU/ml) [P1 ~]	10.00	0.00	(774)	10.00	0.00	(239)	0.04	10.00	0.00	(328)	0.75	10.00	0.00	(335)	0.56	10.00	0.00	(367)	1.00
Immunosenescence																			
Lymphocyte/Monocyte ratio [P3 ~]	3.25	1.64	(427)	3.17	1.80	(246)	0.26	3.29	1.76	(338)	0.60	3.28	1.74	(343)	0.75	3.28	1.69	(374)	0.95
CD4 / Upper CD8 T lymphocye ratio [P3 ~]	2.37	2.78	(405)	2.42	2.56	(239)	0.99	2.43	2.75	(319)	0.89	2.37	2.77	(325)	0.83	2.43	2.66	(355)	0.69
Memory / Naive B lymphocyte ratio [P3 ~]	0.23	0.36	(429)	0.23	0.33	(246)	0.94	0.23	0.33	(339)	0.77	0.23	0.34	(344)	0.77	0.23	0.33	(376)	0.76
Memory / Naive CD4 T lymphocyte ratio [P3 ~]	0.25	0.51	(428)	0.25	0.51	(245)	0.77	0.25	0.55	(338)	0.86	0.25	0.55	(343)	0.63	0.24	0.48	(375)	0.76
Memory / Naive CD8 T lymphocyte ratio [P3 ~]	1.19	3.51	(402)	1.02	2.69	(236)	0.49	1.22	3.34	(316)	0.82	1.19	3.56	(322)	0.83	1.15	3.48	(352)	0.93
Senescent (CD27-/RO-) CD4 T lymphocytes (%) [P3 ~]	1.46	3.32	(429)	1.45	3.37	(246)	0.70	1.58	3.54	(339)	0.83	1.58	3.48	(344)	0.77	1.44	3.15	(376)	0.67
Senescent (CD27-/RO-) CD8 T lymphocytes (%) [P3 ~]	21.81	25.69	(403)	21.52	24.43	(237)	0.98	21.40	25.27	(317)	0.91	21.89	25.70	(323)	0.85	21.81	26.34	(353)	0.92

Informative classical BoA																			
Hand grip strength (kg) [P3 ~]	16.10	9.03	(453)	16.45	8.40	(242)	0.61	16.43	8.68	(336)	0.59	16.60	8.80	(342)	0.27	16.40	9.43	(374)	0.57
Timed up and go test (seconds) [P3 ~]	17.09	12.19	(402)	17.02	12.79	(218)	0.81	16.62	12.39	(304)	0.85	16.60	12.38	(312)	0.81	16.91	11.98	(340)	0.73
Forced expiratory volume in 1 second (l) [P1 ~]	137.00	71.75	(776)	142.50	64.50	(244)	0.10	142.00	66.00	(335)	0.06	142.00	67.50	(341)	**0.03**	142.00	71.75	(372)	0.05
Systolic blood pressure (mmHg) [P3 ^]	143.04	0.96	(462)	144.18	1.31	(245)	0.50	144.16	1.10	(338)	0.44	143.80	1.08	(343)	0.62	143.69	1.05	(376)	0.63
Haematocrit (%) [P3 ^]	0.39	0.00	(427)	0.39	0.00	(246)	0.98	0.39	0.00	(338)	0.98	0.39	0.00	(343)	1.00	0.39	0.00	(374)	0.89
Haemoglobin (g/dl) [P3 ~]	13.00	1.70	(427)	13.00	1.70	(246)	1.00	13.00	1.70	(338)	0.93	13.00	1.70	(343)	0.92	13.00	1.63	(374)	0.83
Red blood cells (x 10^{12}/l) [P3 ^]	4.25	0.02	(427)	4.24	0.03	(246)	0.85	4.24	0.03	(338)	0.84	4.25	0.03	(343)	0.98	4.25	0.02	(374)	0.83
Free triiodothyronine (pmol/l) [P3 ~]	4.40	0.80	(426)	4.35	0.83	(246)	0.12	4.40	0.80	(338)	0.84	4.40	0.80	(343)	0.84	4.40	0.70	(374)	1.00
Vitamin D (nmol/l) [P1 ~]	39.00	37.00	(778)	40.50	38.00	(240)	0.78	39.00	35.00	(331)	1.00	39.00	35.00	(338)	0.80	39.00	34.00	(371)	0.82
Prohormone brain-type natriuretic peptide (pg/ml) [P3 ~]	514.00	791.75	(124)	479.50	787.75	(46)	0.55	503.00	693.00	(85)	0.74	516.00	806.00	(95)	0.88	505.00	798.75	(108)	0.84

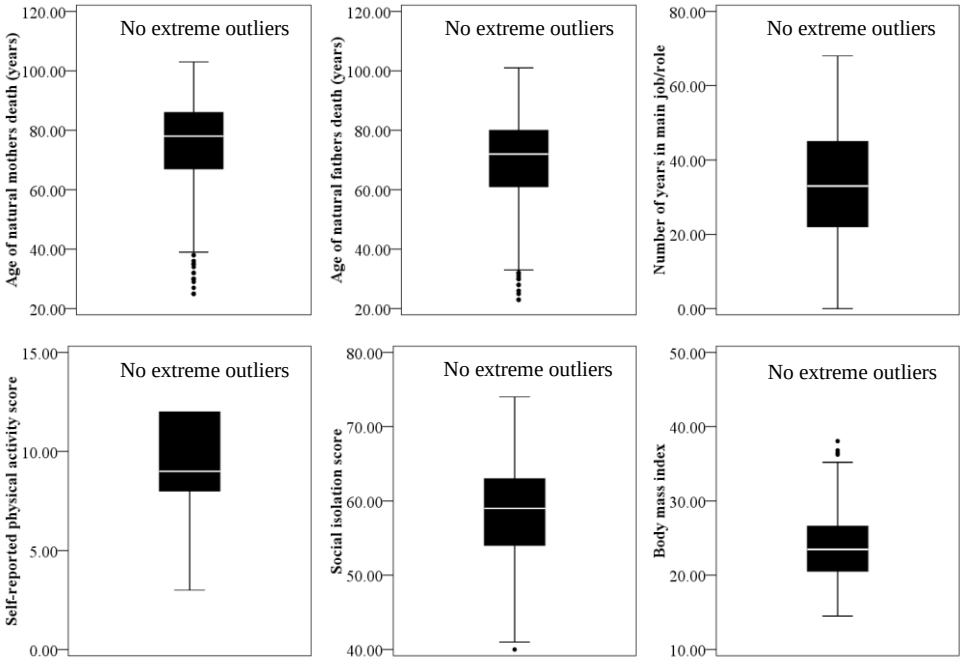
Supplementary Table 13. Descriptive statistics of age-related construct variables of the very old population from the Newcastle 85+ Study. A. Superoxide levels population only, B. Mitochondrial mass population only, C. Mitochondrial membrane potential population only and D. 8-Iso Prostaglandin $F_{2\alpha}$ population only. Continuous data with non-normal distributions are displayed as medians and inter-quartile ranges and continuous data with normal distribution are displayed a means and standard error. ([P1]: Phase 1 data, [P3]: Phase 3 data, ~: non-normal distribution, ^: normal distribution)

Age-related outcomes	All			A.				B.				C.				D.			
	Statistic		n	Statistic		n	p	Statistic		n	p	Statistic		n	p	Statistic		n	p
Disability score [P3]	6.00	7.25	(482)	6.00	7.00	(247)	0.42	6.00	7.00	(339)	0.28	6.00	7.00	(345)	0.22	6.00	7.00	(377)	0.17
SMMSE score [P3]	27.00	5.00	(471)	27.00	5.00	(247)	0.80	27.00	5.00	(339)	0.70	27.00	5.00	(345)	0.95	27.00	5.00	(378)	0.40
Disease Only Morbidity Count [P3]	3.00	2.00	(751)	3.00	2.00	(239)	0.27	2.00	2.00	(328)	0.05	3.00	2.00	(334)	0.09	2.00	2.00	(367)	**0.02**
Passed Away? (censored at 30/04/2012)																			
Yes	49.06	%	(417)	25.81	%	(64)	**0.00**	25.22	%	(86)	**0.00**	25.94	%	(90)	**0.00**	26.65	%	(101)	**0.00**
No	50.94	%	(433)	74.19	%	(184)		74.78	%	(255)		74.06	%	(257)		73.35	%	(278)	

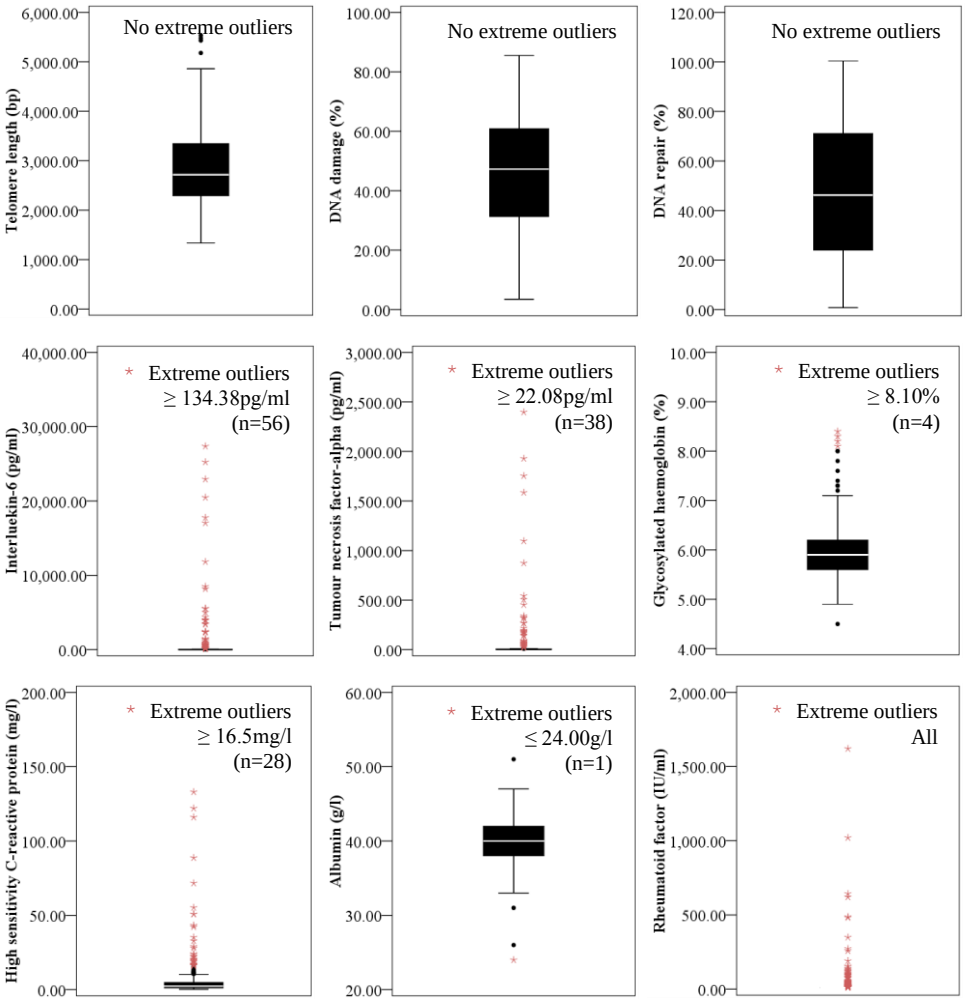
Supplementary Table 14. Descriptive statistics of age-related outcomes of the very old population from the Newcastle 85+ Study. A. Superoxide levels population only, B. Mitochondrial mass population only, C. Mitochondrial membrane potential population only and D. 8-Iso Prostaglandin $F_{2\alpha}$ population only. Discrete data are displayed as medians and inter-quartile ranges since all measures had a non-normal distribution and grouped data are displayed as percentage of population. ([P3]: Phase 3 data)



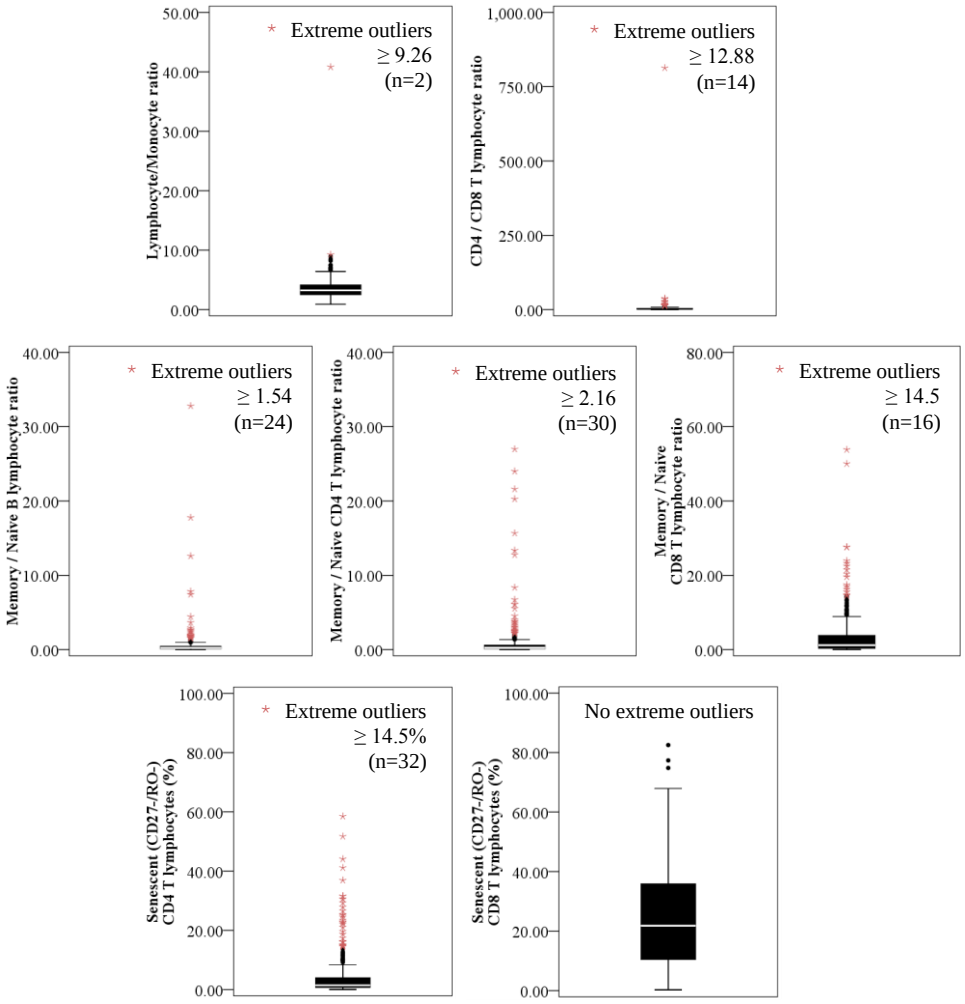
Supplementary Figure 5. Identifying extreme outliers of continuous potential oxidative stress-related BoA of the very old population from the Newcastle 85+ Study for sensitivity analysis. Extreme outliers were values more than 3 times the interquartile range (IQR) below the 25th or above the 75th percentiles, which were identified by default in SPSS boxplots. (All are phase 3 measures)



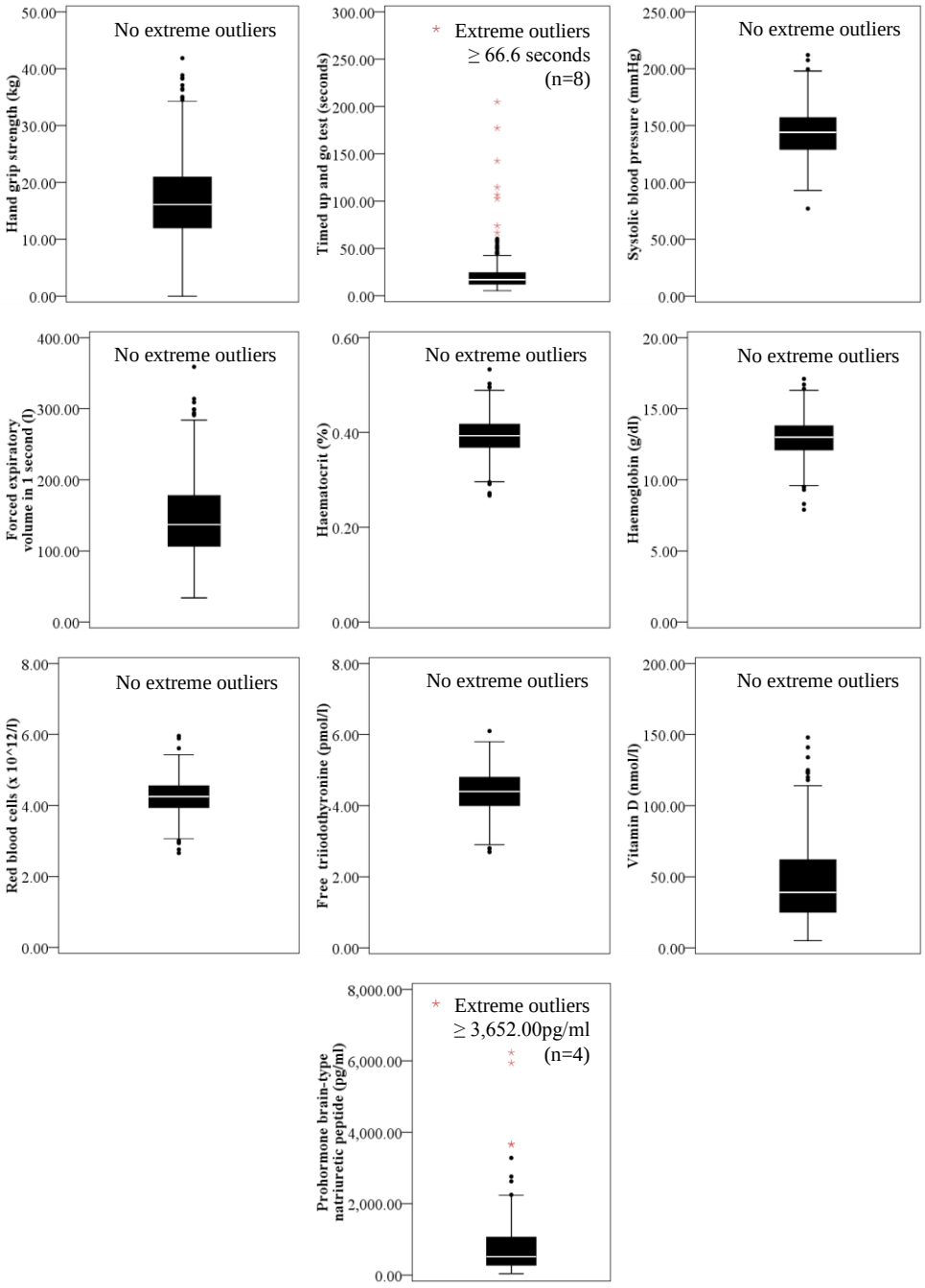
Supplementary Figure 6. Identifying extreme outliers of continuous potential confounders of the very old population from the Newcastle 85+ Study for sensitivity analysis. Extreme outliers were values more than 3 times the interquartile range (IQR) below the 25th or above the 75th percentiles, which were identified by default in SPSS boxplots. (All are phase 3 measures except unchangeable characteristics (age of parents death and number of years in main job/role) which were assessed at phase 1)


Telomere length (bp)
6,000.00
5,000.00
4,000.00
3,000.00
2,000.00
1,000.00
0.00
No extreme outliers
DNA damage (%)
100.00
80.00
60.00
40.00
20.00
0.00
No extreme outliers
DNA repair (%)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
No extreme outliers
Interluekin-6 (pg/ml)
40,000.00
30,000.00
20,000.00
10,000.00
0.00
Extreme outliers
≥ 134.38pg/ml
(n=56)
Tumour necrosis factor-alpha (pg/ml)
3,000.00
2,500.00
2,000.00
1,500.00
1,000.00
500.00
0.00
Extreme outliers
≥ 22.08pg/ml
(n=38)
Glycosylated haemoglobin (%)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
Extreme outliers
≥ 8.10%
(n=4)
High sensitivity C-reactive protein (mg/l)
200.00
150.00
100.00
50.00
0.00
Extreme outliers
≥ 16.5mg/l
(n=28)
Albumin (g/l)
60.00
50.00
40.00
30.00
20.00
Extreme outliers
≤ 24.00g/l
(n=1)
Rheumatoid factor (IU/ml)
2,000.00
1,500.00
1,000.00
500.00
0.00
Extreme outliers
All

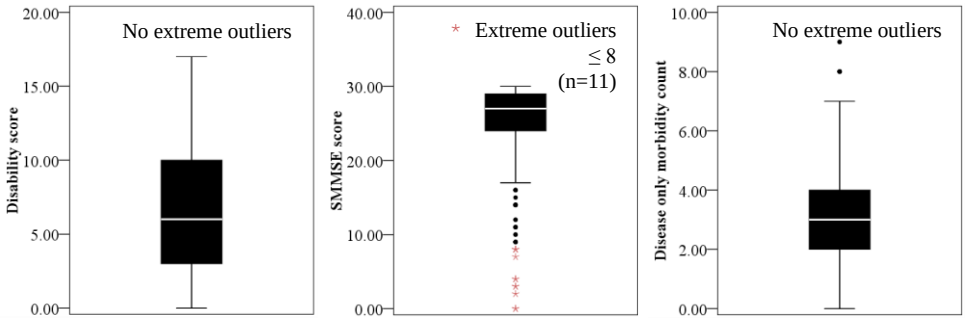
Cont...


Lymphocyte/Monocyte ratio
50.00
40.00
30.00
20.00
10.00
0.00
Extreme outliers
≥ 9.26
(n=2)
CD4 / CD8 T lymphocyte ratio
1,000.00
750.00
500.00
250.00
0.00
Extreme outliers
≥ 12.88
(n=14)
Memory / Naive B lymphocyte ratio
40.00
30.00
20.00
10.00
0.00
Extreme outliers
≥ 1.54
(n=24)
Memory / Naive CD4 T lymphocyte ratio
40.00
30.00
20.00
10.00
0.00
Extreme outliers
≥ 2.16
(n=30)
Memory / Naive CD8 T lymphocyte ratio
80.00
60.00
40.00
20.00
0.00
Extreme outliers
≥ 14.5
(n=16)
Senescent (CD27-/RO-) CD4 T lymphocytes (%)
100.00
80.00
60.00
40.00
20.00
0.00
Extreme outliers
≥ 14.5%
(n=32)
Senescent (CD27-/RO-) CD8 T lymphocytes (%)
100.00
80.00
60.00
40.00
20.00
0.00
No extreme outliers

Cont...



Supplementary Figure 7. Identifying extreme outliers of continuous age-related construct variables of the very old population from the Newcastle 85+Study for sensitivity analysis. Extreme outliers were values more than 3 times the interquartile range (IQR) below the 25th or above the 75th percentiles, which were identified by default in SPSS boxplots. (All are phase 3 measures except rheumatoid factor, forced expiratory volume and vitamin D which were measured at phase 1)



Supplementary Figure 8. Identifying extreme outliers of continuous age-related outcomes of the very old population from the Newcastle 85+Study for sensitivity analysis. Extreme outliers were values more than 3 times the interquartile range (IQR) below the 25th or above the 75th percentiles, which were identified by default in SPSS boxplots. (All are phase 3 measures)

Potential oxidative stress-related BoA	Low (Q1)				Medium (Q2+Q3)				High (Q4)				Total
	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	n
Superoxide levels (AU) [P3]													
PBMCs	≤ 25.5600	63	13.7700	25.5600	25.5601 - 45.7250	123	25.6600	45.7200	≥ 45.7251	62	45.7300	133.4900	248
Lymphocytes	≤ 17.0450	62	10.1100	17.0400	17.0451 - 37.8200	124	17.0500	37.7500	≥ 37.8201	62	37.8900	147.6300	248
Monocyte	≤ 46.0400	62	24.2300	45.9700	46.0401 - 92.0250	124	46.1100	91.8800	≥ 92.0251	62	92.1700	297.2400	248
Mitochondrial mass (AU) [P3]													
PBMCs	≤ 32.0700	86	10.8000	32.0700	32.0701 -51.9300	170	32.1100	51.9300	≥ 51.9301	85	51.9400	107.4700	341
Lymphocytes	≤ 31.1600	86	9.8300	31.1600	31.1600 - 63.0200	170	31.4800	63.0200	≥ 63.0200	85	63.1600	351.1700	341
Monocyte	≤ 43.9900	86	9.4300	43.9900	43.9901 - 83.7000	170	44.8000	83.7000	≥ 83.7001	85	84.6900	239.1000	341
Mitochondrial membrane potential (AU) [P3]													
PBMCs	≤ 2.4670	87	0.4684	2.4670	2.4671 - 3.4503	174	2.4684	3.4503	≥ 3.4504	86	3.4569	18.1070	347
Lymphocytes	≤ 2.9901	87	0.0608	2.9901	2.9902 - 4.9902	174	2.9970	4.9902	≥ 4.9903	86	5.0032	15.8400	347
Monocyte	≤ 2.0155	87	0.3501	2.0155	2.0155 - 3.1763	174	2.0173	3.1763	≥ 3.1763	86	3.2009	9.5704	347
8-Iso Prostaglandin $F_{2\alpha}$ (ng/ml) [P3]	≤ 0.85	95	0.25	0.85	0.86 - 2.93	191	0.87	2.93	≥ 2.94+	93	2.94	18.81	379

Supplementary Table 15. Cut-off values for grouped continuous potential oxidative stress-related BoA. Quartile cut-offs were first identified and values were assigned to Q1, Q2, Q3 or Q4 groups by default in SPSS. Q2 and Q3 were then grouped manually. (Q: Quartile, [P3]: Phase 3 data)

Potential confounders	Low (Q1)				Medium (Q2+Q3)				High (Q4)				Total
	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	n
Age of parents death [P1]													
Mother	≤ 67	204	25	67	68 - 86	439	68	86	≥ 87	167	87	103	810
Father	≤ 61	198	23	61	62 - 80	403	62	80	≥ 81	175	81	101	776
Number of years in main job/role [P1]	≤ 22	177	0	22	23 - 45	327	23	45	≥ 46	162	46	68	666
Self-reported physical activity score [P3]	≥ 9	353	9	12	8	32	8	8	≤ 7	99	3	7	484
Social isolation score [P3]	≤ 54	127	40	54	55 - 63	242	55	63	≥ 64	111	64	74	480
Body mass index [P3]	≤ 21	102	14.50	20.52	22 - 27	226	20.55	27.46	≥ 28	73	27.66	38.05	401

Supplementary Table 16. Cut-off values for grouped continuous potential confounders . Quartile cut-offs were first identified and values were assigned to Q1, Q2, Q3 or Q4 groups by default in SPSS. Q2 and Q3 were then grouped manually. (Q: Quartile, [P1]: Phase 1 data, [P3]: Phase 3 data)

Age-reated construct variables	Low (Q1)				Medium (Q2+Q3)				High (Q4)				Total
	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	Cut-off	n	Minimum	Maximum	n
PBMC telomere length (bp) [P3]	≤2290.3 542103	108	1335.5 452961	2290.3 542103	2290.3542104 - 3345.6611576	215	2294.92 40552	3345.6 611576	≥3345.6 611577	107	3352.5 440824	5534.0 784103	430
PBMC DNA damage (%) [P3]	≤ 31.25 20980	108	3.510 5316	31.25 20980	31.2520981 - 60.2634508	216	31.31 22172	60.90 13181	≥ 60.9 013182	107	61.00 00000	85.49 54035	431
PBMC DNA repair (%) [P3]	≤ 23.98 19005	108	0.800 1581	23.98 19005	23.9819006 - 71.2566201	216	24.06 29685	71.25 66201	≥ 71.2 566202	107	71.28 28880	100.38 24092	431
Interluekin-6 (pg/ml) [P3]	≤3.137 65058	108	0.000 0000	3.137 6506	3.13765059 - 34.54857549	215	3.14 40295	34.54 85755	≥ 34.54 857550	107	34.59 98125	27372.4 342218	430
Tumour necrosis factor-alpha (pg/ml) [P3]	≤1.006 68331	108	0.000 0000	1.006 6833	1.00668332 - 5.39988836	215	1.022 3697	5.399 8884	≥ 5.39 988837	107	5.519 6799	2397.3 236148	430
Glycosylated haemoglobin (%) [P3]	≤ 5.60	113	4.50	5.60	5.61 - 5.90	235	5.70	6.20	≥ 6.21	80	6.30	8.40	428
High sensitivity C-reactive protein (mg/l) [P3]	≤ 1.10	115	0.20	1.10	1.11 - 4.80	213	1.20	4.80	≥ 4.81	106	4.90	133.00	434
Albumin (g/l) [P3]	≤ 38.00	123	24.00	38.00	39.00 - 42.00	226	39.00	42.00	≥ 43.00	85	43.00	51.00	434
Rheumatoid factor (IU/ml) [P1]	≤ 17.7	701	10.00	17.70	17.80 - 870	48	17.90	87.00	≥ 87	25	88.80	1620.00	774
Lymphocyte/Monocyte ratio [P3]	≤ 2.52	107	0.90	2.52	2.53 - 4.16	214	2.52	4.16	≥ 4.17	106	4.18	40.80	427
CD4 / Upper CD8 T lymphocyte ratio [P3]	≤ 1.31	102	0.23	1.31	1.32 - 4.08	202	1.33	4.08	≥ 4.09	101	4.09	813.17	405
Memory / Naive B lymphocyte ratio [P3]	≤ 0.11	115	0.00	0.11	0.12 - 0.46	207	0.12	0.46	≥ 0.47	107	0.47	32.79	429
Memory / Naive CD4 T lymphocyte ratio [P3]	≤ 0.11	111	0.01	0.11	0.12 - 0.62	210	0.12	0.61	≥ .63	107	0.62	26.98	428
Memory / Naive CD8 T lymphocyte ratio [P3]	≤ 0.35	101	0.01	0.35	0.36 - 3.85	201	0.36	3.85	≥ 3.86	100	3.88	53.81	402
Senescent (CD27-/RO-) CD4 T lymphocytes (%[P]) [P3]	≤ 0.74	109	0.04	0.74	0.75 - 4.03	213	0.75	4.03	≥ 4.04	107	4.09	58.50	429
Senescent (CD27-/RO-) CD8 T lymphocytes (%) [P3]	≤10.41	101	0.27	10.41	10.42 - 36.10	202	10.52	36.10	≥ 36.11	100	36.29	82.52	403
Hand grip strength (kg) [P3]	≤12.00	115	0.00	12.00	12.01 - 20.95	225	12.05	20.95	≥ 20.96	113	21.05	41.85	453
Timed up and go test (seconds) [P3]	≤ 12.32	101	5.34	12.32	12.33 - 24.50	203	12.37	24.50	≥ 24.51	98	24.57	177.16	402
Forced expiratory volume in 1 second (l) [P1]	≤ 106.50	194	34.00	106.00	106.51 - 178.00	391	107.00	178.00	≥ 179.00	191	179.00	359.00	776
Systolic blood pressure (mmHg) [P3]	≤ 129.00	119	77.00	129.00	129.01 - 157.00	233	129.50	157.00	≥ 157.01	110	157.50	212.00	462
Haematocrit (%) [P3]	≤ 0.368	107	0.267	0.368	0.369 - .418	216	0.369	0.418	≥ 0.419	104	0.419	0.533	427

Haemoglobin (g/dl) [P3]	≤ 12.10	114	7.90	12.10	12.11 - 13.80	213	12.20	13.80	≥ 13.81	100	13.90	17.10	427
Red blood cells (x 1012/l) [P3]	≤ 3.94	109	2.66	3.94	3.95 - 4.56	213	3.95	4.56	≥ 4.57	105	4.57	5.96	427
Free triiodothyronine (pmol/l) [P3]	≤ 4.00	108	2.70	4.00	4.01 - 4.80	237	4.10	4.80	≥ 4.81	81	4.90	6.10	426
Vitamin D (nmol/l) [P1]	≤ 25.00	210	5.00	25.00	26.00 - 62.00	381	26.00	62.00	≥ 63.00	187	63.00	148.00	778
Prohormone brain-type natriuretic peptide (pg/ml) [P3]	≤ 277.00	31	42.00	276.00	278.00 - 1063.50	62	278.00	1054.00	≥ 1063.51	31	1073.00	6232.00	124

Supplementary Table 17. Cut-off values for grouped continuous age-related construct variables. Quartile cut-offs were first identified and values were assigned to Q1, Q2, Q3 or Q4 groups by default in SPSS. Q2 and Q3 were then grouped manually. (Q: Quartile, [P1]: Phase 1 data, [P3]: Phase 3 data)

Age-related outcomes	Group								Total
	Cut-off	n	Cut-off	n	Cut-off	n	Cut-off	n	n
Disability score [P3]	Fully independent (0)	31	1 - 6	223	7 - 12	138	≥13	90	482
SMMSE score [P3]	Severe cognitive impairment (0-17)	39	Moderate cognitive impairment (18 - 21)	35	Mild cognitive impairment (22 - 25)	86	No cognitive impairment (26-30)	311	471
Disease Only Morbidity Count [P3]	None	36	1 - 2	299	3 - 6	388	≥7	28	751

Supplementary Table 18. Cut-off values for grouped continuous age-related outcomes for sensitivity analysis. ([P1]: Phase 1 data, [P3]: Phase 3 data)

Control no	Date of blood collection 1	Date of blood collection 2	Date of birth	Age at analysis (years)	Gender
1	20/10/2010*	No sample	_	_	_
2	20/10/2010*	27/10/2010*	_	_	_
3	20/10/2010*	27/10/2010*	_	_	_
4	20/10/2010*	27/10/2010*	_	_	_
5	20/10/2010*	27/10/2010*	_	_	_
6	20/10/2010*	27/10/2010*	_	_	_
7	20/10/2010*	No sample	_	_	_
8	22/11/2011	29/11/2011	05/02/1991	22	Male
9	22/11/2011	29/11/2011	08/02/1987	26	Male
10	22/11/2011	29/11/2011	22/02/1990	23	Male
11	22/11/2011	29/11/2011	22/02/1990	23	Male
12	22/11/2011	29/11/2011*	29/11/1986	26	Female
13	22/11/2011	29/11/2011	09/04/1979	34	Male
14	23/11/2011	30/11/2011	22/05/1958	55	Female
15	23/11/2011	01/12/2011*	07/05/1980	33	Male
16	23/11/2011	30/11/2011*	21/10/1961	51	Female
17	23/11/2011	No sample	24/07/1968	44	Female
18	23/11/2011	30/11/2011*	09/12/1986	26	Male
19	23/11/2011	30/11/2011	10/03/1983	30	Male

Supplementary Table 19. Summary of younger control PBMC samples (*: Unsuitable for flow cytometry analysis due to delay of more than 24 hours or error occurring during flow cytometry preparation/analysis/staining,¯:not recorded).

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